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# STUDIES OF THE ENZYMATIC DEAMINATION OF ARA-CYTIDINE—V.

## INHIBITION *IN VITRO* AND *IN VIVO* BY TETRAHYDROURIDINE AND OTHER REDUCED PYRIMIDINE NUCLEOSIDES

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**Abstract**—A reduced pyrimidine nucleoside, 1-(β-D-ribofuranosyl)-4-hydroxytetrahydro-2(1H)-pyrimidinone, termed tetrahydrouridine, was found to be a potent and specific inhibitor of the enzymatic deamination of 1-β-D-arabinofuranosylcytosine (ara-cytidine, ara-C) by preparations of human liver. Kinetic studies were consistent with a model of partial (or regulatory-type) inhibition in which the inhibited enzyme was still able to form product, but at a reduced rate. Enzyme inhibition was dependent upon a preincubation reaction with tetrahydrouridine, and the substrate appeared to interact preferentially with uninhibited enzyme. The uninhibited and *fully* inhibited deamination reactions had measured $K_m$ constants of $1.6 \times 10^{-4}$ M and $> 1 \times 10^{-2}$ M respectively; the ratio of their $V_{max}$ values was $> 4$. The dissociation constant ($K_i$) of the enzyme–inhibitor complex was approximately $10^{-4}$–$10^{-5}$ M. Tetrahydrouridine also markedly inhibited deaminase preparations from mouse kidney, rhesus monkey liver and actinomycete mycelium. Several other pyrimidine nucleosides, catalytically reduced by the same process that yields tetrahydrouridine, were also potent inhibitors of the deaminase. In preliminary studies *in vivo* in two dogs and two rhesus monkeys, tetrahydrouridine markedly inhibited the deamination of ara-C at nontoxic doses.

ARA-C,*† A USEFUL anticancer and antiviral drug, is subject to a rapid enzymatic deamination in man to form a biologically inactive product, [1–4] and this finding prompted us to look for compounds which could inhibit this reaction. $H_4$-U and other reduced pyrimidine nucleosides, the subject of this paper,‡ are the third type of inhibitor to be found; the other two types of inhibitors are reported elsewhere.[5–8]

$H_4$-U was detected initially in a crude reaction mixture prepared by the catalytic reduction of cytidine in water.[9] The purification of $H_4$-U, as well as work with other reduced pyrimidine nucleosides, has been followed with an enzyme assay.[6] This paper reports on the discovery of this last type of inhibitor, on its kinetics of enzyme inhibition, on its activity toward a variety of deaminases, and on its deaminase-inhibiting activity *in vivo*. The isolation and chemical data relating to $H_4$-U are the

* Previous papers in this series have used the name cytosine arabinoside. The generic name is cytarabine.

† The abbreviations used are: ara-C and ara-cytidine for 1-β-D-arabinofuranosylcytosine; ara-U and ara-uridine for 1-β-D-arabinofuranosyluracil; $H_2$-U for 5,6-dihydrouridine; $H_4$-U for 3,4,5,6-tetrahydrouridine [or 1-(β-D-ribofuranosyl)-4-hydroxytetrahydro-2(1H)-pyrimidinone]; $H_4$-C for 3,4,5,6-tetrahydrocytidine.

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subject of a separate paper.[9,10] The biological evaluation of $H_4$-U in conjunction with ara-C in an L-1210 (mouse leukemia) system will be reported elsewhere.*

## MATERIALS AND METHODS

*Materials.* The preparation of reagents, buffers and homogenates of human liver has been described earlier.[1] Except where otherwise noted, reduction mixtures and reduced nucleosides were prepared by Dr. A. R. Hanze of The Upjohn Co., using a rhodium-on-alumina catalyst.[9] $H_2$-U was purchased from Calbiochem., Los Angeles, Calif.

*Competitive efficacy (C.E.) test.* This test has been described earlier.[6] The C.E. value of a test compound is a relative measure of how well that compound is able to compete with ara-C-$^3$H for the active site of the human liver deaminase under certain specified reaction conditions. The larger the C.E. value, the more inhibitory the compound. Unlabeled ara-C (ara-C-$^1$H) is used in place of test compound in order to construct a standard curve and to standardize test results. The molar ratios of ara-C-$^1$H to ara-C-$^3$H can be correlated *directly* with the amount of deaminase inhibition.

C.E. values are expressed in terms of ara-C-$^1$H equivalents/$\mu$mole of test compound. Thus, a C.E. value of 10 means that 1 $\mu$mole of the test compound was as inhibitory as 10 $\mu$mole of ara-C-$^1$H. By definition, ara-C-$^1$H has a C.E. value of 1·0.

