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STUDIES OF THE ENZYMATIC DEAMINATION OF ARA-CYTIDINE—IV.

INHIBITION BY AN ACRIDINE ANALOGUE AND ORGANIC SOLVENTS

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Abstract—A substituted acridine, 9-[*p*-(2-amino-1-hydroxyethyl)anilino]-6-chloro-2-methoxyacridine, termed acridine 1, was found to be an effective and specific inhibitor of the enzymatic deamination of 1-β-D-arabinofuranosylcytosine (ara-cytidine, ara-C) by preparations of human liver. Kinetic studies were indicative of and consistent with a model of competitive inhibition in which ara-C and acridine 1 formed a substrate–inhibitor complex which, in turn, was the actual inhibitor of the deaminase. Supporting evidence for this mechanism came from two sources: (1) comparison of stereomodels of ara-C and acridine 1 showed major areas of overlap between the two molecules; (2) structure–activity studies showed that analogues of acridine 1 whose structures had little or no overlap with the ara-C molecule were inactive as inhibitors. The measured K_m value for the uninhibited deamination reaction was $1{\cdot}3 \times 10^{-4}$ M. The dissociation constants for the substrate–inhibitor and the enzyme-substrate-inhibitor complexes were $1{\cdot}2 \times 10^{-4}$ M and $1{\cdot}0 \times 10^{-5}$ M respectively. Organic solvents were inhibitory to the deaminase at concentrations as low as 2 per cent (v/v). A survey of 14 solvents suggested that the inhibition was related, in part at least, to the structures of the solvents.

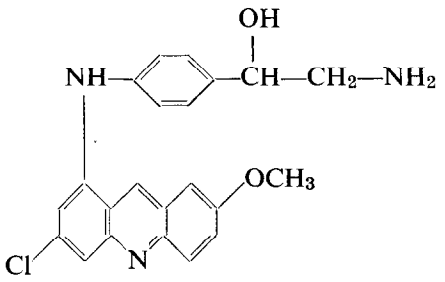
THE RAPID enzymatic deamination of ara-C* in man[1–4] (R. W. Talley and C. G. Smith, unpublished data, 1963) prompted us to look for compounds which could specifically inhibit this deamination reaction. The first compound found, N^4-hydroxy-5-methyl-2′-deoxycytidine, was shown to be a competitive inhibitor of the enzyme.[5, 6] Follow-up studies by Dollinger *et al.*[7, 8] and by Burchenal *et al.*[9] have confirmed the inhibitory activity of this compound and have extended this observation to include other newly synthesized N^4-hydroxypyrimidine nucleosides. This paper† reports the discovery of a second type of inhibitor, a substituted acridine compound termed acridine 1, which was found to inhibit the enzyme by forming an inhibitory complex with the substrate. Also reported here are some pertinent test data for some organic solvents, which were found to be inhibitory to the deaminase during the structure–activity studies.

MATERIALS AND METHODS

Materials. The preparation of reagents, buffers and homogenates of human liver has been described earlier.[1] Acridines, quinolines and analogues of the R^1 portion of

* Ara-C (or ara-cytidine) is an abbreviation for 1-β-D-arabinofuranosylcytosine. Previous papers in this series have used the name cytosine arabinoside. The generic name is cytarabine.

† Presented in part at the Medicinal Chemistry Section of the Fall Meeting of the American Chemical Society, Chicago, Ill., 10–15 September, 1967.



Acridine 1

9-[*p*-(2-amino-1-hydroxyethyl)anilino]-6-chloro-2-methoxyacridine

acridine 1 were prepared or procured as indicated in Table 1. Aqueous solutions of these compounds were prepared in 10^{-3} to 10^{-4} M HCl. Solvents (see Table 2) were obtained commercially, and aqueous solutions of these materials were adjusted to pH 7·5 to 8·5 prior to use.

Competitive efficacy (C.E.) test. This test also has been described earlier.[6] The C.E. value of a test compound is a 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.

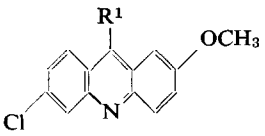
C.E. values are expressed in terms of ara-C-^{1}H-equivalents/μmole of test compound. Thus, a C.E. value of 10 means that 1 μmole of the test compound was as inhibitory as 10 μmole ara-C-^{1}H. By definition, ara-C-^{1}H has a C.E. value of 1·0.

