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 mM 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 mM 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 mM and 0.03 mM 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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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-hydroxypropylanino)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).

TABLE I

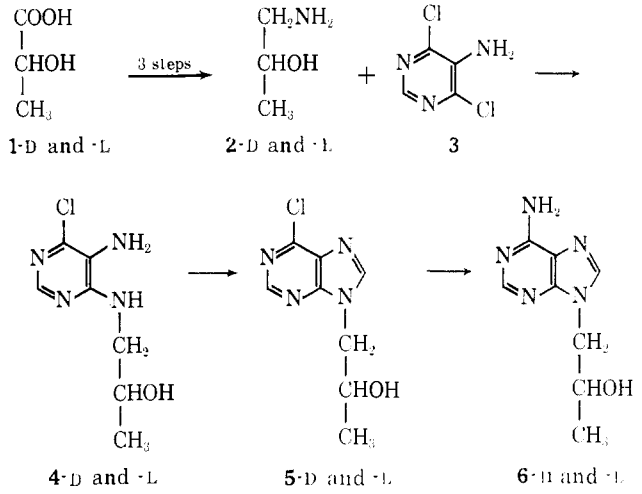
PHYSICAL CONSTANTS AND ANALYTICAL RESULTS ON SOME PYRIMIDINES AND PURINES

Compd	Recrystln solvent	Mp, °C	Yield, %	Ultraviolet: λ_{max}, mμ ($\epsilon \times 10^{-3}$)		Formula	Carbon, %		Hydrogen, %		Nitrogen, %		Chlorine, %		$[\alpha]^{25}D$, deg (solvent)
				1 N HCl	H_2O		Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found	
4-D	H_2O	87–88	58.9	305 (10.8)	290 (8.05) 263 (7.90)	$C_7H_{11}ClN_4O \cdot H_2O$	38.08	38.09	5.93	5.93	25.39	25.43	16.06	16.04	−27.6 ± 0.4 (EtOH)
4-L	H_2O	87–89	66.8	305 (10.8)	290 (8.05) 263 (7.90)	$C_7H_{11}ClN_4O \cdot H_2O$	38.08	38.05	5.93	5.91	25.39	25.64	16.06	16.11	+25.9 ± 0.7 (EtOH)
5-D	*a*	164–167	77.5	265 (8.35)	265 (8.35)	$C_6H_9Cl_2N_4O$	38.57	38.80	4.05	4.29	22.49	22.22	28.47	28.81	−33.3 ± 0.3 (MeOH)
5-L	*a*	167–169	92.4	265 (8.30)	265 (8.30)	$C_8H_{16}Cl_2N_4O$	38.57	38.70	4.05	4.20	22.49	22.25	28.47	28.20	+33.1 ± 0.3 (MeOH)
6-D	EtOH	192–195	56.4	260 (1.35)	260 (1.35)	$C_8H_{11}N_5O$	49.73	49.78	5.73	5.84	36.25	36.47			−44.3 ± 0.3 (EtOH)
6-L	EtOH	193–195	59.6	260 (1.31)	260 (1.32)	$C_8H_{11}N_5O$	49.73	49.53	5.73	5.71	36.25	36.20			+44.0 ± 0.4 (EtOH)

a Acetonitrile + ether containing dry HCl.

tions similar to those used for the D isomers. The optical rotation of D-(−)- and L-(+)-9-(2-hydroxypropyl)adenines (**6-D** and **6-L**) did not change after three recrystallizations of the pure products, indicating a high optical purity of these compounds.

CHART I



Experimental Section[6]

The physical data and analyses are given in Table I.

D-(−)- and L-(+)-5-Amino-4-chloro-6-(2-hydroxypropylamino)pyrimidine (4-D and 4-L).—A solution of 2.44 g (32.5 mmoles) of **2-D** or **2-L**, 5.35 g (32.5 mmoles) of **3**, and 3.34 g (33.0 mmoles) of triethylamine in 50 ml of 1-butanol was heated under reflux for 20 hr. The volatile materials were removed *in vacuo*, and the tan residue was crystallized from water (25 ml) and gave the crude product as a monohydrate.