*Kinetic studies.* In all experiments except the preincubation studies, the inhibitor and enzyme were preincubated in buffer prior to the addition of substrate. Preincubation mixtures were prepared in 12-ml centrifuge tubes in an ice bath; they contained $H_4$-U in the indicated concentrations, 250 $\mu$mole glycylglycine buffer at pH 8·0, 0·2 ml of a centrifuged 25% homogenate of normal human liver prepared in KR buffer and distilled water to a total volume of 0·5 ml. The tubes were preincubated for 15–20 min at 37°, chilled in an ice bath and 0·1 ml ara-C-$^3$H (5 $\mu$c) in the indicated concentrations was added per tube. The tubes were reincubated at 37° and the tube contents were assayed for radioactivity as described previously.[11] The reaction velocity for each substrate concentration was calculated from the initial, linear portion of each deamination curve; these velocities then were corrected for the lower incubation temperatures which prevailed during the first 15 sec of incubation. The measured temperatures of the reaction mixtures were 0° at 0 sec, 27° at 5 sec, 33° at 10 sec and 36° at 15 sec. It is important to note that the shortest incubation time used in these experiments was 4–8 times longer than the time of temperature equilibration.

*Animal studies.* The dog studies were performed by the Southern Research Institute, Birmingham, Ala., under contract with The Upjohn Company. The dog and monkey data were taken from personal communications with Dr. L. B. Mellett and Mr. T. L. Mulligan of the Southern Research Institute; more detailed information will be presented in a manuscript being prepared by these investigators. Toxicity studies in rats and mice were performed at The Upjohn Co.

In the dog and monkey work, ara-C-$^3$H (Schwarz BioResearch, Inc., Orangeburg, N.Y.; 50 mg/kg, *ca.* 50 $\mu$c/animal) and $H_4$-U (The Upjohn Company; 100 mg/kg) were administered i.v. Bloods and urines were sampled periodically from 0 to 48 hr. Ara-C was assayed microbiologically by the method of Pittillo and Hunt;[12] the combination of ara-C plus ara-U was assayed radiosotopically by a modification of the method of Dixon and Adamson;[13] the radioisotopic data from urine were

* J. S. Evans, in preparation.

confirmed chromatographically with Whatman No. 1 paper and a 1-butanol–water solvent system (84:16, v/v).

Toxicity tests were carried out in 18–22 g female CF-1 mice (Carworth Farms, Kalamazoo, Mich.) and in 90–110 g male (Wistar origin) Upjohn rats. $H_4$-U was administered in saline i.p. on a daily basis.

## EXPERIMENTAL AND RESULTS

*Inhibition of the deaminase by $H_4$-U.* The C.E. data (below) showed good inhibitor activity for $H_4$-U against the deaminase present in human liver, but it did not indicate whether this compound was a specific inhibitor of the deaminase or whether its activity resulted from a nonspecific interaction with proteins. The specific nature of the inhibition was demonstrated by experiments in which 1 $\mu$mole $H_4$-U was preincubated with 0·2 ml of a 25% homogenate of rat liver for 45 min at 37° prior to assay in a standard C.E. test. Under these conditions, the inhibitory activity of $H_4$-U was not diminished. (Rat liver homogenate contains no measurable deaminase activity.[1]) Pure $H_4$-U has a C.E. value of 7400.

*Inhibition of the deaminase by other reduced pyrimidine nucleosides.* The catalytic reduction of various pyrimidine nucleosides such as ara-C, deoxycytidine, uridine, deoxyuridine and thymidine has yielded reaction mixtures with C.E. values ranging from 150 to 650. The variations in inhibitor activity of these reaction mixtures are primarily the result of differences in the amount of hydrogenation and the amount of hydrolysis (see Hanze[9]); they do not reflect structure–activity differences between the different reduced nucleosides. The important conclusion to be drawn from these C.E. data is that all of the tested nucleosides, when reduced, became potent deaminase inhibitors. The best inhibitor previously described had a C.E. value of only 24.[6]

Dihydronucleosides were not nearly as good inhibitors as $H_4$-U. and it is not known how much of their activities are due to contamination by the tetrahyronucleosides. $H_2$-U had a C.E. value of 3 as compared to a C.E. value of 7400 for $H_4$-U. Dihydrothymidine, crystallized from a crude reduction mixture, had a C.E. value of 28, as compared to the crude mother-liquor with a C.E. value of 450.

*Preincubation requirement.* Enzyme inhibition was dependent upon a preincubation reaction with $H_4$-U. The reaction began immediately and was complete in approximately 4 min (Table 1). Preincubation of $H_4$-U alone or in combination with the substrate was without effect.

*Inhibition kinetics.** Double reciprocal plots of $1/v$ v. $1/S_t$ (Lineweaver and Burk[14]) gave a family of nonlinear curves which appear to have different intercepts at $1/v$ and different initial slopes (Fig. 1A). Fig. 1B, which is an enlargement of the high-substrate portion of Fig. 1A, confirms this speculation and shows that the intercepts and slopes were directly related to the concentrations of the inhibitor. The correlation between

