Enzyme Inhibitors. XVII. Kinetic Studies on the Irreversible Inhibition of Adenosine Deaminase[1]

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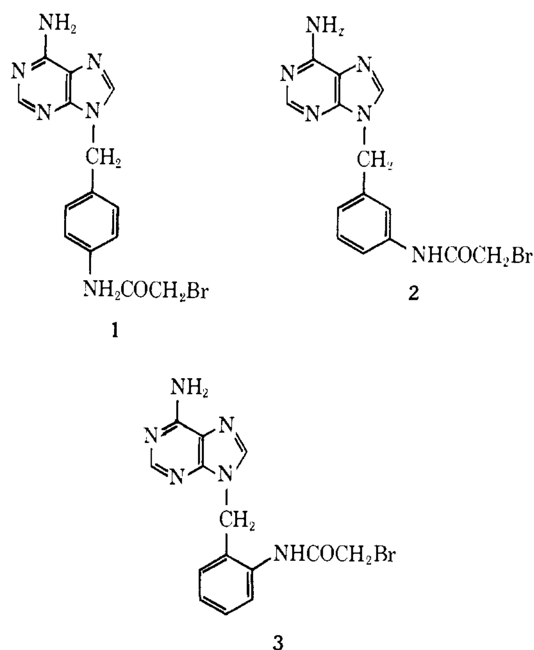
Received December 15, 1966

A kinetic method has been developed for the evaluation of the reversible enzyme-inhibitor dissociation constant (K_i) and of the first-order rate constant for the alkylation of an enzyme by an active-site-directed irreversible inhibitor. This method should be applicable to all irreversible enzyme inhibitors that proceed through an initial reversible enzyme–inhibitor complex. It has been shown that irreversible inactivation of adenosine deaminase caused by 9-(*p*-bromoacetamidobenzyl)adenine (**1**) and by 9-(*o*-bromoacetamidobenzyl)adenine (**3**) is not a random bimolecular process but proceeds by a first-order reaction in an initial reversible enzyme-inhibitor complex. Using this new method, the K_i of **1** and **3** were found to be 1.4×10^{-5} *M* and 43×10^{-5} *M*, respectively, in excellent agreement with the values obtained previously by the usual double reciprocal plot method. Furthermore, it was found that **3** alkylates the enzyme about seven times more rapidly than does **1**. The first-order rate constant (k_2) for the alkylation in the reversible E–I complex for **1** = 1.1×10^{-2} min^{-1} and for **3** = 7.7×10^{-2} min^{-1}.

During the past several years, a number of laboratories have been interested in the design and synthesis of irreversible inhibitors of certain enzymes.[2-4] In studies on esterases and irreversible inhibitors, equations have been derived which allow one to evaluate K_a (the affinity constant) and k_p (the phosphorylation rate constant) or k_{2c} (the carbamoylation rate constant of the enzyme).[5] In this paper we wish to present a new mathematical description of the irreversible inhibition of an enzyme which allows one to determine K_i and k_2 (the first-order rate constant for the alkylation of the enzyme by the inhibitor).

Several recent papers have described our research on the irreversible inactivation of calf intestinal mucosa adenosine deaminase by 9-(*p*-, *m*- and *o*-bromoacetamidobenzyl)adenines.[6] It was found that the *para* and *ortho* derivatives (**1** and **3**) were much more effective irreversible inhibitors of adenosine deaminase than was the *meta* isomer (**2**). It was also possible to measure the initial reversible inhibition of adenosine deaminase by these compounds, and it was found that the effectiveness of reversible inhibition decreases in the following order: *para* isomer (**1**) > *meta* isomer (**2**) > *ortho* isomer (**3**). Consequently the potency of these compounds as irreversible inhibitors is not merely a reflection of their ability to form a reversible complex with the enzyme.



A second factor which must be considered when comparing irreversible inhibitors of enzymes is the chemical reactivity of the alkylating or acylating group of the inhibitor. 4-(*p*-Nitrobenzyl)pyridine has been used previously to compare the chemical reactivity of alkylating groups.[7] Using 4-(*p*-nitrobenzyl)pyridine we compared the reaction rates of iodoacetamide with **1**, **2**, and **3**. If the rate with iodoacetamide was normalized to 1, then the rates with **1**, **2**, and **3** were 3.0, 4.1, and 5.6, respectively. Since this order of reactivity does not represent the effectiveness of **1**, **2**, and **3** as irreversible inhibitors of adenosine deaminase, it is apparent that a more subtle effect or a combination of several effects determine the effectiveness of an irreversible inhibitor of the enzyme. In order to gain more insight into the mechanism of this reaction, a kinetic study of the irreversible inactivation of adenosine deaminase by **1**, **2**, and **3** was undertaken.