D-(−)- and L-(+)-9-(2-Hydroxypropyl)-6-chloropurine Hydrochloride (5-D and 5-L).—To a solution of 2.00 g (9.05 mmoles) of **4-D** or **4-L** in 29 ml of triethyl orthoformate was slowly added 12.8 mmoles of concentrated HCl with rapid stirring. After 48 hr, complete loss of the pyrimidine absorption at 305 mμ was observed and the solution was concentrated to one-half volume. Acetonitrile (15 ml) was added and HCl was passed through the chilled solution. The white precipitate was collected and washed with ether.

D-(−)- and L-(+)-9-(2-Hydroxypropyl)adenine (6-D and 6-L).—A mixture of 1.00 g (4.02 mmoles) of **5-D** or **5-L** in 15 ml of liquid ammonia was heated in a steel bomb at 65° for 18 hr. The NH_3 was evaporated at room temperature, and the white residue was dried *in vacuo*. The solid mixture was triturated with 100 ml of boiling acetone and filtered while hot to remove the insoluble NH_4Cl. The filtrate was evaporated to one-half volume and refrigerated overnight. The white precipitate was collected by filtration.

Reagents and Assay Procedure.—Adenosine deaminase (type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The assay procedure for reversible inhibitors has been described previously[4] and is a modification of the general procedure of Kaplan.[7] The measurements of the initial rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6.

(6) The infrared spectra were determined on a Perkin-Elmer Model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer Model 202 spectrophotometer; the enzyme studies were done on a Gilford Model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected. The optical rotations were taken on a Perkin-Elmer Model 141 polarimeter in solution of 1% concentration. The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

(7) N. O. Kaplan, *Methods Enzymol.*, **2**, 173 (1955).

Results and Discussion

An examination of Table II reveals that adenosine deaminase exhibits a significant stereoselectivity in the formation of an enzyme–inhibitor complex with L-(+)-9-(2-hydroxypropyl)adenine (**6**-L). Initially one might be tempted to speculate that if adenosine deaminase exhibits a stereoselectivity in the formation of an enzyme–inhibitor complex, it would be for the D isomer of 9-(2-hydroxypropyl)adenine since the D isomer has the same absolute configuration at the carbon atom bearing the hydroxyl group as does the carbon atom at the 2′ position of the ribose moiety of adenosine. However, if one studies molecular models of certain similar conformations of adenosine, **6**-D and **6**-L, a fact becomes apparent which offers a logical explanation for the stereoselectivity of adenosine deaminase for the L isomer (**6**-L) over the D isomer (**6**-D). An examination of Chart II shows that if the adenine moiety and the 2′-hydroxyl group of the inhibitor bind to the same points on the enzyme that are utilized by the same groups on adenosine, two situations can occur depending upon which inhibitor is complexing with the enzyme.[8] The D isomer (**6**-D) has the same absolute configuration at the 2′ carbon as adenosine; thus, the methyl group of **6**-D projects into the same region that would normally be occupied by the ribose moiety of the substrate. Since this region on the enzyme would be expected to be hydrophilic in nature, the methyl group of **6**-D would cause some repulsion to binding. That some repulsion to binding is caused by the methyl group of **6**-D can be seen by comparing the $[I]/[S]_{0.5}$ of **6**-D to the $([I]/[S])_{0.5}$ of 9-(2-hydroxyethyl)adenine(**7**).

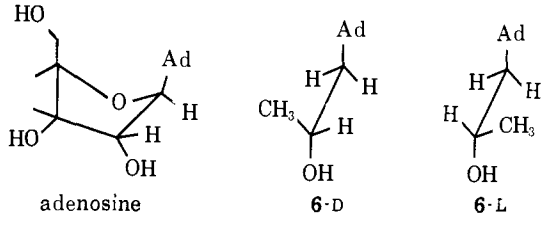
Table II

Index of Inhibition and Partial Inhibition of Adenosine Deaminase by Some 9-(Hydroxyalkyl)adenines