* The following nomenclature was used in describing the kinetics of inhibition: $E_t$ = *total* molar concentration of the active centers of the deaminase; $E$ = molar concentration of the *free* active centers of the deaminase; $S_t$ = *total* molar concentration of ara-C substrate; $S$ = molar concentration of *free* ara-C substrate; $I_t$ = *total* molar concentration of $H_4$-U inhibitor; $I$ = *total* molar concentration of *free* $H_4$-U inhibitor; $ES$ = molar concentration of enzyme–substrate complex; $EI$ = molar concentration of enzyme–inhibitor complex; $EIS$ = molar concentration of the enzyme–inhibitor–substrate complex; $v_o$, $v_{part}$, $v_i$ = uninhibited, partially inhibited, and fully-inhibited reaction velocities respectively, as measured in moles of ara-U product per min; $V_o$, $V_{part}$, $V_i$ = maximum velocities of $v_o$, $v_{part}$ and $v_i$ respectively; $i/(I - i)$ (nomenclature of Reiner[15]) can be calculated as $(v_o - v_{part})/v_{part}$; $K_m$ and $K_{mi} = (k_{-1} + k_2)/k_1$ and $(k_{-5} + k_6)/k_5$ respectively.

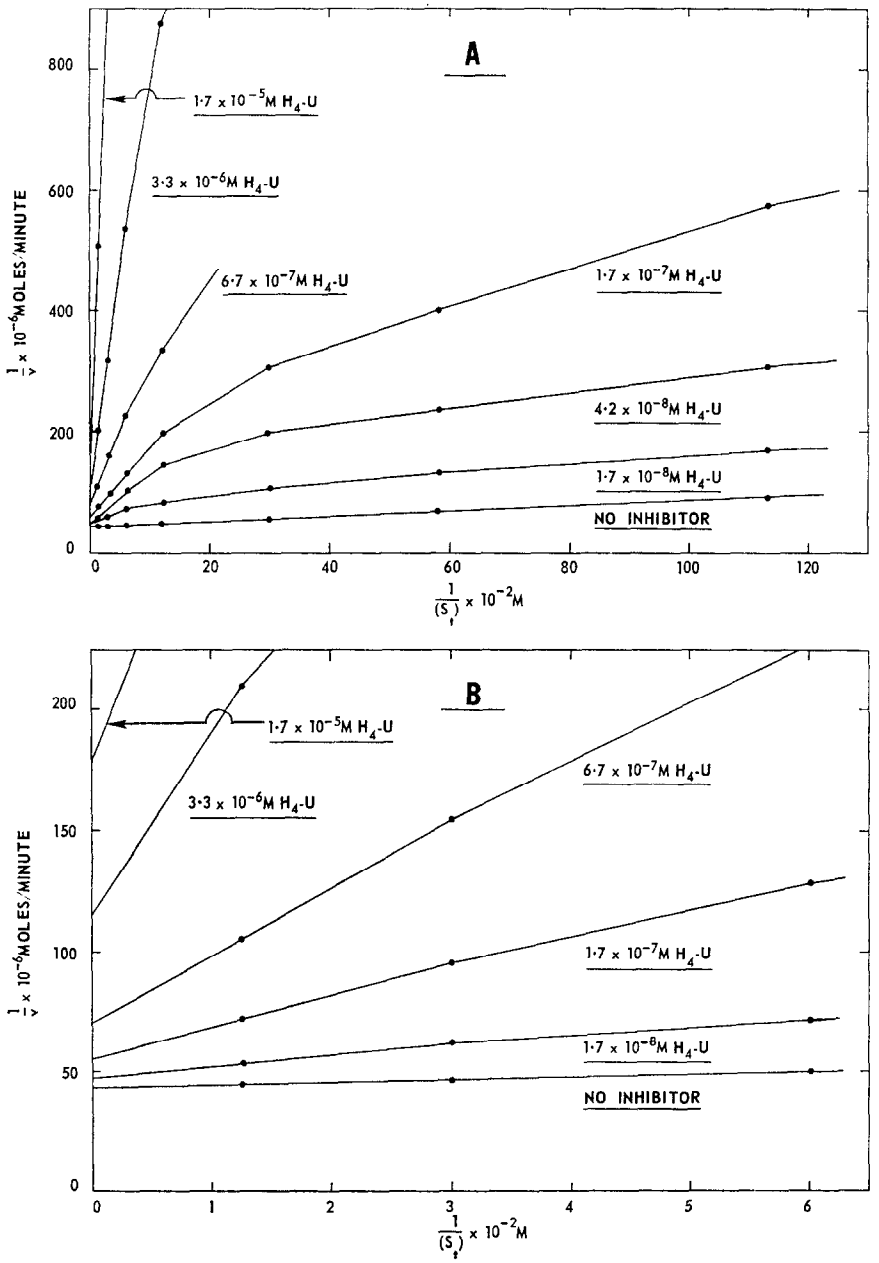



FIG. 1. Inhibition of the enzymatic deamination of ara-C by $H_4$-U. Ara-C and $H_4$-U were present in the indicated concentrations. $H_4$-U and the enzyme were preincubated for 15–20 min at 37° prior to the addition of ara-C. Experimental procedures are described in Methods. Each point in the figure is the average of 2 or more replicates. (A) Lineweaver–Burk data for all levels of ara-C and $H_4$-U tested; (B) an enlarged portion of Fig. 1A showing the initial slopes and intercepts at $1/v$.

