MOLECULAR PHARMACOLOGY, 14, 199-209

Studies on the Effects of Infusion of Enzyme Inhibitors on Mouse Adenosine Deaminase

PAUL P. TROTTA, MARY P. AHLAND, GEORGE F. BROWN, AND M. EARL BALIS

Memorial-Sloan-Kettering Cancer Center, New York, New York 10021

(Received June 30, 1977)
(Accepted August 22, 1977)

SUMMARY

TROTTA, PAUL P., AHLAND, MARY P., BROWN, GEORGE F. & BALIS, M. EARL (1978) Studies on the effects of infusion of enzyme inhibitors on mouse adenosine deaminase. *Mol. Pharmacol.*, 14, 199-209.

Two inhibitors of adenosine deaminase, 9-*erythro*-(2-hydroxy-3-nonyl)adenine (EHNA) and 9-β-D-arabinofuranosyl-6-hydroxylaminopurine (ara-HA), which represent relatively tight and weak binding substrate analogues, respectively, were infused into mice over a period of 5 days. Nine different tissues were excised, and adenosine deaminase activity levels, as well as selected kinetic parameters and electrophoretic variants, were characterized. Isoelectric focusing demonstrated two variants (adenosine deaminases I and II) that focused in the pH range 4.65–4.75 and exhibited an approximate apparent molecular weight of 36,000 by gel filtration. The ratio of these two forms, which differed from tissue to tissue, was unaffected by the inhibitor infusions. Striking increases in adenosine deaminase activity were observed after the infusions and were found to vary with the tissue as well as the nature and dose of inhibitor. Activities in the lung, stomach, liver, and jejunum increased by about 8-, 3-, 3-, and 2-fold, respectively, after infusion of no more than 0.6 mg/ml of EHNA. Other tissues displayed significant but smaller increases. The effects of ara-HA were generally smaller than those of EHNA, except in the jejunum, ileum, and thymus. Inhibition of adenosine deaminase *in vitro* by EHNA, and to a lesser extent by ara-HA, also varied from tissue to tissue, but did not correlate with the ratio of adenosine deaminases I and II. Adenosine deaminase partially purified from mice treated with a higher dose of EHNA (5.0–7.5 mg/ml) displayed a significantly lower K_i value for EHNA; smaller decreases were noted in the K_m for adenosine. The most striking change was observed in the K_i of adenosine deaminase II for EHNA, which changed from 14.9 nM (control) to 3.1 nM (after infusion). By classical Ackermann-Potter plots EHNA was shown not to be a titrating or stoichiometric inhibitor either before or after the infusions. EHNA in all cases showed classical competition with substrate. The data may be of value in the development of combined chemotherapy regimens involving adenosine deaminase inhibitors as well as in understanding the molecular basis of the role of adenosine deaminase in regulating cell division.

This investigation was supported by Grant CA-14906 from the National Cancer Institute through the National Large Bowel Cancer Project, by United States Public Health Service Grants CA-08748, CA-18856, and CA-18428, and by American Cancer Society Grant BC-202.

INTRODUCTION

Adenosine deaminase (EC 3.5.4.4) catalyzes the deamination of adenosine, or adenosine analogues, to inosine, or the corresponding 6-oxy derivatives. This en-

0026-895X/78/0141-0199$02.00/0

zyme has become a recent focus of attention as the principal system *in vivo* that catalyzes the deamination, and consequent inactivation, of a number of potent antiviral and antitumor agents, such as 9-β-D-arabinofuranosyl-6-aminopurine, 9-β-D-xylofuranosyl-6-aminopurine, formycin A, 3′-deoxyadenosine, and 2′,3′-dideoxyadenosine (1–7). A number of investigators have suggested the simultaneous administration of adenosine deaminase inhibitors to enhance the chemotherapeutic efficiency of these analogues (7–10). In support of this, LePage and co-workers (11) have recently reported that the tightly binding inhibitor 2′-deoxycoformycin significantly potentiates ara-A[1] therapy of mice bearing L1210 leukemia; a similar enhancement of cytoxicity against L1210 cells in culture has also been shown (12). The enhancement of the toxicity of 3′-deoxyadenosine by the same inhibitor also supports the validity of the approach (13). It is therefore important to understand the *in vivo* response of adenosine deaminase to the administration of inhibitors.

The inhibition of adenosine deaminase is of additional significance because of recent data that support a causal relationship between the apparent absence of this enzyme and at least one variety of severe combined immunodeficiency disease (14, 15). Studies in our laboratory suggest that the apparent absence of this enzyme in at least some patients might be related to the presence of a genetically programmed, tightly binding inhibitor that is dissociable upon dilution (16, 17). In support of this general mechanism of immunosuppression, prolonged survival of allografts in mice receiving the adenosine deaminase inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine (18) or 2′-deoxycoformycin (19) has recently been reported.

The present study documents changes in levels and kinetic properties of mouse adenosine deaminase in response to the infusion of inhibitors. The two inhibitors chosen, EHNA (20) and ara-HA (21), represent classes of analogues with relatively high and low affinity, respectively. Our data demonstrate a dose-dependent increase in activity, as well as an increase in the apparent affinity of the enzyme for the infused inhibitor subsequent to infusion. The most dramatic effects were observed with the relatively tightly binding EHNA. These data may be of eventual usefulness in the development of combined chemotherapy regimens involving adenosine deaminase inhibitors, as well as in understanding the molecular basis of the regulatory role of this enzyme.