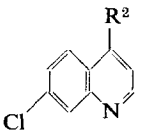
Kinetic studies. Incubation mixtures were prepared in 12-ml centrifuge tubes in an ice bath; they contained ara-C-^{3}H (5 μc/tube) in indicated concentrations, acridine 1 in indicated concentrations, 250 μmole glycylglycine buffer at pH 8·0, 0·2 ml of a centrifuged 25% homogenate of normal human liver prepared in Krebs–Ringer buffer, and distilled water to a total vol. of 0·6 ml. The tubes were incubated 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 8 times longer than the time of temperature equilibration.

EXPERIMENTAL AND RESULTS

Inhibition of the deaminase by acridine 1. The initial screening data showed that acridine 1 had good inhibitor activity in the C.E. test, but the data 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 μmole of acridine 1 was

TABLE 1. CHEMICAL STRUCTURES, SOURCES AND DEAMINASE-INHIBITING ACTIVITIES OF SOME ACRIDINES, QUINOLINES AND ANALOGUES OF THE R^1 OF ACRIDINE 1





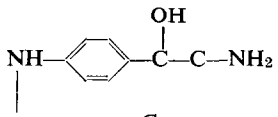
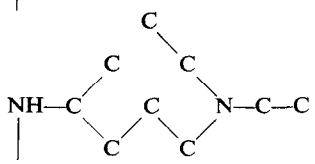
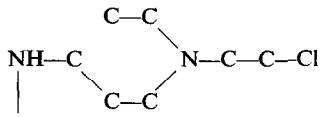
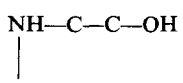
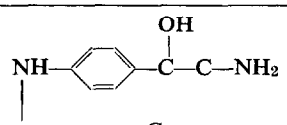
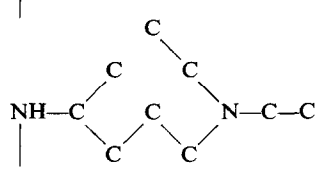
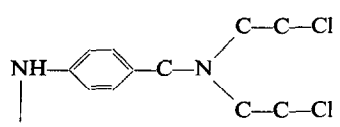
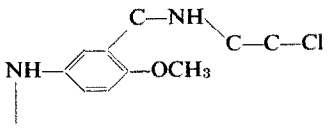
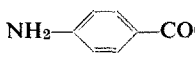
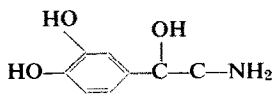
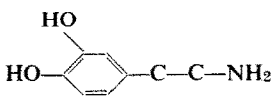
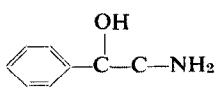
Compound	R^1	C.E.*	Source
1.		13·6	J. H. Burckhalter†
2.		0·6	Winthrop Laboratories
3.		0·7	Inst. for Cancer Res.‡
4.		0·6	The Upjohn Company§
5.		< 0·5‖	The Upjohn Company§
6.		< 0·5‖	Aldrich Chem. Co.
7.		< 0·5‖	Aldrich Chem. Co.
	R^2		
8.		2·1	J. H. Burckhalter†
9.		< 0·2	Winthrop Laboratories
10.		< 0·2	J. H. Burckhalter†

TABLE 1.—*continued.*

Compound	R^2	C.E.*	Source
11.		< 0·2	J. H. Burckhalter†
	Analogues of the R^1 of acridine 1		
12.		< 0·1	Calbiochem.
13.		< 0·1	Aldrich Chem. Co.
14.		< 0·1	Merck & Co.
15.		< 0·1	Aldrich Chem. Co.

* C.E. = competitive efficiency value; see Methods for details.

† These compounds were obtained through the kindness of Dr. J. H. Burckhalter at the University of Michigan, College of Pharmacy, Ann Arbor, Mich. Compounds 1 and 8 were synthesized by the method of Nobles *et al.*[10]

‡ This compound was obtained (by Dr. M. K. Bach at The Upjohn Company) through the kindness of Dr. H. J. Creech at the Institute for Cancer Research, Philadelphia, Pa.

§ Compounds 4 and 5 were synthesized by the Upjohn Chemistry Department according to the procedures described in *Chem. Abstr.* **26**, 4684, ref. 2 (1932); **37**, 378, ref. 6 (1943), respectively.

‖ More precise determinations were prevented by the relative aqueous insolubility of these compounds.

preincubated with 0·2 ml of a 25% homogenate of rat liver for 20–30 min at 37° prior to assay in a standard C.E. test. Under these conditions, the inhibitory activity of acridine 1 was not diminished. (Rat liver homogenate contains no measurable deaminase activity.[1]) The measured C.E. value for acridine 1 is 13·6.