TABLE 1. EFFECT OF PREINCUBATION TIME ON THE REACTION BETWEEN $H_4$-U AND THE DEAMINASE*

| Preincubation (37°) | | Incubation (37°) | | Inhibition of ara-cytidine deamination† (%) |
|---|---|---|---|---|
| Components | Min | Addition | Min | |
| Enzyme + buffer | 0·2 | + Ara-cytidine | 1·0 | 0 |
| | 6·0 | | 1·0 | 0 |
| $H_4$-U + enzyme + buffer | 0·2 | + Ara-cytidine | 1·0 | 32 |
| | 1·2 | | 1·0 | 82 |
| | 2·0 | | 1·0 | 95 |
| | 4·0 | | 1·0 | 103 |
| | 6·0 | | 1·0 | 100 |

* Assay procedures are described in Methods. Ara-cytidine and $H_4$-U were used at concentrations of $8{\cdot}8 \times 10^{-5}$ M and $1{\cdot}7 \times 10^{-7}$ M respectively.

† Per cent inhibitions are expressed in terms of inhibition attainable under partial inhibition kinetics. In this instance, 0% inhibition corresponded to 8·7 mμmole ara-uridine product; 100% inhibition, the maximum attainable, corresponded to 1·4 mμmole product.

the increasing slopes and intercepts with the increasing inhibitor concentrations was statistically significant at the 0·001 level. These data are consistent with a mechanism of either partial or complete noncompetitive* inhibition.

The complete non-competitive mechanism was eliminated from consideration by a diagnostic plot of $I_t$ v. $i/(1-i)$ shown in Fig. 2. Here, curves were obtained with increasing slopes, whereas curves with decreasing slopes would have been obtained had the inhibition been complete noncompetitive.[15] In addition, the diagnostic plots shown in Fig. 3A and 3B confirmed the partial nature of this inhibition. Partial inhibition, in these plots, is characterized by "Lineweaver–Burk intercepts and slopes", which increase nonlinearly with increasing concentrations of inhibitor until the enzyme becomes fully inhibited, and then the slope and intercept curves each approach a maximum value;[15] this is the pattern seen in both figures. The dashed lines drawn in all of the diagnostic plots show how a complete noncompetitive inhibitor would have appeared.

Partial inhibition can be described by the following stoichiometric scheme. It can be seen that the inhibited enzyme is still able to form product, but at a reduced rate. (For a detailed kinetic and mathematical analysis of this type of inhibition, see Reiner.[15])

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} E + \text{products} \tag{1}$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} EI \tag{2}$$

$$ES + I \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} EIS \tag{3}$$

$$EI + S \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} EIS \overset{k_6}{\rightarrow} EI + \text{products} \tag{4}$$

The overall measured velocity of product formation at any specified concentration of substrate and inhibitor is the sum of the respective velocities of the uninhibited and

* This type of inhibition is more accurately described as complete inclusive inhibition of the enzyme.[15]

the fully inhibited enzyme reactions. The equation for this summation of velocities is shown in eq. 7, below.* The dissociation constant ($K_i$ or $K_3$) of the enzyme–inhibitor complex was estimated from the following approximate equation:

$$K_i = \frac{(E)(I)}{(EI)} \cong \frac{K_m(I)}{K_{mi}} \left[\frac{V_{\text{part}} - V_i}{V_o - V_{\text{part}}}\right]$$

The derivation of this equation is presented below.*

The uninhibited and fully inhibited deamination reactions had measured $K_m$ constants of $1{\cdot}6 \times 10^{-4}$ M and $> 1 \times 10^{-2}$ M respectively; the ratio of the $V_{\max}$ values was $> 4$. The dissociation constant ($K_i$) of the enzyme–inhibitor complex was estimated to be approximately $10^{-4}$ to $10^{-5}$ M.

Returning to Fig. 1A, the nonlinear character of the Lineweaver–Burk plots at moderate and low substrate concentrations is consistent (i) with the proposed experimental model (see derivation of Botts and Morales[16]), and (ii) with the different affinities of the uninhibited and fully inhibited forms of the enzyme for the substrate (see Discussion). The possibility that these non-linear Lineweaver–Burk plots resulted

* Derivation of $K_i$:

I. At high $S$, $E$ and $EI$ are small, and

$$E_t \cong ES + EIS \tag{5}$$

This is equivalent to

$$\frac{ES}{E_t} + \frac{EIS}{E_t} \cong 1 \tag{6}$$

II. The total measured velocity of the partially inhibited enzyme reaction is equal to the sum of the respective velocities of the uninhibited (eq. 1) and fully-inhibited (eq. 4) enzyme reactions:

$$\begin{aligned} v_{\text{part}} &= v_o + v_i \\ &= k_2(ES) + k_6(EIS) \\ &= \left(\frac{ES}{E_t}\right)\left(k_2 E_t\right) + \left(\frac{EIS}{E_t}\right)\left(k_6 E_t\right) \\ &= \left(\frac{ES}{E_t}\right) V_o + \left(\frac{EIS}{E_t}\right) V_i \end{aligned} \tag{7, 8}$$