[1] The abbreviations used are: ara-A, 9-β-D-arabinofuranosyl-6-aminopurine; EHNA, 9-*erythro*-(2-hydroxy-3-nonyl)adenine; MTT, 3-(4,5-Dimethyl Thiazolyl-)-2,5-diphenyl ara-HA, 9-β-D-arabinofuranosyl-6-hydroxylaminopurine.

MATERIALS AND METHODS

Xanthine oxidase (crystalline suspension in ammonium sulfate; from buttermilk), nucleoside phosphorylase (crystalline suspension in ammonium sulfate; from calf spleen), bovine serum albumin, imidazole, MTT tetrazolium, and phenazine methosulfate were purchased from Sigma. Adenosine was obtained from SBR (Orangeburg, N.Y.), and [8-^{14}C]adenosine (water–ethanol solution, 1:1; 54.6 mCi/mmole), from New England Nuclear. Ampholines were purchased from LKB Instruments. Agar (special grade) and sucrose (enzyme grade) were purchased from Schwarz/Mann; acrylamide and bisacrylamide, from Bio-Rad; and potassium phosphate, from Mallinckrodt. Thin-layer cellulose plates were purchased from Eastman Kodak. Ara-HA was a generous gift from Dr. A. Giner-Sorolla of the Sloan-Kettering Institute (Rye, N. Y.); EHNA was a generous gift from Dr. Howard Schaeffer of Burroughs Wellcome (Research Triangle Park, N.C.).

Enzyme assays. For the determination of K_i values for EHNA and K_m values for adenosine, enzyme was first incubated at 37° for 30 min in the presence of bovine serum albumin (0.5 mg/ml) and potassium phosphate (110 mM, pH 7.0). In the absence of bovine serum albumin significant amounts of activity were lost over this time period. The reaction was started by the addition of 0.1 volume of 0.1 mM adenosine. The rate of deamination was monitored by continuous recording at 37° at

265 nm. A change in millimolar extinction coefficient of 8.33 (22) was employed to calculate micromoles of adenosine deaminated. The tissue distribution of adenosine deaminase was determined by a modification of the assay of Hopkinson and coworkers (23), in which the inosine produced is converted to uric acid by nucleoside phosphorylase (0.1 unit/ml) and xanthine oxidase (0.2 unit/ml) (16). Appropriate background blanks were always subtracted to eliminate errors due to contaminating adenosine deaminase in commercial preparations of nucleoside phosphorylase and xanthine oxidase.

A radioactivity assay was also employed, particularly in the determination of the inhibition by ara-HA. The high concentration of the latter necessary to produce inhibition interfered with the spectrophotometric assays. Incubation was performed at 37° in potassium phosphate (100 mM, pH 7.0) at the substrate concentrations and for the time periods indicated in the individual experiments. The reaction was stopped by boiling for 2 min, and the radioactive products, inosine and hypoxanthine, were separated from the substrate on thin-layer cellulose plates, using water-saturated 1-butanol–concentrated ammonium hydroxide (99:1) as the developing solvent. Substrate and product spots were cut out and radioassayed by liquid scintillation.

A unit of activity is defined as the amount of enzyme that deaminates 1 μmole of substrate under the specified steady-state assay conditions. For determination of specific activity, protein was quantitated by the method of Lowry *et al.* (24), using bovine serum albumin as standard.

Isoelectric focusing. Analytical focusing was performed in a flat bed of 5% polyacrylamide containing 2% ampholines, pH 4–6, and 5% sucrose in an LKB Multiphor 2117 apparatus. The anode and cathode were composed of 1 M H_2SO_4 and 0.50% (w/v), pH 5–7 ampholines, respectively. A sample of approximately 0.02 ml was soaked into a 5 × 10 mm cellulose acetate strip, which was applied to the surface of the gel. Focusing proceeded for approximately 2 hr to a final voltage of about 900 V (maximum power output, 40 W). Immediately after termination of the run, the gel was overlaid with a thin layer of 1% agar containing adenosine (1.5 mM), MTT tetrazolium (0.1 mg/ml), phenazine methosulfate (0.1 mg/ml), xanthine oxidase (0.0075 unit/ml), and nucleoside phosphorylase (0.005 unit/ml) in 25 mM potassium phosphate, pH 7.5, essentially as described by Spencer and co-workers (25). After a 30–60-min incubation at 37° in the dark, blue bands corresponding to adenosine deaminase activity appeared. A faint blue background was also visible as a result of the contaminating adenosine deaminase in the xanthine oxidase and nucleoside phosphorylase added.

Preparative isoelectric focusing was performed in an LKB Uniphor 7900 apparatus in the pH range 3–5. A 5–50% linear sucrose gradient of approximately 160 ml containing 1% (w/v) ampholines was produced with a Pharmacia P-3 peristaltic pump. The sample to be focused was applied in the 50% sucrose solution. The cathode and anode solutions were composed of 2.9% (v/v) ethanolamine in 64% sucrose and 1% (v/v) H_2SO_4, respectively. Runs were allowed to proceed for 48 hr to a final voltage of 1000–1200 V (maximum power output, 3 W). Fractions of approximately 1.0 ml were collected.