Stereomodel comparisons of molecular structures. Dreiding stereomodels* of ara-C and acridine 1 showed major areas of congruency and coincidence between the two molecules. A stylized representation of these structures is shown in Fig. 1. As can be seen: the 4-aminopyrimidine group resembles and coincides with the anilino group; the first two oxygen and carbon atoms of the pentofuranose are coincident with the hydroxyl, amino and ethyl groups, respectively; and the 5′-hydroxymethyl portion of ara-C lines up precisely with the 2-methoxy function of acridine 1. Thus, it would appear that the structure of acridine 1 is quite similar to that of ara-C.

* Swissco, Greenville, Ill.

Structure-activity correlates for acridine 1. The finding that acridine 1 structurally resembled ara-C prompted us to test several structurally related acridines, quinolines and analogues of the R^1 portion of acridine 1. Unfortunately, only a limited study was feasible, since many important compounds were readily soluble only in organic solvents, and (as noted below) organic solvents cannot be used with the deaminase.

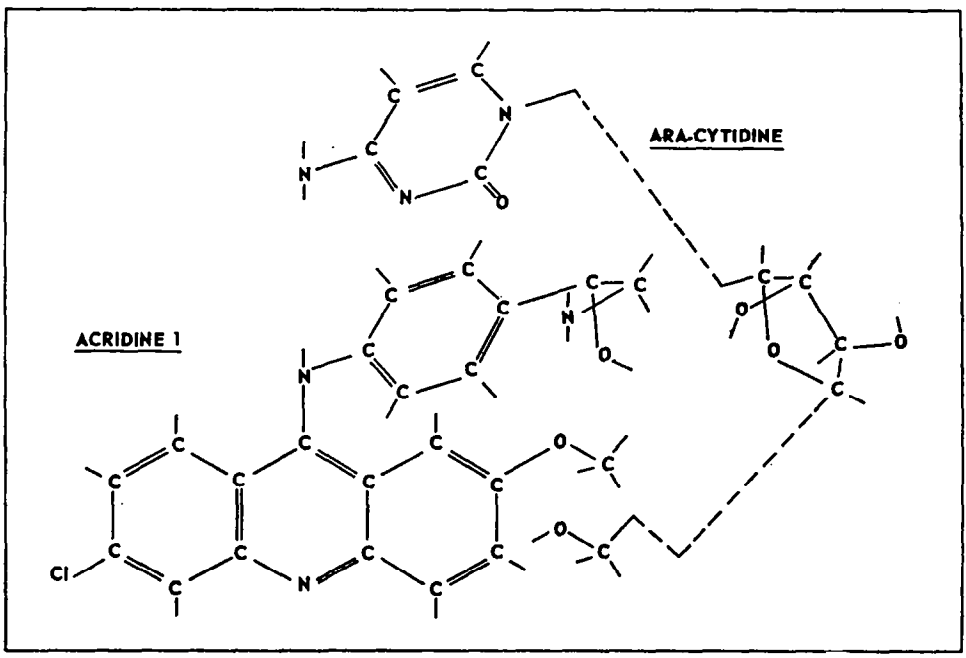


FIG. 1. A stylized representation of the structures of ara-C and acridine 1. Polaroid photographs were taken of Dreiding stereomodels of the two compounds and the above figure was traced from the photographs. Attempts were made to retain the three-dimensional character of the models. See the text for discussion of the structural congruencies and coincidences between the two models.

Nonetheless, the data which were obtained (Table 1) do support the conclusion that almost the entire acridine 1 structure is required for effective inhibition of the deaminase: (1) Modification of the benzyl-alcohol substituent of acridine 1, as in acridines 2–4, markedly reduced deaminase inhibition; replacement or elimination of the benzyl-alcohol substituent, as in acridines 5–7, completely abolished inhibitor activity. A similar pattern is seen with quinolines 8–11. (2) Elimination of the third ring and the 2-methoxy group from acridine 1 (to form quinoline 8) reduced the inhibition of the deaminase better than 6-fold. A similar pattern of activity loss is observed when acridines 2 and 3 are compared with quinolines 9 and 10. (3) Analogues of the R^1 portion of acridine 1 (compounds 12–15) were inactive, confirming again the need for an acridine or quinoline ring structure. Other analogues of R^1 were not available for testing.

Stereomodels of compounds 2–15 showed much less overlap with the stereomodel of ara-C as compared with the stereomodel of acridine 1.