When an enzyme is irreversibly inhibited by an alkylating or acylating agent, it would appear that the two most probable mechanisms for the inactivation are (A) a bimolecular attack of the inhibitor on the enzyme or (B) the initial formation of a reversible complex through which covalent bond formation occurs.

These two mechanisms may be distinguished kinetically since, in the case of the bimolecular mechanism

(1) This investigation was supported by Grant T-337A from the American Cancer Society, by a Public Health Service research grant (5-R01-GM-09775-05), by a research career program award (5-K3-CA-18718-05) from the National Cancer Institute, and a training grant (5-T1-GM-555-05) from the Division of Medical Sciences, U. S. Public Health Services, Bethesda, Md.

(2) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

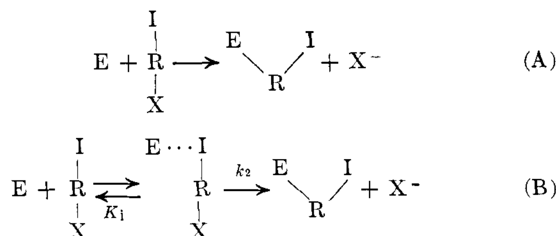
(3) G. Schoelmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).

(4) W. B. Lawson and H. J. Schramm, *ibid.*, **4**, 377 (1965).

(5) (a) A. R. Main, Science, **144**, 992 (1964); (b) A. R. Main and F. Iverson, *Biochem. J.*, **100**, 525 (1966); (c) A. R. Main and F. L. Hastings, *Science*, **154**, 400 (1966).

(6) (a) H. J. Schaeffer and E. Odin, *J. Med. Chem.*, **9**, 576 (1966); (b) H. J. Schaeffer and R. N. Johnson, *J. Pharm. Sci.*, **55**, 929 (1966); (c) H. J. Schaeffer and E. Odin, *J. Med. Chem.*, **10**, 181 (1967).

(7) (a) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955); (b) T. J. Bardos, N. Datta-Gupta, P. Hebborn, and D. J. Triggle, *J. Med. Chem.*, **8**, 167 (1965); (c) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **2**, 21 (1965).



(A), an increase in the concentration of the inhibitor would result in a corresponding increase in the rate of inactivation at all concentrations of inhib tor. However, in the second case where an initial reversible enzyme–inhibitor complex is formed, increases in the concentration of inhibitor would result in an increase in the rate of irreversible inactivation only until the enzyme was saturated with inhibitor. Further increases in the concentration of inhibitor would not affect the rate of inactivation.[8] This phenomenon has been discussed previously by Baker, *et al.*,[9] who have described it as a "rate saturation effect." It must be emphasized that the observance of apparent first-order irreversible inactivation of an enzyme does not usually differentiate mechanism A from B since in the experimental design of most irreversible inactivations, the concentration of inhibitor is significantly greater than the concentration of enzyme; thus, both mechanisms would exhibit apparent first-order kinetics. In order to differentiate these mechanisms, it is necessary to determine if a rate saturation effect is present or absent.

Baker, *et al.*,[9] have derived eq 3 to evaluate this rate saturation effect. Briefly, the derivation is outlined below for an irreversible inhibitor that forms an initial reversible E–I complex.

$$K_i = [E][I]/[EI] \quad (1)$$

$$[E_t] = [E] + [EI] \quad (2)$$

Substituting (2) into (1) gives (3)

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \quad (3)$$

where [E] is the free enzyme concentration, [I] the concentration of the inhibitor, [EI] the concentration of the reversible enzyme–inhibitor complex, K_i the dissociation constant of this complex, and $[E_t]$ the total enzyme concentration.