At high $S$, $v_{\text{part}} = V_{\text{part}}$ and $EIS \cong (E_t - ES)$ (eq. 5).
Substitution of these values into eq. 8 yields the following:

$$V_{\text{part}} = \left(\frac{ES}{E_t}\right) V_o + \left(\frac{E_t - ES}{E_t}\right) V_i$$

which can be rewritten in two ways dependent upon eq. 6:

$$\left(\frac{ES}{E_t}\right) = \frac{V_{\text{part}} - V_i}{V_o - V_i} \tag{9}$$

$$\left(\frac{EIS}{E_t}\right) = \frac{V_o - V_{\text{part}}}{V_o - V_i} \tag{10}$$

III. At high $S$, the stoichiometric scheme presented in the text shows that:

$$E \sim \left(\frac{K_m}{S}\right)\left(\frac{ES}{E_t}\right) \text{ and } EI \sim \left(\frac{K_{mi}}{S}\right)\left(\frac{EIS}{E_t}\right)$$

The ratio of these two proportionalities is:

$$\frac{E}{EI} = \frac{K_m(ES/E_t)}{K_{mi}(EIS/E_t)} \tag{11}$$

Finally, the approximate $K_i$ is obtained by substituting eq. 9 and eq. 10 into eq. 11, and then by multiplying both sides of the equation by I:

$$K_i = \frac{(E)(I)}{(EI)} \cong \frac{K_m(I)}{K_{mi}} \left[\frac{V_{\text{part}} - V_i}{V_o - V_{\text{part}}}\right]$$