Drug infusion. CD-1 female albino mice (Charles River, Willington, Mass.) weighing approximately 20 g were maintained on a diet of Purina rat chow and tap water ad libitum. Infusion was performed by catheterization through the tail into the abdominal cavity, using procedures developed in this laboratory and previously described (26). Drugs (in 0.9% NaCl) were infused at a rate of 0.8 ml/day for 5 days. The animals were killed by cervical fracture, and the appropriate tissues were excised, cooled immediately on Dry Ice, and homogenized as 50% (w/v) solutions in a glass homogenizer in 250 mM sucrose–20 mM imidazole HCl, pH 7.0. Studies were performed as soon as feasible on the clear supernatants obtained by centrifugation at 10,000 × *g* for 30 min. The concentrations of EHNA and ara-HA were

determined spectrally, employing millimolar extinction coefficients of 14.8 (at 263 nm) for EHNA[2] and 10.4 (at 267 nm) for ara-HA (21).

RESULTS

Tissue distribution of adenosine deaminase activity and its electrophoretic variants. Specific adenosine deaminase activity was determined in nine tissues; the average data from a number of animals are listed in Table 1 in descending order. The highest activities were in the rapidly dividing tissues of the intestinal tract and the thymus, whereas the lowest were characteristic of lung, liver, and kidney. This order is in reasonable agreement with published data obtained in other species, including humans (27-29). Sephadex G-100 chromatography indicated further that most of the activity had an apparent molecular weight of 36,000 (data not shown). The enzyme from the mouse therefore falls in the previously described type C category (30), similar to the results obtained in the rat (31, 32). These data distinguish mouse adenosine deaminase from the human enzyme, which is characterized by a substantial quantity of type A enzyme (mol wt > 200,000) in various tissues (28).

Analytical isoelectric focusing of various tissue preparations revealed two major electrophoretic variants, termed I and II for the more cathodal and anodal of the variants, respectively, with isoelectric points in the range 4.65-4.75. They are distinguishable from rat adenosine deaminases I and II, which have pI values about 0.2 unit higher (31, 32). A particular distribution of variants appeared to be characteristic of each of the tissues examined. Thus, for example, the ileum exhibited only traces of the more anodal variant (II), whereas approximately equal amounts of the two forms were observed in the colon. No consistent correlation could be drawn between the activity level characteristic of a given tissue and the ratio of electrophoretic variants.

Inhibition of adenosine deaminase activity in vitro by EHNA and ara-HA. Each of the supernatants from the tissue homogenates was exhaustively dialyzed against 50 mM imidazole-100 mM sodium chloride, pH 7.0, and then tested for its sensitivity to EHNA and ara-HA (Table 2). A concentration of ara-HA between 1 and 2 mM generally produced about 50% inhibition of activity. Only relatively small differences were noted in the tested tissues, with the exception of the jejunal enzyme, which was not inhibited under the conditions employed. A much more striking tissue dependence of the extent of inhibition was obtained with EHNA. In this case the extent of inhibition ranged from 45% at an EHNA concentration of 7.5 nM with the liver enzyme, to no substantial inhibition of the kidney enzyme when the EHNA concentration was as high as 25 nM. The responses of other tissues were intermediate between these extremes.

No strict correlation was apparent between the tissue distribution of the two electrophoretic variants (Table 1) and the extent of inhibition observed. Indeed, as discussed later (Table 3), the inherent sensitivities to inhibition by EHNA of partially purified adenosine deaminases I and II from pooled tissues are quite similar, as judged by the K_i values. These observations suggest that although the isoelectric points of the two forms are similar from tissue to tissue, structural differences may exist that are not expressed in terms of charge alteration. This conclusion is fur-

TABLE 1

Tissue distribution of mouse adenosine deaminase

Activity was determined by converting the inosine produced to uric acid, as described in MATERIALS AND METHODS. Values are means and standard errors for the number of animals shown.

Tissue	No. of animals	Specific adenosine deaminase activity
		unit/mg protein
Jejunum	7	0.472 ± 0.055
Thymus	11	0.267 ± 0.056
Ileum	10	0.110 ± 0.015
Spleen	10	0.067 ± 0.004
Colon	11	0.060 ± 0.007
Stomach	10	0.028 ± 0.006
Kidney	12	0.012 ± 0.002
Liver	10	0.011 ± 0.002
Lung	9	0.0077 ± 0.0004

[2] H. Schaeffer, personal communication.

TABLE 2

Sensitivity of mouse adenosine deaminase to inhibition by EHNA and ara-HA

In studies with EHNA, activity was determined at an adenosine concentration of 30 μM by following the production of uric acid at 293 nm, as described in MATERIALS AND METHODS. The assay was started by the addition of enzyme; no prior incubation of enzyme and inhibitor was performed. The enzyme preparations employed for both EHNA and ara-HA studies were crude homogenates (see MATERIALS AND METHODS) exhaustively dialyzed against 50 mM imidazole HCl, 100 mM sodium chloride, pH 7.0. For studies with ara-HA, activity was measured by incubation with [8-^{14}C]adenosine (20 μM) for 15 min under the conditions described in MATERIALS AND METHODS, followed by separation of the radioactive products by thin-layer cellulose chromatography. Inhibitor was added directly to the assay mixture without prior incubation.