Because, as we have described earlier, an irreversible inactivation of an enzyme depends on both K_i and on k_2 (see eq B), we have derived an equation which separates these two kinetic parameters in order to gain a more complete understanding of the reaction mechanism. For an irreversible inactivation of an enzyme that proceeds through an initial reversible complex and follows apparent first-order kinetics, the rate of inactivation (R) is described by eq 4.

$$R = k_2[E\text{–}I] \quad (4)$$

Substituting (3) into (4) gives (5).

$$R = k_2 \frac{[E_t]}{\frac{K_i}{[I]} + 1} \quad (5)$$

Since

$$R/[E_t] = k_{obsd} \quad (6)$$

where k_{obsd} equals the observed first-order rate constant for enzyme inactivation, then

$$k_{obsd} = \frac{k_2}{\frac{K_i}{[I]} + 1} = \frac{k_2[I]}{K_i + [I]} \quad (7)$$

Taking the reciprocal of (7) gives (8). Thus, if a series

$$\frac{1}{k_{obsd}} = \frac{K_i}{k_2}\frac{1}{[I]} + \frac{1}{k_2} \quad (8)$$

of irreversible enzyme inactivations are performed at various inhibitor concentrations, a plot of $1/k_{obsd}$ against $1/[I]$ should give a straight line whose intercept would be $1/k_2$ and whose slope would be K_i/k_2. A similar equation has been derived for the reaction of isopropyl methylphosphonofluoridate (sarin) with a variety of substituted catechols.[10]

Experimental Section

Adenosine deaminase (Type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co.

The adenosine deaminase inactivation procedure is a modification of a method described in the literature.[6a,11] A solution of the enzyme was prepared such that a 50-μl aliquot when diluted in phosphate buffer to 3.1 ml which was 0.081 m*M* in adenosine gave the desired initial velocity of enzymic reaction. Equal volumes of this enzyme solution were then mixed with equal volumes of phosphate buffer containing 10% dimethyl sulfoxide (DMSO) or solutions of the irreversible inhibitor in phosphate buffer containing 10% DMSO. These solutions were incubated at 37°, and at various intervals 0.5-ml samples were removed and immediately cooled to 0°. The amount of enzyme remaining in each aliquot was determined at 25° in triplicate experiments by using a 100-μl sample of each aliquot diluted with phosphate buffer which was 0.081 m*M* in adenosine. It was shown that the irreversible inhibitor did not inactivate the enzyme during the few minutes it was kept at 0°. Each point on the plots is an average of three determinations; each inactivation experiment was repeated at least twice.

Results and Discussion

In Figures 1 and 2 are shown some of the apparent first-order rates of irreversible inhibition of adenosine deaminase by the *para* and *ortho* compounds (**1** and **3**). The *meta* derivative (**2**) was also capable of causing irreversible inactivation of adenosine deaminase, but the rates were too low to allow an accurate kinetic evaluation due to considerable thermal inactivation of the enzyme over a 24–48-hr period. In the case of **1** and **3**, the irreversible inactivation of adenosine deaminase was relatively rapid; therefore, the thermal inactivation of the enzyme was ignored. The apparent first-order rate constants for the irreversible inhibition of adenosine deaminase were calculated from the slopes of the lines shown in Figures 1 and 2 and a complete

(8) This argument implies that the initial concentration of inhibitor was insufficient to saturate the enzyme. If the initial concentration of inhibitor saturated the enzyme, then further increases would not increase the rate. In this case one could decrease the initial concentration of inhibitor to observe the variation in inactivation rate.

(9) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(10) (a) M. A. Schwartz, Ph.D. Thesis, University of Wisconsin, 1959; (b) T. Higuchi, Progress Report submitted to Armed Forces Chemical Center, Edgewood, Md. (May 20, 1959); (c) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanism," W. A. Benjamin, Inc., New York, N. Y., 1966, p 143.