Inhibition by solvents. As noted earlier, organic solvents cannot be used with the deaminase. When these are present in reaction mixtures, the enzyme is inhibited

markedly. Table 2 shows the solvents tested, their inhibitory activities and some of their dielectric constants. The data in the last column of the table indicate that the inhibitory activities of the solvents cannot be ascribed solely to differences in dielectric constants. A discussion of structure–activity relationships is presented later.

TABLE 2. INHIBITORY ACTIVITIES AND DIELECTRIC CONSTANTS OF SOLVENTS TESTED FOR EFFECT ON THE ENZYMATIC DEAMINATION OF ARA-CYTIDINE

Compound	Solvent	Inhibition of Deaminase (%)*	Dielectric constant ($\Sigma_{25}°$)†	Ratio of % inhibition to $\Sigma_{25}°$‡
16.	Water	0	78·4	0
17.	Methanol	20·9	32·6	0·64
18.	*N,N*-Dimethylformamide	57·1		
19.	Ethanol	49·9	24·3	2·0
20.	2-Aminoethanol	19·3		
21.	1,2-Ethanediol	31·0	37·0	0·84
22.	2-Methoxyethanol	50·2		
23.	2-Ethoxyethanol	49·3		
24.	1,2-Dimethoxyethane	47·7		
25.	1-Propanol	78·8	20·1	3·9
26.	2-Propanol	60·2	18·3	3·3
27.	1,2-Propanediol	56·7	31·0	1·8
28.	1,3-Propanediol	55·0	34·0	1·6
29.	1,2,3-Propanetriol	21·5	42·0	0·51
30.	Pyridine	< 95§	12·3	> 7·7

* Assay procedures were those described for the C.E. test, except that the solvents were tested in 0·69 m-mole amounts in a total volume of 0·5 ml. At this concentration, ethanol (the standard for comparison) inhibited the deamination approximately 50 per cent. S.D's of these measured inhibitions averaged ± 1·5.

† Dielectric constants were obtained from the *Handbook of Chemistry and Physics*.[12]

‡ The force between two charges is inversely proportional to the dielectric constant of the medium; hence, the inverse of $\Sigma_{25}°$ is used here.

§ Pyridine inhibited the deaminase 68 and 33 per cent at the 0·23 and 0·07 m-mole levels, respectively.

The inhibition of the deaminase by solvents was dose-related. Fig. 2 shows the percentage inhibition obtained when ethanol concentrations present in the enzyme reaction mixtures were varied between 0 and 20% (v/v). Significant inhibition was measured at ethanol concentrations as low as 2 per cent, and a 50 per cent inhibition was measured at a concentration of about 8 per cent.

*Inhibition kinetics.** Double reciprocal plots of $1/v$ v. $1/S_t$ (Lineweaver and Burk[13]) gave a family of nonlinear curves corresponding to the different concentrations of inhibitor (Fig. 3A). Fig. 3B, which is an enlargement of the high substrate portion of Fig. 3A, confirms the nonlinearity of the inhibitor curves by showing that all of these curves approach a common intercept at $1/V_{max}$. Two additional diagnostic plots, $(1/v_i - 1/v_0)$ v. I_t and $i/(1 - i)$ v. I_t (Fig. 4, A and B, respectively), gave families of

* 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 acridine 1 inhibitor; I = molar concentration of free acridine 1 inhibitor; ES = molar concentration of enzyme–substrate complex. SI = molar concentration of substrate–inhibitor complex; ESI = molar concentration of the inhibited enzyme–substrate complex; v_i, v_0 = inhibited and uninhibited reaction velocities, respectively, as measured in moles of ara-uridine product/min; $i/(1 - i)$ = nomenclature of Reiner,[14] and can be calculated as $(v_0 - v_i)/v_i$.

straight-line curves, which had common intercepts at their respective origins and slopes inversely related to S_t. Miscellaneous studies showed that the rates of product formation were not influenced by the preincubation of enzyme, substrate, inhibitor or their combinations, nor were they affected by the length of the incubation time. Thus, it was concluded that enzyme, substrate and inhibitor were not inactivated under