Tissue	EHNA		Ara-HA	
	Concentration	Inhibition	Concentration	Inhibition
	nM	%	*mM*	%
Liver	7.5	48.0	1.6	48.4
Spleen	12.5	42.3	1.6	46.8
Stomach	12.5	25.0	1.6	53.0
Jejunum	12.5	17.1	2.3	0[a]
Ileum	25.0	46.6	1.6	43.6
Colon	25.0	39.6	1.6	47.7
Thymus	25.0	43.5	1.6	49.4
Lung	25.0	16.9	1.6	57.3
Kidney	25.0	0[b]	1.6	43.6

[a] Activation of up to 30% was noted on some samples.

[b] Activation of up to 100% was noted on some samples.

ther supported by changes in the kinetic parameters of the enzyme after inhibitor infusion, which were not accompanied by changes in isoelectric point (see below). However, the possibility also exists that variation results from binding of EHNA to other components of the homogenate, thereby reducing the effective inhibitor concentration.

Effect of inhibitor infusion on specific adenosine deaminase activity in various tissues. Striking changes in the enzyme activity were observed to be dependent upon dose, tissue, and the infused inhibitor. The greatest increases following EHNA infusion were observed in the lung (almost 8-fold), stomach (greater than 3-fold), liver (greater than 3-fold), and jejunum (greater than 2-fold) (Fig. 1.) In each case these effects occurred over the approximate dose range of 0–0.60 mg/ml. The other tissues examined displayed relatively negligible changes except thymus (Fig. 1). In the latter tissue the enzyme displayed a unique drop to about one-fifth of the control value, followed by a return to the control level at the higher doses. Interestingly, the infusion of ara-HA produced a similar biphasic response in the activity from the thymus (Fig. 1). However, in general the effects of ara-HA infusion were substantially smaller, with the exception of the jejunum and ileum, where the respective activities obtained after ara-HA infusion were approximately equal to and greater than those observed with the EHNA-treated animals.

Analytical isoelectric focusing of tissue homogenates after inhibitor infusion generally resulted in patterns that were virtually indistinguishable from the control distribution of variants (Fig. 2). Several runs indicated no consistent change in this pattern as a function of inhibitor concentration. Thus, whatever the mechanism for activity alterations, both adenosine deaminases I and II were affected in a roughly equivalent manner.

Effect of EHNA infusion on selected catalytic properties. Since EHNA infusion had produced the greatest effects on activity levels in certain tissues, it was also of interest to ascertain whether this compound could alter catalytic properties. We were cognizant of the fact, as recently documented by Cha and co-workers (33, 34), that classical steady-state kinetic analysis may not be applicable in the presence of a tightly binding inhibitor, since slow rates of association-dissociation may produce a non-steady-state condition. It was therefore necessary to ascertain whether EHNA behaved as a classical stoichiometric inhibitor against the mouse adenosine deaminase.

Enzyme was pooled from the nine tissues listed in Table 1 and partially purified by preparative isoelectric focusing as described in MATERIALS AND METHODS. Each of the variants was then incubated at 37° for 30 min with various concentrations of EHNA in 100 mM potassium phosphate,