(11) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

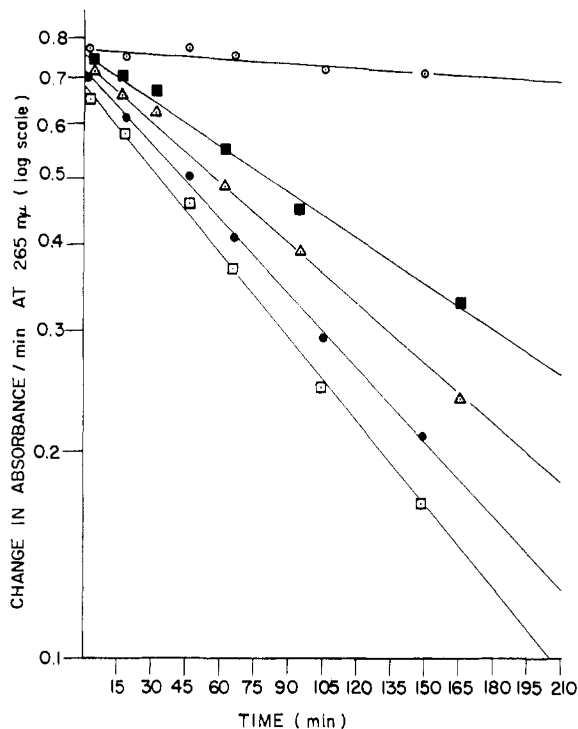


Figure 1.—Comparison of the irreversible inhibition of adenosine deaminase by 9-(*p*-bromoacetamdiobenzyl)adenine (**1**): O, enzyme control; ■, 0.012 mM **1**; Δ, 0.020 mM **1**; ●, 0.040 mM **1**; □, 0.060 mM **1**. The abscissa is time of incubation at 37°.

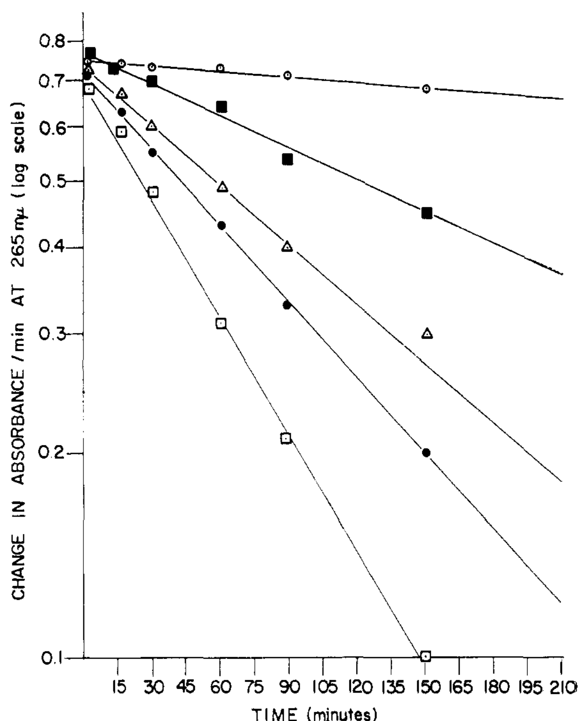


Figure 2.—Comparison of the irreversible inhibition of adenosine deaminase by 9-(*o*-bromoacetamidobenzyl)adenine (**3**): O, enzyme control; ■, 0.020 mM **3**; Δ, 0.040 mM **3**; ●, 0.60 mM **3**; □, 0.10 mM **3**. The abscissa is time of incubation at 37°.

listing of the data is given in Table I. A plot of $1/k_{obsd}$ *vs.* $1/[I]$ is shown in Figure 3. An examination of Figure 3 reveals that for the *para* derivative (I) the slope of the line is 1.24 and the intercept is 91. From these data it can be calculated that for **1** the $K_i = 1.4 \times 10^{-5}\ M$ and $k_2 = 1.1 \times 10^{-2}\ \text{min}^{-1}$. In the

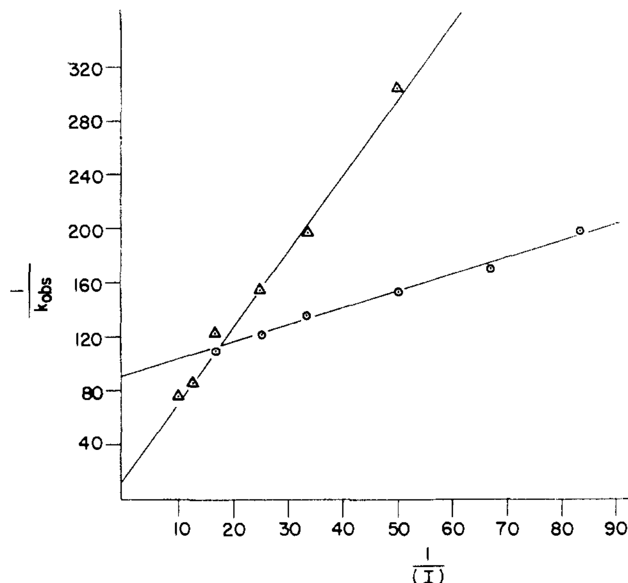


Figure 3.—Plot of $1/k_{obsd}$ for irreversible inhibition of adenosine deaminase *vs.* $1/[I]$ (mM): O, 9-(*p*-bromoacetamidobenzyl)-adenine (**1**); Δ, 9-(*o*-bromoacetamidobenzyl)adenine (**3**).