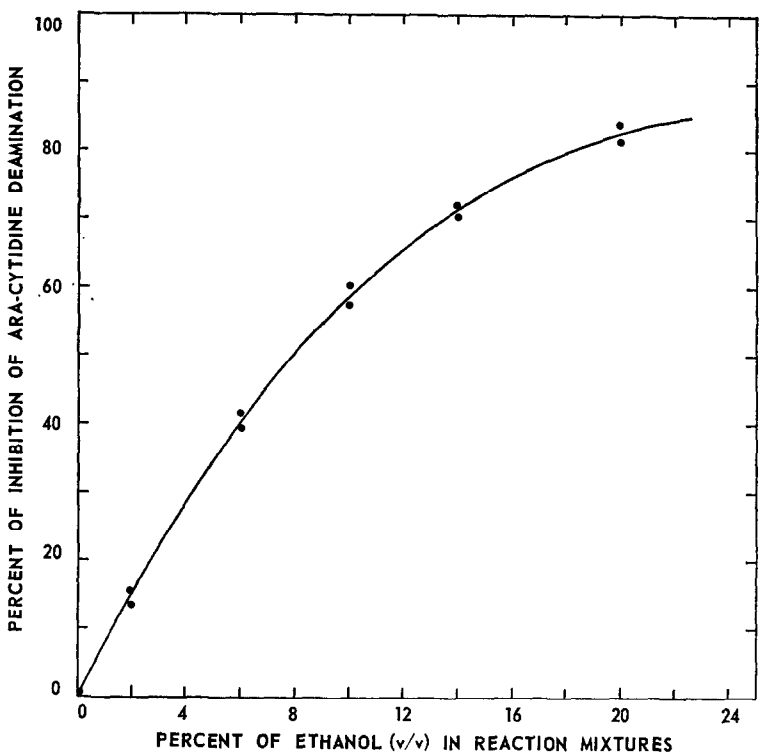


FIG. 2. The dose-related inhibitory effect of ethanol on the enzymatic deamination of ara-C. Assay procedures were those described for the C.E. test; ethanol concentrations are shown in the figure.

preincubation and incubation conditions, and that no rate-limiting intermediate was involved in the inhibition of the enzyme (see Discussion). All of these data, including those in the foregoing sections, are indicative of and consistent with a model of competitive inhibition* in which a substrate–inhibitor complex, rather than just inhibitor, is the actual inhibitor of the enzyme. Secondarily, the substrate–inhibitor complex also would be expected to inhibit the enzyme indirectly at low substrate concentrations by decreasing the effective levels of substrate.

* This type of inhibition is more accurately described as complete exclusive inhibition of the free enzyme.[14]