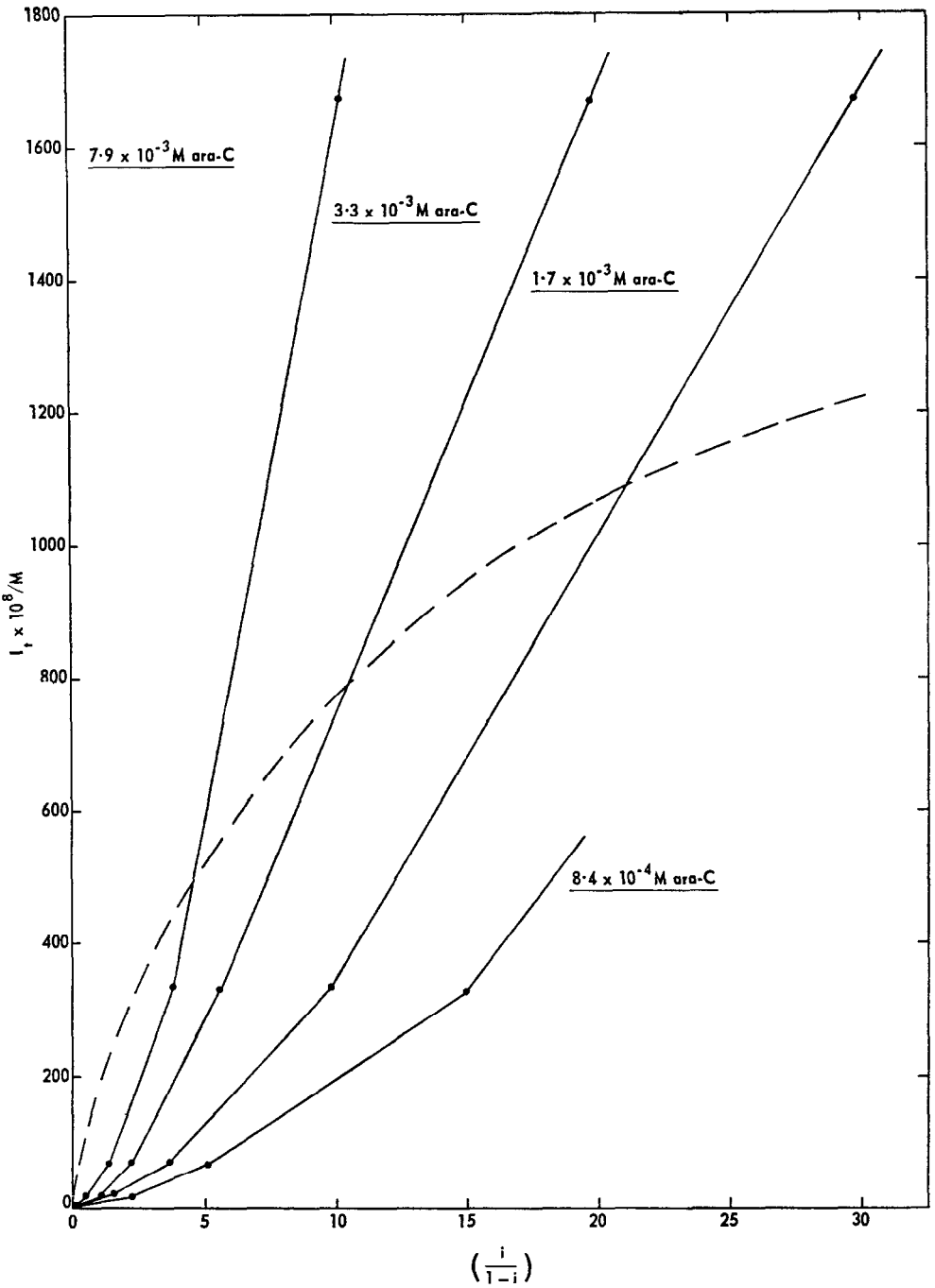



FIG. 2. A diagnostic plot of inhibitor concentration as a function of the reaction velocities of the ara-C deamination. Ara-C and $H_4$-U were present in the indicated concentrations. $H_4$-U and the enzyme were preincubated for 15–20 min at 37° prior to the addition of ara-C. See the text for experimental procedures. Each point in the figure is the average of at least 2 replicates. The equations which led to this plot are described by Reiner.[15]

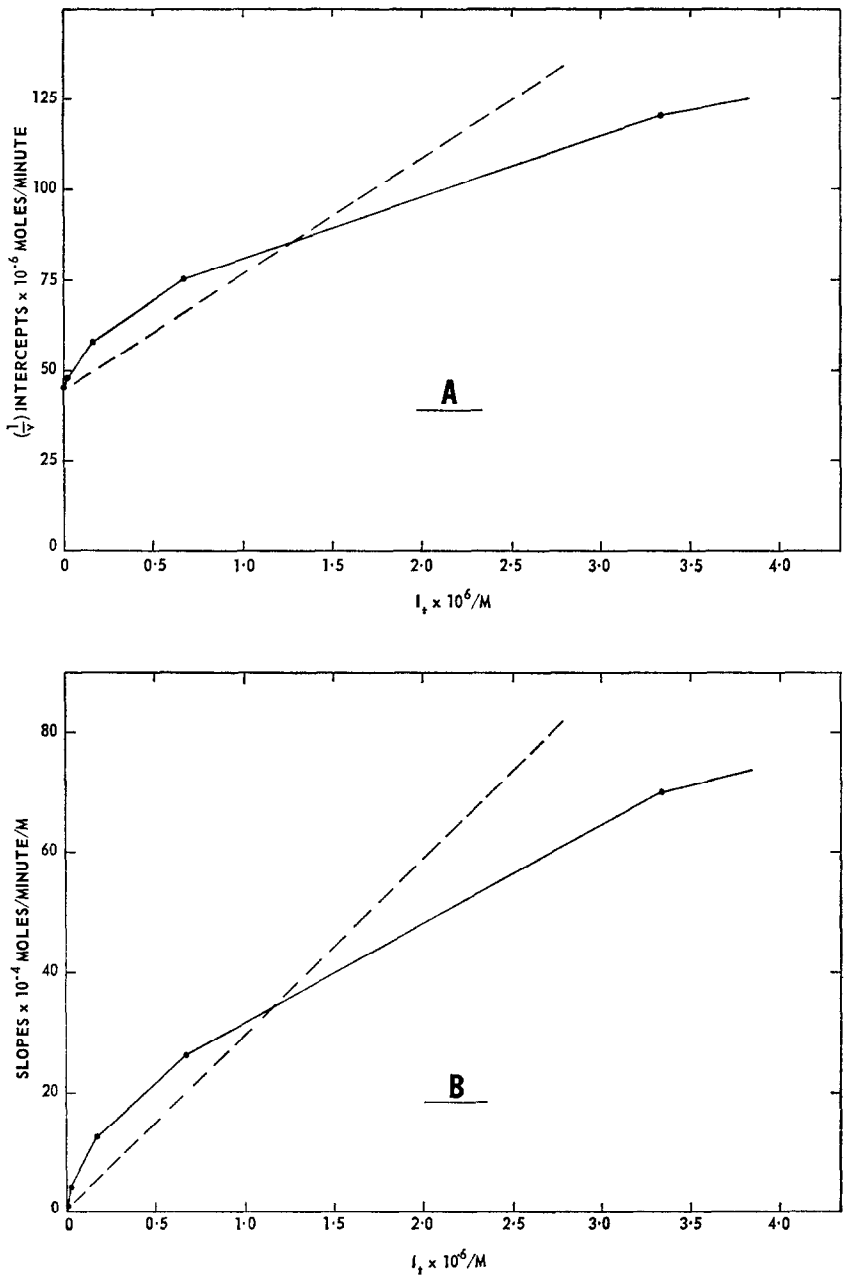



FIG. 3. Diagnostic plots of the intercept and slope data obtained from Fig. 1B. See Fig. 1 and the text for experimental details. (A) A plot of the intercepts at $1/V$ as a function of inhibitor concentration; (B) a plot of the slopes of the curves at $1/V$ as a function of inhibitor concentration.

from the formation of a substrate–inhibitor complex was eliminated from consideration by one of the diagnostic plots presented earlier (Fig. 2); inhibition by a substrate–inhibitor complex would have been characterized in this type of plot by linear origin–intercept curves.[15]

No interaction between catalytic and/or regulatory sites on the enzyme could be detected by using Hill-style plots[17] based on the equations of Scarano *et al.*[18] If multiple sites exist, they do not appear to interact with one another to any measurable extent.