case of the *ortho* derivative (**3**), the slope of the line is 5.57 and the intercept is 13. Therefore, $K_i = 43 \times 10^{-5}\ M$ and $k_2 = 7.7 \times 10^{-2}\ \text{min}^{-1}$. We have previously determined that both **1** and **3** are competitive inhibitors of adenosine deaminase by the Lineweaver–Burk method and found that the K_i of **1** $= 1.3 \times 10^{-5}\ M$ and the K_i of **3** $= 44 \times 10^{-5}\ M$.[6a,c] The determi-

Table I

Kinetic Constants for the Irreversible Inhibition of Adenosine Deaminase by 9-(*p*-Bromoacetamidobenzyl)- (**1**) and 9-(*o*-Bromoacetamidobenzyl)adenine (**3**)

mM concn	$t_{1/2}$, min	$k_{obsd} \times 10^4$ min^{-1}	$1/k_{obsd}$	$1/[I]$, mM
		para isomer (**1**)		
0.060	75.5	9.18	109	16.7
0.040	83.7	8.28	121	25.0
0.030	94.0	7.38	135	33.3
0.020	106	6.53	153	50.0
0.015	118	5.87	170	66.7
0.012	137	5.06	198	83.3
		ortho isomer (**3**)		
0.100	52.0	13.3	75.2	10.0
0.080	58.5	11.8	84.4	12.5
0.060	84.5	8.20	122	16.7
0.040	107	6.45	155	25.0
0.030	137	5.04	198	33.3
0.020	204	3.40	294	50.0

nation of the inhibitor constant (K_i) by the irreversible inhibition experiment is therefore in excellent agreement with our previous data. We believe that these data offer strong experimental support to the mechanism as outlined in eq B, especially in view of the fact that iodoacetamide did not irreversibly inhibit adenosine deaminase even at concentrations of 1.6 mM. It is also clear that the alkylation reaction (k_2) between **3** and the enzyme is approximately seven times faster than in the case of **1** and the enzyme, even though **3** is not even twice as reactive as **1** when measured with 4-(*p*-nitrobenzyl)pyridine as the nucleophilic reagent.

Finally, the results of the irreversible inactivation of adenosine deaminase by **1** and **3** represent an interesting example which must be considered when a comparison is made between two irreversible inhibitors of an enzyme. If the irreversible inactivation of an enzyme is performed only with a single concentration of each inhibitor, it is possible to draw an erroneous conclusion concerning the relative effectiveness of the inhibitors. For example, if the irreversible inhibition of adenosine deaminase were performed with 0.1 m*M* concentrations of **1** or **3**, it would be found that **3** inactivates the enzyme more rapidly than **1**. If, however, a similar experiment were performed at 0.03 m*M* concentration of inhibitor, it would be found that **1** is more effective than **3**. This apparent reversal of potency of **1** and **3** as irreversible inhibitors of adenosine deaminase occurs because the observed first-order loss of enzyme activity is a function of both K_i and k_2. When the irreversible inactivations are carried out at concentrations of inhibitor which do not saturate the enzyme, the amount of the total enzyme, $[E_t]$, in the reversible enzyme–inhibitor complex is dependent on K_i. The amounts of E_t in the reversible E–I complex at 0.10 m*M* and 0.03 m*M* concentrations of **1** are $0.88[E_t]$ and $0.70[E_t]$, respectively, whereas in the case of **3**, the amounts of E_t in the reversible E–I complex at the same concentrations are $0.18[E_t]$ and $0.067[E_t]$. Thus, for **3**, a much larger percentage change in the concentration of the reversible E–I complex will occur than in the case of 1 which, in turn, would result in an apparent reversal of effectiveness of these compounds as irreversible inhibitors of adenosine deaminase. From these data it is clear that in comparing irreversible inhibitors of an enzyme, the observed first-order inactivation rates should not be employed unless the K_i's of the compounds are equal. Rather, the comparison should be made by the procedure outlined in this paper so that both K_i and k_2 are evaluated.