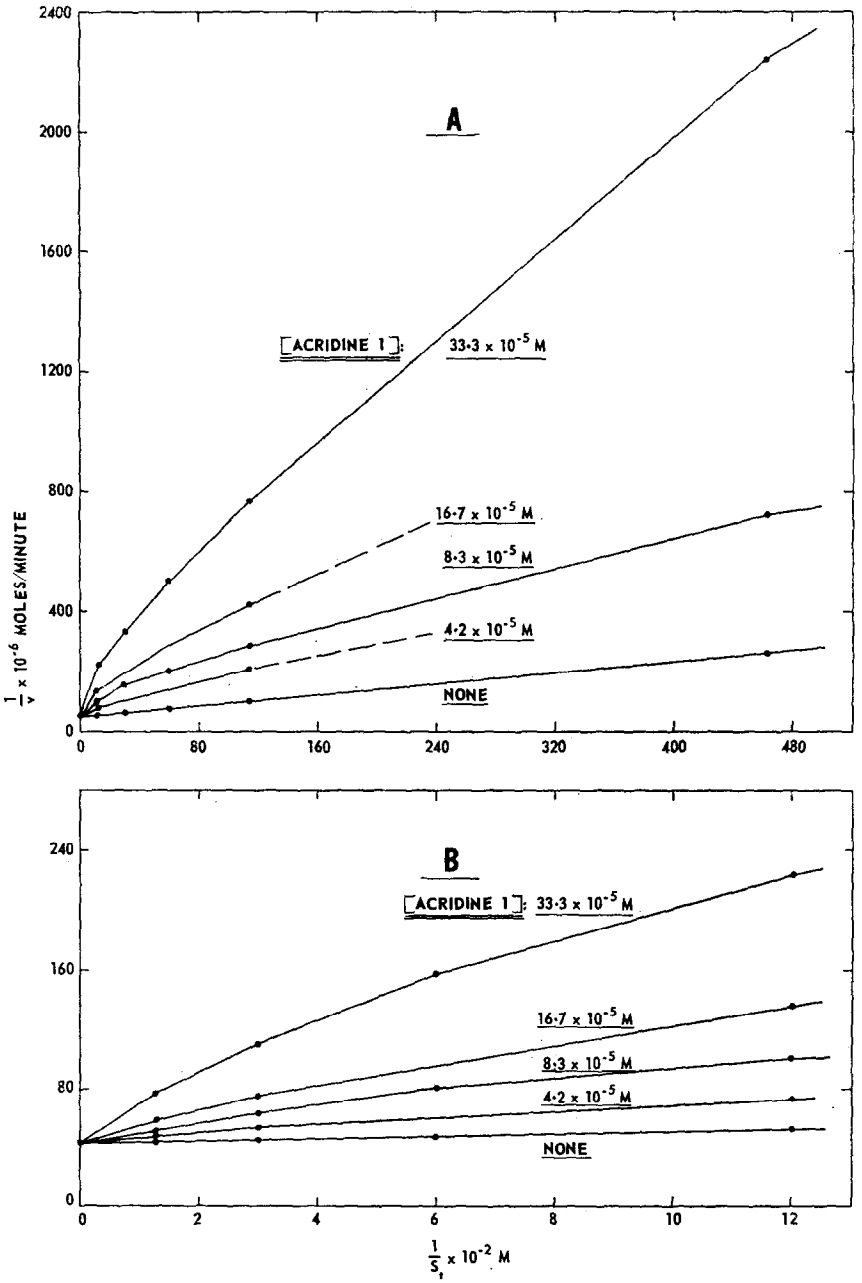


FIG. 3. The inhibition of the enzymatic deamination of ara-C by acridine 1. See Methods for experimental procedures. Ara-C and acridine 1 were present in the indicated concentrations. Each point in the figure is the average of two or more replicates. (A) Lineweaver–Burk data for all levels of S_t tested; (B) enlargement of Fig. 3A showing the data for only high levels of S_t.