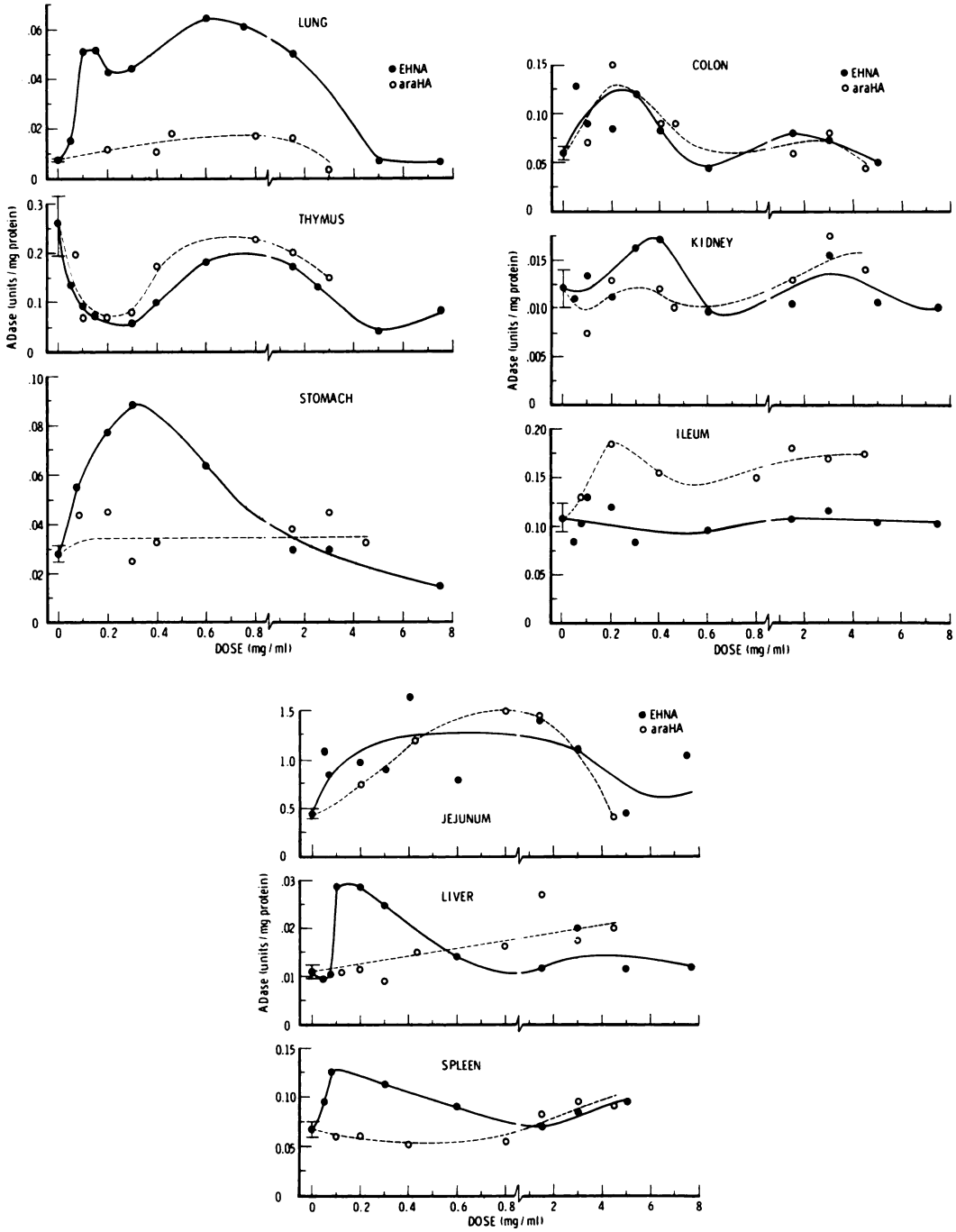

FIG. 1. *Effects of infusion of EHNA and ara-HA on specific adenosine deaminase (ADase) activity in various mouse tissues*

The inhibitors, at the concentrations shown on the abscissae, were infused into mice for 5 days at a constant rate of 0.8 ml/day. Control values for each tissue were obtained by averaging data from several animals as indicated in Table 1. Data for the various inhibitor concentrations were obtained by averaging the results from duplicate mice, or represent single-animal experiments. Other details are described under MATERIALS AND METHODS. p values for the increases in activity relative to controls, calculated from Student's t-test, were: $p < 0.01$ for lung, stomach, jejunum, and liver of EHNA-treated mice; $p < 0.05$ for spleen and colon of EHNA-treated mice and for lung, jejunum, and liver of ara-HA-treated mice; and $p < 0.20$ for ileum of EHNA-treated mice. The decrease in activity observed in the thymus was significant at $p < 0.025$ after EHNA infusion and at $p < 0.20$ after ara-HA treatment.

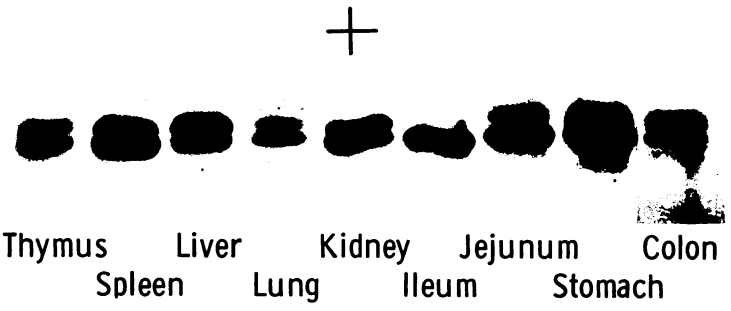

FIG. 2. *Analytical isoelectric focusing on polyacrylamide gel of mouse adenosine deaminase*
Crude mouse tissue homogenates were subjected to analytical isoelectric focusing in a pH 4–6 gradient, and the gel was stained for enzymatic activity as described under MATERIALS AND METHODS.

pH 7.0. The enzymatic reaction was initiated by addition of substrate. Ackermann-Potter plots (35) were constructed in which the measured velocity of the reaction was plotted against the enzyme concentration (Fig. 3). The series of straight lines establish that in this system EHNA does not behave as an extremely tightly binding or titrating inhibitor. The same results were obtained with enzyme isolated from EHNA-infused mice (Fig. 3, inset). This behavior is in marked contrast to that of 2′-deoxycoformycin, which produced a marked curvature in the Ackermann-Potter plot when tested against human erythrocyte adenosine deaminase (34).

In light of these data, the inhibition of mouse adenosine deaminase by EHNA was further analyzed by constructing Lineweaver-Burk plots (Fig. 4). The closeness of the extrapolated y intercept on each of the plots in the presence and absence of inhibitor indicates that EHNA behaves primarily as a classical competitive inhibitor. The data in Fig. 4c and d indicate that this is true after EHNA infusion as well. A similar conclusion was obtained from Eadie-Hofstee plots (data not shown). As shown in Table 3, the K_i values extracted from these plots for adenosine deaminases I and II were quite similar (11.1 and 14.9 nM for enzymes I and II, respectively). After EHNA infusion, however, changes were observed in the K_i value for enzyme II, which dropped to one-fifth of its original value, while that for enzyme I was decreased by about one-half. The high values for the correlation coefficients of the slopes of the Eadie-Hofstee plots support a high precision in the determination of these parameters. As shown in Fig. 4, the K_m for adenosine was also lowered as a result of EHNA infusion, but to a much smaller extent than the K_i value.