Enzyme Inhibitors. XVIII. Studies on the Stereoselectivity of Inhibition of Adenosine Deaminase by DL-, D-, and L-9-(2-Hydroxypropyl)adenine[1a]

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Received December 24, 1966

Previous studies on the inhibition of adenosine deaminase with compounds that contain an asymmetric center utilized the corresponding racemic compounds. In order to determine if adenosine deaminase exhibits a stereoselectivity in complexation with one enantiomer of a DL mixture, the syntheses of D-(−) and L-(+) isomers of 9-(2-hydroxypropyl)adenine (**6-D** and **6-L**) were undertaken because the DL racemate of this compound has previously been shown to be a good reversible inhibitor of this enzyme. Enzymic evaluation of L-(+)-9-(2-hydroxypropyl)-adenine (**6-L**) and D-(−)-9-(2-hydroxypropyl)adenine (**6-D**) revealed that **6-L** is a much better inhibitor of adenosine deaminase than is **6-D**; the $([I]/[S])_{0.5}$ of **6-L** = 0.148, whereas the $([I]/[S])_{0.5}$ of **6-D** = 1.48. A rationalization for this difference in inhibitory properties is presented. Calculations based on the $([I]/[S])_{0.5}$ of **6-L** compared to the $([I]/[S])_{0.5}$ of 9-(2-hydroxyethyl)adenine reveal that the free energy of binding of the methyl group of **6-L** is −1.15 kcal, a value which cannot be accounted for on the basis of hydrophobic forces alone. Thus, the positive involvement of van der Waals forces is invoked. Based on these and other data, it is concluded that there is a specific methyl binding region on adenosine deaminase which forms a unique "tight fit" or "lock and key" type of fit with the methyl group of L-(+)-9-(2-hydroxypropyl)adenine (**6-L**).

It is well-known that many enzymes exhibit stereoselectivity when complexes are formed between the enzyme and a molecule that contains one or more asymmetric centers. Recently, it has been suggested that calf intestinal mucosal adenosine deaminase has both polar and nonpolar areas which are important for binding the substituents at the 9 position of a 6-substituted purine derivative.[2]

Since many of the compounds which have been found to inhibit adenosine deaminase contain an asymmetric center, the possibility existed that the enzyme was only combining with one of the enantiomers of a DL mixture. It became desirable, therefore, to determine if adenosince deaminase exhibits either a specificity or selectivity[3] when combining with an inhibitor molecule. In order to study this problem, we decided to prepare the optically active forms of 9-(2-hydroxypropyl)adenine, a good reversible inhibitor of adenosine deaminase, and evaluate these compounds as reversible inhibitors of this enzyme.

Chemistry.—The general method of synthesis of D- and L-9-(2-hydroxypropyl)adenines was patterned after the method utilized for the preparation of the racemic compound.[4] Optically pure D-(−)-1-amino-2-propanol (**2-D**) was obtained from D-(−)-lactic acid (**1-D**) in three steps by a modification of a previously reported procedure[5] (see Chart I). Treatment of **2-D** with 5-amino-4,6-dichloropyrimidine (**3**) resulted in the formation of D-(−)-5-amino-4-chloro-6-(2-hydroxypropylamino)pyrimidine (**4-D**). Cyclization of **4-D** with a mixture of triethyl orthoformate and concentrated HCl gave the required 6-chloropurine derivative (**5-D**), which upon treatment with liquid ammonia gave D-(−)-9-(2-hydroxypropyl)adenine (**6-D**). The series of L isomers was obtained by a sequence of reac-

(1) (a) This investigation was supported by Grant T-337A from the American Cancer Society, by a Public Health Service research grant (5-R01-GM-09775-05), by a research career program award (5-K3-CA-18718-05) from the National Cancer Institute, and a training grant (5-T1-GM-555-05) from the Division of Medical Sciences, U. S. Public Health Services, Bethesda, Md. (b) Recipient of 1966 Lusford Richardson Pharmacy Award.

(2) H. J. Schaeffer and R. Vince, *J. Med. Chem.*, **8**, 507 (1965).

(3) The term selectivity implies that the inhibition of an enzyme occurs mainly with one enantiomer of a DL pair, whereas specificity implies that the activity is exclusively in one enantiomer of a DL pair.

(4) H. J. Schaeffer, D. Vogel, and R. Vince, *J. Med. Chem.*, **8**, 502 (1965).

(5) D. E. Wolf, W. H. Jones, J. Valiant, and K. Folkers, *J. Am. Chem. Soc.*, **72**, 2820 (1950).