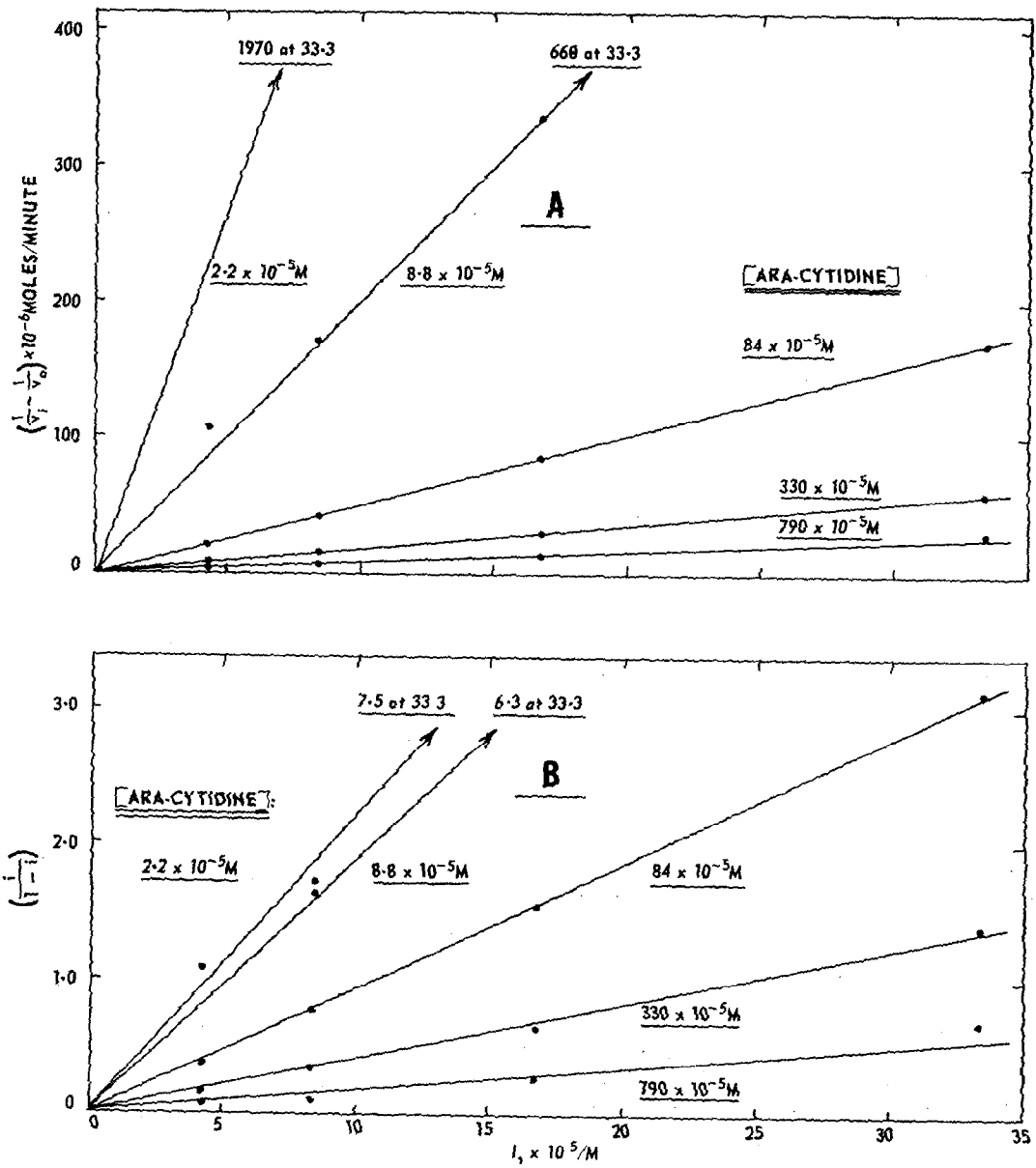


FIG. 4. Diagnostic plots (*A and B*) for the inhibition of the enzymatic deamination of ara-C by acridine 1. Experimental procedures were those described in Methods. Acridine 1 and ara-C concentrations were as shown. Each point is the average of two or more replicates. See the text for the derivation and analyses of the equations which led to these plots.

Inhibition by a substrate–inhibitor complex can be described by the following stoichiometric scheme

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

$$S + I \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} SI$$

$$E + SI \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} ESI$$

with the conservation equations

$$E_t = E + ES + ESI$$

$$I_t = I + SI + ESI$$

Under steady state conditions, the concentrations of the intermediates and enzyme remain constant:

$$\frac{d(ES)}{dt} = \frac{d(E)}{dt} = \frac{d(SI)}{dt} = \frac{d(ESI)}{dt} = 0$$

and the velocity of product formation can be described in the following reciprocal form

$$\frac{1}{v} = \frac{1}{k_2(ES)} = \frac{K_1 + S_t + [(K_1)(S_t)(I_t)/(K_4)(K_3 + S_t)]}{(\mathrm{V_{max}})(S_t)} \tag{1}$$

where $K_1 = (k_{-1} + k_2)/k_1$, $K_3 = k_{-3}/k_3$, $K_4 = k_{-4}/k_4$, and $\mathrm{V_{max}} = k_2 E_t$.

When Michaelis–Menten equilibrium assumptions prevail, $K_1 \cong K_m$. At $I_t = 0$, $v_i = v_0$; as $S_t \to$ high concentrations and $I_t =$ a finite constant, then $v_i \to \mathrm{V_{max}}$; as $S_t \to$ low concentrations and $I_t =$ a finite constant, then $v_i \to 0$ nonlinearly.

Two additional equations can be derived from equation 1

$$\left[\frac{1}{v_i} - \frac{1}{v_0}\right] = (I_t)\left[\frac{K_1}{K_4(K_3 + S_t)(\mathrm{V_{max}})}\right] \tag{2}$$

$$\left[\frac{i}{(1-i)}\right] = (I_t)\left[\frac{K_1 S_t}{K_4(K_3 + S_t)(K_1 + S_t)}\right] \tag{3}$$

Equation 2 is one that we have developed as a diagnostic tool and as a technique for rapidly calculating the dissociation constants K_3 and K_4; plots of $(1/v_i - 1/v_0)$ v. I_t are straight-line curves (for *SI*-type inhibition) with slopes equal to $K_1/K_4(K_3 + S_t)(\mathrm{V_{max}})$. Equation 3 is a form investigated in detail by Reiner;[14] it is included here because of its important diagnostic significance. Of all the types of inhibition studied by Reiner, only inhibition by an *SI* complex was characterized by nonlinear curves in a double reciprocal plot, and by linear, origin-intercept curves in an $i/(1-i)$ v. I_t plot.

The constants (with 95 per cent confidence limits) found for the deamination reaction are shown below. K_1 was calculated from equation 1 using velocity data from the uninhibited enzyme reaction ($I_t = 0$)$_t$ K_3 and K_4 were calculated from equations 2 and 3.