DISCUSSION

It is well established that a number of mammalian enzyme levels can be stimulated *in vivo* by administration of substrate, substrate analogues, or hormones (for reviews, see refs. 36–38). We conclude from the present study that adenosine deaminase is among this group, and that the magnitude of the response is dependent on the tissue examined as well as on the dose and nature of the infused analogues. An interesting feature of the response of certain tissues, in particular lung, liver, and stomach, is the finding that only the lower doses of EHNA were effective in stimulating activity. Although the reason for this type of response, as well as for the biphasic response observed in the thymus, is not understood, the data do suggest an antagonism between mechanisms of activation and inhibition.

The present data do not establish whether the observed activity increases can be attributed to the synthesis of new enzyme protein. Recent studies in this laboratory (31, 32) indicate that the increase in adenosine deaminase observed in the intestinal mucosa of the rat can be

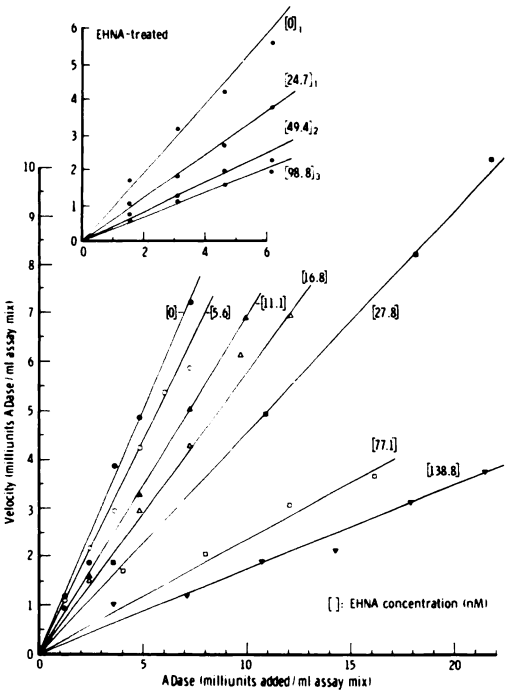

FIG. 3. *Ackermann-Potter plots of mouse adenosine deaminase (ADase) activity after incubation with EHNA*

Various concentrations of enzyme were first incubated at 37° with the indicated concentrations of EHNA in a final volume of 1.0 ml containing potassium phosphate (110 mM, pH 7.0) and bovine serum albumin (0.5 mg/ml). To 0.9 ml of the solution was added 0.1 ml of 1.0 mM adenosine to initiate the assay, which was monitored at 265 nm as described under MATERIALS AND METHODS. The data shown in the inset were obtained using enzyme from mice infused with 5.0 or 7.5 mg/ml of EHNA. Activities were determined by the radioactive assay described under MATERIALS AND METHODS under incubation conditions similar to those used in the spectrophotometric assay. All enzyme preparations in these experiments were partially purified by preparative isoelectric focusing. The control enzyme represented mainly variant I, whereas the enzyme from EHNA-infused mice was predominantly variant II. The correlation coefficients, calculated for the least-squares regression line passing through the origin, were at least 0.97, except for a value of 0.92 for the data obtained in the presence of 11.1 nM EHNA.

correlated with the preferential synthesis of a particular electrophoretic variant. The inhibitory effect of cycloheximide or puromycin on both the activity increase and the appearance of the new variant strongly suggested the participation of active protein synthesis (32, 34). The data we have obtained in the mouse, however, indicate that after inhibitor infusion the electrophoretic pattern of adenosine deaminase variants remains essentially unchanged. Thus, if new enzyme protein was being induced, each variant was being stimulated to approximately the same extent. It should also be noted that our previous studies with actinomycin D in the rat have indicated that adenosine deaminase is synthesized from a relatively unstable messenger (32, 39). Thus an effect on mRNA turnover provides a reasonable mechanism for the enhanced synthesis.

The alternative mechanism—involving interruption in degradation—must also be considered. One interpretation is that the infused inhibitor binds preferentially to one conformational form of the enzyme to form a complex that is relatively resistant to proteolysis, as has been reported for the complex *in vivo* of methotrexate and dihydrofolate reductase (40). Alternatively, specific proteases responsible for adenosine deaminase degradation might be inhibited as a result of the altered metabolic condition in the presence of lowered enzyme activity. Inhibited or absent adenosine deaminase activity has been observed to result in an elevated ratio of the adenine to guanine nucleotide pool levels in mammalian cells (41–44). These changes might influence rates of protein synthesis and/or degradation.

The altered affinity of adenosine deaminase for EHNA and for adenosine after EHNA infusion can be explained by either of the general mechanisms discussed above. Thus new enzyme protein that displays an active site with a higher affinity for substrate or substrate analogues may be induced, or a previously existing variant with such a characteristic may be stabilized by the presence of the inhibitor. It should be noted, however, that the altered affinity constants were obtained from animals that had been treated with a relatively high dose of EHNA (i.e., 5.0 and 7.5 mg/ml). These concentrations exceeded the dose range that produced the maximal response in terms of activity stimulation (see Fig. 1). Thus the mecha-