*$H_4$-U inhibition of other deaminases.* Deaminase preparations from mouse kidney, rhesus monkey liver and actinomycete mycelium (*Streptomyces lincolnensis*) were prepared and tested in the same manner as that described for human liver. At an ara-C concentration of $1{\cdot}7 \times 10^{-3}$ M and an $H_4$-U concentration of $10^{-5}$ to $10^{-6}$ M, the rate of product formation was inhibited $> 95$ per cent with all of the enzyme preparations. This result is comparable to that found for the deaminase present in human liver.

*$H_4$-U activity* in vivo. Preliminary studies with two Beagle dogs and two rhesus monkeys (performed, as indicated earlier, at the Southern Research Institute) have shown $H_4$-U to be an extremely effective inhibitor of the deaminase *in vivo*. When the dogs were pretreated with $H_4$-U 30 min prior to the administration of ara-C, higher and more persistent serum levels of ara-C were obtained; the measured half-lives of ara-C in the sera of the two dogs were increased 2·4 and 1·7 times respectively. The urinary excretion of ara-C and ara-U also were altered markedly for at least 48 hr.

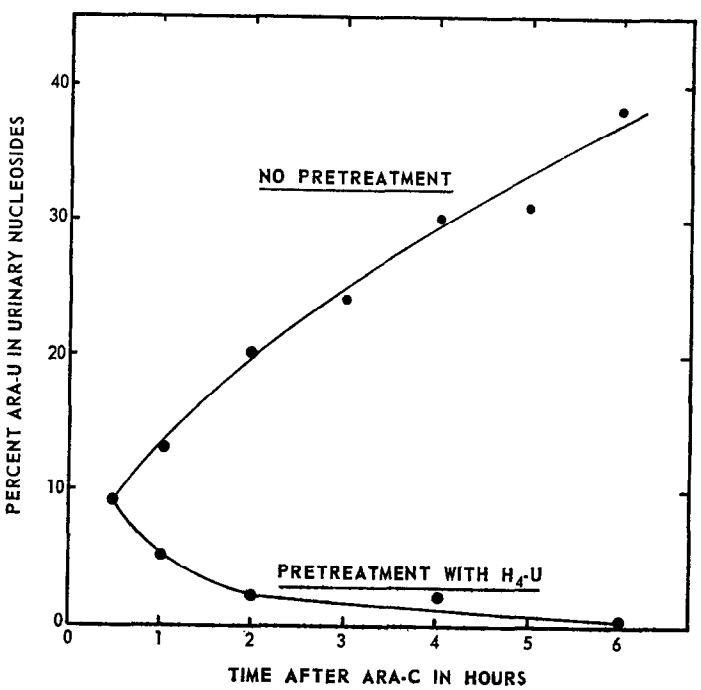



FIG. 4. The urinary excretion of ara-U in a Beagle dog with and without pretreatment with $H_4$-U. The data for a second Beagle dog were almost identical. Each animal served as its own control. See the text for experimental details. (The data were contained in a personal communication from Dr. L. B. Mellett and Mr. T. L. Mulligan, who plan to publish these and related results in detail elsewhere.)

The data for the first 6 hr are shown in Fig. 4; the excretion of ara-U became negligible while the excretion of ara-C increased.

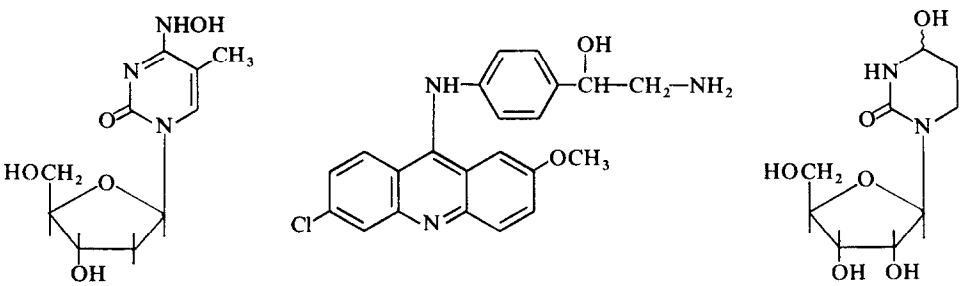
A similar pattern of inhibition also was seen in the rhesus monkeys after pretreatment with $H_4$-U. The inhibitor effect was most marked through the first 4 hr but persisted through a 24-hr period, during which time about 45 per cent of the isotope recovered from the urine appeared as ara-C, while about 39 per cent appeared as ara-U. This is in contrast to the urinary excretion pattern seen in untreated rhesus monkeys where essentially no ara-C was found even at early sampling times.

$H_4$-U was neither lethal nor noticeably toxic to rats or mice at a dose of 10 mg/kg given daily for 7 consecutive days. Similarly, no acute toxic manifestations were observed in the dogs or monkeys at the single dose level of 100 mg/kg. Higher doses will be tested as adequate amounts of $H_4$-U become available.