$$K_1 = (k_{-1} + k_2)/k_1 = (1{\cdot}3 \pm 0{\cdot}3) \times 10^{-4}\,\text{M}$$

$$K_3 = \frac{k_{-3}}{k_3} = \frac{(S)(I)}{(SI)} = (1{\cdot}2 \pm 0{\cdot}9) \times 10^{-4}\,\text{M}.$$

$$K_4 = \frac{k_{-4}}{k_4} = \frac{(E)(SI)}{(\text{ESI})} = (1{\cdot}0 \pm 0{\cdot}2) \times 10^{-5}\,\text{M}.$$

$$V_{max} = k_2 E_t = 0{\cdot}45\ \mu\text{mole/min/g wet wt. liver.}$$

DISCUSSION

The nonlinear curves obtained in the $1/v$ v. $1/S_t$ plots (Fig. 3) were consistent with the substrate–inhibitor type of enzyme inhibition just described. However, similar nonlinear plots also would have been obtained had either of the following two events occurred: 1) the destruction of acridine 1 as a function of incubation time, or of ara-C concentration, or of both; or 2) the formation of a rate-limiting intermediate which reduced the *apparent* reaction velocities at short incubation times. These two possibilities were eliminated from consideration, however, by the results of the pre-incubation experiments cited earlier. Thus, by difference, only the substrate–inhibitor mechanism appeared to be consistent with the data from the double reciprocal plot. Confirmatory evidence for this postulated mechanism came from several sources.

The diagnostic plots shown in Fig. 4 provided the needed kinetic confirmation: 1) Both plots showed the straight-line relations, origin intercepts and slope–substrate relationships predicted by equations 2 and 3. 2) In Fig. 4B, the slopes of the lines for the two lowest substrate levels which were tested appeared to approach a maximum; this result was predicted by Reiner[14] from equation 3. 3) The combination of nonlinear curves in a Fig. 3 plot coupled with linear, origin–intercept curves in a Fig. 4B plot was reported by Reiner[14] for only the substrate–inhibitor type of inhibition.

The substrate–inhibitor mechanism also received strong indirect support from previous substrate specificity studies, and from the stereomodel and structure–activity studies reported here. In the first instance, previously reported data[5, 6] were unequivocal in demonstrating the very specific substrate requirements of the deaminase. Yet, in this present study, a simple benzyl-alcohol analogue coupled to a 2-methoxy-acridine was able to effectively and specifically inhibit this same enzyme. These two results are incongruous unless one postulates a substrate-mediated inhibition.

Construction of stereomodels showed that the structures of ara-C and acridine 1 had major areas of overlap, and that a substrate–inhibitor complex was feasible. The measured dissociation constant for this complex was relatively small ($1{\cdot}2 \times 10^{-4}$ M), and this indicated that a substrate–inhibitor complex was probable. Finally, those analogues of acridine 1 which would not be expected to form stable substrate–inhibitor complexes with ara-cytidine were inactive as inhibitors, Thus, even the

nonkinetic data are indicative of and consistent with a substrate–inhibitor type of mechanism.

Several miscellaneous but important questions are raised by the data presented in this paper. Answers to these questions could provide useful biochemical information and could lead to more effective inhibitors of the enzyme. 1) What is the nature of the substrate–inhibitor complex and why is it bound more tightly to the enzyme than is the free ara-cytidine? 2) Can the acridine 1 requirement be met by some structural analogue, for example, analogues with a 4-methoxybutyl group or a 3-(2-methoxyethyl)-quinolyl group substituted for the acridine ring portion of acridine 1? 3) Can the ara-C requirement be replaced by some nucleoside such as cytidine or by the N^4-hydroxyl or 5-methyl analogues of cytidine or ara-C?

In a previous paper,[1] the suggestion was made that ara-C blood levels might be prolonged in man by orally administering a deaminase inhibitor while systemically administering ara-C. This suggestion was based on two assumptions: 1) that the oral treatment would produce a locally high level of inhibitor in the liver where the bulk of the deaminase activity is located; and 2) that the inhibitor would interact *directly* with the enzyme. With a substrate–inhibitor type of inhibitor, however, the oral treatment would have to include the coadministration of ara-C (or some other active nucleoside), for without the substrate, there can be no inhibition.

The alkyl solvents in Table 2 can be divided into one of three groups depending upon their activities and structures. Solvents in the first group were much less inhibitory than ethanol. and they were characterized by having one hydroxyl or amino substituent on every carbon atom (solvents 17, 20, 21 and 29). Solvents in the second group were about as inhibitory as ethanol, and they were observed to have either one unsubstituted carbon atom or at least one of their hydroxyl or amino groups blocked by methyl or ethyl groups (solvents 19, 27 and 28, and solvents 18, 22, 23 and 24, respectively). The third group was much more inhibitory than ethanol, and these solvents had two unsubstituted carbom atoms each (solvents 25 and 26). These observations suggest that there may be specific relationships between the solvent structures and their inhibitory activities. This speculation also is consistent with the lack of correlation found between dielectric constants and enzyme inhibition (Table 2).

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