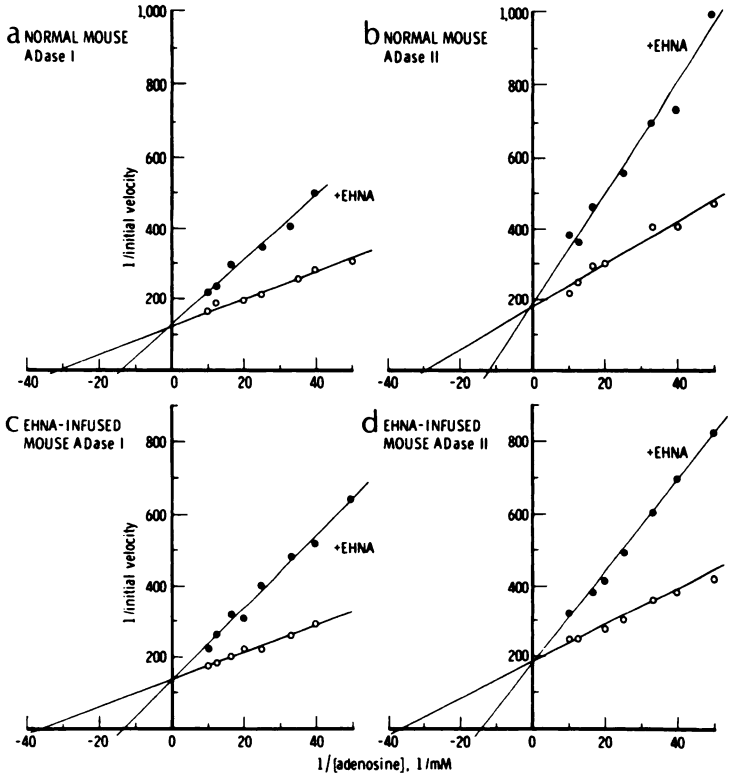

FIG. 4. *Lineweaver-Burk plots of adenosine deaminase (ADase) activity in the presence and absence of EHNA*

Activity was determined by the spectrophotometric assay at 265 nm as described under MATERIALS AND METHODS and Fig. 3. Enzyme was obtained by preparative isoelectric focusing of the pooled tissues listed in Table 1. a. Control adenosine deaminase I. b. Control enzyme variant II. c. Enzyme I obtained from mice infused with 5.0 or 7.5. mg/ml of EHNA. d. Enzyme II obtained from the same mice as in Fig. 4c. Correlation coefficients determined for the least-squares regression line indicated a high degree of association between the x and y values and are summarized in Table 3.

TABLE 3

Effect of EHNA infusion on K_i for EHNA and K_m for adenosine of mouse adenosine deaminase

Partially purified enzyme was obtained by preparative isoelectric focusing of the pooled homogenates from tissues listed in Fig. 1, as described in MATERIALS AND METHODS. Both K_i and K_m values were obtained from analysis of Eadie-Hofstee plots in the presence and absence of EHNA. Also shown are the correlation coefficients (r) for the slopes from which the kinetic parameters were calculated.

Enzyme variant	K_i for EHNA				K_m for adenosine			
	Normal	r	Treated	r	Normal	r	Treated	r
	nM		*nM*		*μM*		*μM*	
I	11.1	0.95[b]	6.0	0.93	33.5	0.98	27.1	0.96
II	14.9	0.95	3.1	0.98	35.3	0.95	23.3	0.98

nisms for the two effects may be distinct, and may also involve different populations of adenosine deaminase molecules.

Evidence that mammalian adenosine deaminase activity may be regulated by induction and/or derepression comes from a recent report of a kindred exhibiting a dominantly transmitted hemolytic anemia associated with a marked reduction in adenine nucleotides (45) and a 45–70-fold

elevation in adenosine deaminase activity, which was characterized as a typical phenotype with normal kinetic parameters (46). These investigators therefore suggested a defect in the normal feedback mechanisms of induction and suppression of adenosine deaminase synthesis. The changes reported in the mammalian enzyme as a function of diet (47) may also reflect aspects of the same control system.

It is hoped that the data presented here may be of eventual use in the rational design of combination chemotherapy regimens involving adenosine deaminase inhibitors. It has already been established that combination therapy of EHNA with the antimetabolite ara-A is successful in extending the survival time of mice bearing the Ehrlich ascites carcinoma (7). The inhibitor 2'-deoxycoformycin also significantly potentiates the cytotoxicity of ara-A against L1210 leukemic cells (11, 12).

The knowledge that an organism can react to these inhibitors with greater or modified adenosine deaminase activity and that these responses are organ-specific should be of value if this therapy is extended to humans. The possibility that similar phenomena may occur with other inhibitors should also be borne in mind.