9-Substituted adenine[a]	mM concn for 50% inhib (I_{50})[b]	$([I]/[S])_{0.5}$
DL-2-Hydroxypropyl (**6**-DL)	0.016 ± 0.002[c]	0.245 ± 0.02[c]
D-2-Hydroxypropyl (**6**-D)	0.098 ± 0.005	1.48 ± 0.008
L-2-Hydroxypropyl (**6**-L)	0.0098 ± 0.0005	0.148 ± 0.004
2-Hydroxyethyl (**7**)	0.070 ± 0.004	1.1 ± 0.05[d]
DL-2-Hydroxybutyl (**8**)	0.078 ± 0.003	1.2 ± 0.04[d]
DL-2-Hydroxyoctyl (**9**)	0.209 ± 0.001	2.8 ± 0.02[d]

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 mM. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 mM. In those cases where a larger value is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/Vi *vs.* [I]. [c] Average deviation. [d] Data from H. J. Schaeffer and C. F. Schwender, *J. Pharm. Sci.*, **56**, 207 (1967).

Chart II



In the case of the L isomer (**6**-L), if the adenine moiety and the 2′-hydroxyl group bind to the same points on the enzyme as do the corresponding groups on **6**-D, it can be seen that the methyl group of **6**-L will project away from this hydrophilic region and would not cause repulsion to binding. If the methyl group of **6**-L were not contributing to binding, the compound should have an $([I]/[S])_{0.5}$ equal to 9-(2-hydroxyethyl)adenine (**7**). However, **6**-L is bound much more tightly to the enzyme than is **7**. In fact, calculations based on the differences of the free energy of binding of **6**-L and **7** to adenosine deaminase show that the methyl group of **6**-L makes a contribution to binding of −1.15 kcal.[9] Cohn and Edsall[10] have shown by means of quantitative solubility measurements that the transfer of a methyl or methylene group from an aqueous to a nonaqueous phase is accompanied by a free-energy change of approximately −730 cal. In an evaluation of the contribution of the highly distance-specific van der Waals forces, Salem[11] has suggested that the maximum free-energy contribution to binding which could be observed for a methyl group is −600 cal. Belleau and Lacasse[12] have presented an excellent discussion of these two types of forces in connection with a study of complex formation with acetylcholinesterase and on the basis of this study have shown that the positive involvement of van der Waals attractions is of rare occurrence, at least in the case of acetylcholinesterase. The present inhibition studies on adenosine deaminase establish that there is a positive involvement of van der Waals forces in the complexation of the methyl group of **6**-L. This methyl binding site is probably a cavity on the enzyme which may preexist or which may be formed by a conformational change of the enzyme. That the enzyme forms a "tight fit" with the methyl group of **6**-L can be seen by comparing certain DL inhibitors with **6**-DL. For example, when the methyl group of **6**-DL is compared to an ethyl group as in DL-9-(2-hydroxybutyl)adenine (**8**), it is seen that the compound with a terminal ethyl group (**8**) in place of the terminal methyl group, as in **6**-DL, is a weaker inhibitor than **6**-DL. This phenomenon is even more obvious when the comparison is made between DL-9-(2-hydroxyoctyl)adenine (**9**) and **6**-DL. In this case, **9** is not one-tenth as active as a reversible inhibitor as is **6**-DL. Thus, the preparation of inhibitors that have larger groups in the area of a specific methyl binding region results in weaker inhibitors.[13] These data taken in conjunction with the change in free energy of binding of **6**-L relative to **7** establish that there is a specific methyl binding site on the enzyme and that there is a "tight fit" or classical "lock and key" type of fit for this methyl group.

(8) These drawings are not to be interpreted to represent the actual conformation which binds to adenosine deaminase. Rather, they are drawn in this perspective so that the effect of the methyl group in **6**-D and **6**-L may be easily visualized.

(9) The differences in the free energy of binding of **6**-L and **7** were calculated by the following equation: $\Delta F = -2.303RT \log [I_{50}(\mathbf{7})/I_{50}(\mathbf{6}\text{-L})]$.

(10) E. J. Cohn and J. T. Edsall, "Proteins, Aminoacids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chapter 9.

(11) L. Salem, *Can. J. Biochem. Physiol.*, **40**, 1287 (1962).

(12) B. Belleau and G. Lacasse, *J. Med. Chem.*, **7**, 768 (1964).

(13) It is also possible that the area on the enzyme beyond the methyl binding region may be quite polar and thereby repels the longer chain compounds.