## DISCUSSION

To date, three types of deaminase inhibitors have been found. All three types of inhibitors are conformationally similar to the ara-C substrate, all three types of inhibitors are specific for the same enzyme, and yet, each of the three types of inhibitors appears to act by a different mechanism of inhibition. Certainly, this is a most unique biochemical situation.

The hydroxyamino nucleoside inhibitor did not form a detectable intermediate during the deaminase reaction, and it appeared to compete directly with ara-C for the active site of the enzyme.[5,6] The acridine inhibitor formed a moderately stable complex with ara-C ($K_i \cong 10^{-4}$ M), and it was this complex which competed with free substrate for the active site of the enzyme.[7,8] $H_4$-U inhibition required a preincubation reaction with the enzyme, and this resulted in a noncompetitive "regulation" of the rate of enzyme action. The structures of the three inhibitors are shown below.




The $K_m$ and $V_{max}$ data taken together indicated that the uninhibited and $H_4$-U-inhibited enzyme molecules had very different affinities for the substrate. The respective $K_m$ values (given by the expressions $(k_{-1} + k_2)/k_1$ and $(k_{-5} + k_6)/k_5$) are a measure of two variables: (i) the affinities of the different enzyme forms for the substrate ($E + S \rightleftarrows ES$ and $EI + S \rightleftarrows EIS$), and (ii) the rates of product formation by these different enzyme–substrate complexes. The $V_{max}$ values, on the other hand, are just a measure of the second variable, the rates of product formation. Since the $V_{max}$ values in these experiments were not much different for the uninhibited and fully inhibited enzyme, then, by difference, the disparate $K_m$ values must be indicative of very different affinities of the two enzyme forms for the substrate.

The experimental model which is proposed to explain the observed interaction of the enzyme with $H_4$-U and ara-C is a complex case of partial inhibition: (i) The inhibitor and enzyme interact to give a mixture of uninhibited and inhibited enzyme molecules; (ii) the inhibited enzyme molecules form product, but at a reduced rate; (iii) the enzyme–substrate complex also can interact with inhibitor to form an inhibited enzyme–substrate complex. As such, the model predicts the nonlinear Lineweaver–Burk plots, which were seen at low and moderate concentrations of substrate and inhibitor. The model also predicts that *linear* Lineweaver–Burk plots will be obtained: (i) at either very high or zero concentrations of inhibitor, when only one type of enzyme molecule is present; and (ii) at very high substrate concentrations, when both types of enzyme molecules are fully saturated with substrate. Experimentally, linear plots were observed under the predicted conditions with the single exception that it was not technically possible to check for linearity at high concentrations of inhibitor.

The test data, showing that $H_4$-U was active in the rhesus monkeys, were especially significant. This species not only seems to have deaminase activity present in every body tissue, but it has among the highest deaminase levels of any animal tested. Thus, the rhesus monkey would seem to constitute the ultimate test *in vivo* for deaminase inhibitors destined for human use.

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## REFERENCES

1. G. W. Camiener and C. G. Smith, *Biochem. Pharmac.* **14**, 1405 (1965).
2. R. V. Loo, M. J. Brennan and R. W. Talley, *Proc. Am. Ass. Cancer Res.* **6**, 41 (1965).
3. R. J. Papac, W. A. Creasey, P. Calabresi and A. D. Welch, *Proc. Am. Ass. Cancer Res.* **6**, 50 (1965).
4. W. A. Creasey, R. J. Papac, M. E. Markiw, P. Calabresi and A. D. Welch, *Biochem. Pharmac.* **15**, 1417 (1966).
5. G. W. Camiener, *Proc. Am. Ass. Cancer Res.* **8**, 9 (1967).
6. G. W. Camiener, *Biochem. Pharmac.* **16**, 1691 (1967).
7. G. W. Camiener and R. V. Tao, *Biochem. Pharmac.* **17**, 1411 (1968).
8. R. V. Tao and G. W. Camiener, *Abstr. Pap. Am. chem. Soc.*, 154th natn. Meetings (1967).
9. A. R. Hanze, *J. Am. chem. Soc.*, **89**, 6720 (1967).
10. A. R. Hanze, *Abstr. Pap. Am. chem. Soc.*, 154th natn. Meetings (1967).
11. G. W. Camiener, *Biochem. Pharmac.* **16**, 1681 (1967).
12. R. F. Pittillo and D. E. Hunt, *Proc. Soc. exp. Biol. Med.* **124**, 636 (1967).
13. R. L. Dixon and R. H. Adamson, *Cancer Chemother. Rep.* **48**, 11 (1965).
14. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
15. J. M. Reiner, *Behaviour of Enzyme Systems.* Burgess, Minneapolis, Minn. (1959).
16. J. Botts and M. Morales, *Trans. Faraday Soc.* **49**, 696 (1953).
17. A. V. Hill, *J. Physiol., Lond.* **40**, iv (1910).
18. E. Scarano, G. Geraci and M. Rossi, *Biochemistry, N.Y.* **6**, 192 (1967).