REFERENCES

1. Klenow, H. (1961) *Biochem. Biophys. Res. Commun.*, 5, 156–159.
2. Brink, J. J. & LePage, G. A. (1964) *Cancer Res.*, 24, 312–318.
3. Brink, J. J. & LePage, G. A. (1965) *Can. J. Biochem.*, 43, 1–15.
4. Ellis, D. B. & LePage, G. A. (1965) *Mol. Pharmacol.*, 1, 231–238.
5. Schabel, F. M., Jr. (1968) *Chemotherapy*, 13, 321–338.
6. Giziewicz, J., DeClercq, E., Luczak, M. & Shugar, D. (1975) *Biochem. Pharmacol.*, 24, 1813–1817.
7. Plunkett, W. & Cohen, S. S. (1975) *Cancer Res.*, 35, 1547–1554.
8. Doering, A., Keller, J. & Cohen, S. S. (1966) *Cancer Res.*, 26, 893–897.
9. Koshiura, R. & LePage, G. A. (1968) *Cancer Res.*, 28, 1014–1018.
10. Chao, D. L. & Kimball, A. P. (1972) *Cancer Res.*, 32, 1721–1724.
11. LePage, G. A., Worth, L. S. & Kimball, A. P. (1976) *Cancer Res.*, 36, 1481–1485.
12. Cass, C. E. & Au-Yeung, T. H. (1976) *Cancer Res.*, 36, 1486–1491.
13. Johns, D. G. & Adamson, R. H. (1976) *Biochem. Pharmacol.*, 25, 1441–1444.
14. Meuwissen, H. J., Pickering, R. J., Pollara, B. & Porter, I. H., eds. (1975) *Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency*, Academic Press, New York.
15. Hitzig, W. H. (1973) in *Immunologic Disorders in Infants and Children* (Stiem, E. R. & Fulginiti, V. A., eds.), pp. 218–228, Saunders, Philadelphia.
16. Trotta, P. P., Smithwick, E. M. & Balis, M. E. (1976) *Proc. Natl. Acad. Sci. U. S. A.*, 73, 104–108.
17. Smithwick, E. M. & Trotta, P. P. (1977) in *Proceedings of the International Workshop on Bone Marrow Transplantation*, Tarrytown, N. Y., in press.
18. Lum, C. T., Sutherland, D. E. R. & Najarian, J. S. (1977), *N. Engl. J. Med.*, 296, 819.
19. Chassin, M. M., Chirigos, M. A., Johns, D. G. & Adamson, R. H. (1977) *N. Engl. J. Med.*, 296, 1232.
20. Schaeffer, H. & Schwender, C. F. (1974) *J. Med. Chem.*, 17, 6–8.
21. Giner-Sorolla, A. (1969) *J. Heterocycl. Chem.*, 6, 405–406.
22. Kalckar, H. M. (1947) *J. Biol. Chem.*, 167, 429–443.
23. Hopkinson, D. A., Cook, P. J. L. & Harris, H. (1969) *Ann. Hum. Genet.*, 32, 361–367.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
25. Spencer, N., Hopkinson, D. A. & Harris, H. (1968) *Ann. Hum. Genet.*, 32, 9–14.
26. Born, C. T. & Moller, M. L. (1974) *Lab. Anim. Sci.*, 24, 355–358.
27. Brady, T. G. & O'Donovan, C. I. (1965) *Comp. Biochem. Physiol.*, 14, 101–120.
28. Ma, P. F. & Magers, T. A. (1975) *Int. J. Biochem.*, 6, 281–286.
29. Van der Weyden, M. B. & Kelley, W. N. (1976) *J. Biol. Chem.*, 251, 5448–5456.
30. Ma, P. F. & Fisher, J. R. (1968) *Comp. Biochem. Physiol.*, 27, 687–694.
31. Trotta, P. P. & Balis, M. E. (1977) *Cancer*, in 40, 2592–2599, 1977.
32. Trotta, P. P. & Balis, M. E. (1977) *Cancer Res.*, 37, 2297–2305.
33. Cha, S. (1975) *Biochem. Pharmacol.*, 24, 2177–2185.
34. Cha, S., Agarwal, R. P. & Parks, R. E., Jr. (1975) *Biochem. Pharmacol.*, 24, 2187–2197.
35. Ackermann, W. W. & Potter, V. R. (1949) *Proc. Soc. Exp. Biol. Med.*, 72, 1–9.
36. Knox, W. E., Auerbach, V. H. & Lin, E. C. C.

(1956) *Physiol. Rev.*, **36**, 164–254.

37. Schimke, R. T. (1969) *Curr. Top. Cell Regul.*, **1**, 77–124.
38. Schimke, R. T. & Doyle, D. (1970) *Annu. Rev. Biochem.*, **39**, 929–976.
39. Imondi, A. R., Lipkin, M. & Balis, M. E. (1970) *J. Biol. Chem.*, **245**, 2194–2198.
40. Bertino, J. R., Cashmore, A., Fink, M., Calabresi, P. & Lefkowitz, E. (1965) *Clin. Pharmacol. Ther.*, **6**, 763–770.
41. Green, H. & Chan, T. S. (1973) *Science*, **182**, 836–838.
42. Agarwal, R. P., Crabtree, G. W., Parks, R. E., Jr., Nelson, J. A., Keightley, R., Parkman, R., Rosen, F. S., Stern, R. C. & Polmar, S. H. (1976) *J. Clin. Invest.*, **57**, 1025–1035.
43. Agarwal, R. P., Sagar, S. M. & Parks, R. E., Jr. (1975) *Biochem. Pharmacol.*, **24**, 693–701.
44. Parks, R. E., Jr., Crabtree, G. W., Kong, C. M., Agarwal, R. P., Agarwal, K. C. & Scholar, E. M. (1975) *Ann. N. Y. Acad. Sci.*, **255**, 412–434.
45. Paglia, D. E., Valentine, W. N., Tartaglia, A. P. & Konrad, P. N. (1970) *Blood*, **36**, 837.
46. Valentine, W. N., Tartaglia, A. P. & Gilanz, F. (1977) *Science*, **195**, 783–785.
47. Tritsch, G. L., Nechaev, A. & Paolini, N. (1977) *Fed. Proc.*, **36**, 1155.