A Transition State in Pieces: Major Contributions of Entropic Effects to Ligand Binding by Adenosine Deaminase†

Warren M. Kati,‡ Scott A. Acheson,§ and Richard Wolfenden*

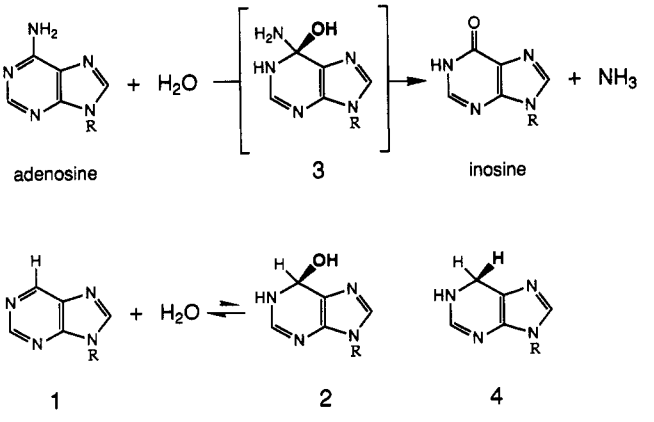
Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Received January 7, 1992; Revised Manuscript Received April 9, 1992

ABSTRACT: Nebularine undergoes hydration at the active site of adenosine deaminase, in a reaction analogous to a partial reaction in the displacement of ammonia from adenosine by water, to generate an inhibitory complex that captures much of the binding affinity expected of an ideal transition-state analogue. Enzyme affinities of several compounds related to nebularine 1,6-hydrate, and to its stable analog 2′-deoxycoformycin, were compared in an effort to identify the structural origins of strong binding. Binding of the stable transition-state analog inhibitor 2′-deoxycoformycin was rendered 9.8 kcal/mol less favorable by removal of substituent ribose, 9.7 kcal/mol less favorable by inversion of the 8-hydroxyl substituent of the diazepine ring, and 10.0 kcal/mol less favorable by removal of atoms 4–6 of the diazepine ring. Binding of the unstable transition-state analog nebularine hydrate was rendered at least 9.9 kcal/mol less favorable by removal of the 6-hydroxyl group and 10.2 kcal/mol less favorable by removal of atoms 1–3 of the pyrimidine ring. In each case, the enzyme exhibited only modest affinity ($K_d \geq 10^{-2}$ M) for the "missing piece", indicating that incorporation of 2 binding determinants within a single molecule permits an additional 7–12 kcal/mol of intrinsic binding energy to be manifested as observed binding energy. These results are consistent with earlier indications that adenosine deaminase may use 10.5 kcal/mol of the intrinsic free energy of binding of the two substrates to place them in positions appropriate for reaction at the active site, overcoming the unfavorable entropy change of −35 eu for the equilibrium of 1,6-hydration of purine ribonucleoside and reducing the equilibrium constant for attainment of the transition state in deamination of adenosine. Thus, adenosine deaminase may achieve up to 8 orders of magnitude of its catalytic power by converting the nonenzymatic, bimolecular, hydration reaction to a monomolecular reaction at its active site. Several new 6-substituted 1,6-dihydropurine ribonucleosides, prepared by photoaddition of formate and by low-temperature addition of organolithium reagents to a derivative of purine ribonucleoside, exhibited K_i values of 9–1400 μM against adenosine deaminase, in accord with the active site's considerable tolerance of bulky leaving groups in substrates. Inhibition by one diastereomer of 6-carboxy-1,6-dihydropurine ribonucleoside was found to be time-dependent, progressing from a weakly bound to a more strongly bound complex.

Transition-state analog inhibitors offer a useful structural tool for studying enzyme mechanisms, because examination of their inhibitory complexes can supply evidence concerning binding interactions that are likely to be involved in catalysis. In the case of adenosine deaminase, the competitive inhibitor nebularine, lacking a substituent at C-6, is not bound in the form that is abundant in free solution (**1**, Scheme I), but rather as a 1,6-addition compound (Kurz & Frieden, 1987). This bound addition compound has been identified as nebularine 1,6-hydrate (**2**, Scheme), resembling the hydrated reaction intermediate in brackets, by comparison of its UV and NMR spectra with those of the relatively stable analog in which its N-1 hydrogen atom has been replaced by a methyl group (Jones et al., 1989), and X-ray diffraction from single crystals of the enzyme–inhibitor complex confirms this assignment (Wilson et al., 1991). The K_i value of nebularine 1,6-hydrate, 3×10^{-13} M (Jones et al., 1989), is much lower than that of the product inosine, 1×10^{-4} M, and is not far removed from the maximal dissociation constant (ca. 10^{-16} M) of the enzyme–substrate complex in the transition state for deamination of adenosine, estimated from the rate enhancement that this enzyme products (Frick et al., 1987).

Scheme I: Reaction Catalyzed by Adenosine Deaminase, Proceeding through a Hydrated Tetrahedral Intermediate (**3**), and Competitive Inhibitors Formed by Covalent Hydration (**2**) and Photochemical Reduction (**4**) of Purine Ribonucleoside (**1**)[a]



[a] R = ribose.

In principle, adenosine deaminase might scavenge nebularine 1,6-hydrate directly from solution. The onset of

† This work was supported by Research Grant GM-18325 from the National Institutes of Health.

* To whom correspondence should be addressed.

‡ Present address: Department of Molecular and Cell Biology, Pennsylvania State University, University Park, PA 16802.

§ Present address: Glaxo Research, Glaxo, Inc., 5 Moore Dr., Research Triangle Park, NC 27709.

0006-2960/92/0431-7356$03.00/0

inhibition is much too rapid, however, to be understood in terms of any mechanism that would require that the enzyme combine directly with a species as rare as nebularine 1,6-hydrate (Kurz & Frieden, 1987). Thus, it seems reasonable to infer that enzyme-bound **2** is usually formed by hydration of nebularine within the active site, in a rapid reaction that serves as an analogue of a partial reaction in deamination of adenosine. If the enzymatic hydration of nebularine resembles a partial reaction in enzymatic hydrolysis of adenosine, then it would be of interest to understand the forces involved in stabilizing nebularine 1,6-hydrate, since these forces are presumably analogous to the forces used by the enzyme in stabilizing intermediates in the hydrolysis of adenosine. In an initial approach to analyzing these forces, we observed that 1,6-dihydropurine ribonucleoside (**4**), in which a hydrogen atom is substituted for the hydroxyl group at C-6 of **2**, was bound relatively weakly (K_i = 5.4 × 10^{-6} M) (Kati & Wolfenden, 1989a). Comparison of the binding affinities of **2** and **4** showed that the 6-hydroxyl group appeared to contribute –9.8 kcal/mol to the binding affinity of hydrate **2**. The remarkable magnitude of this contribution to binding affinity suggested that the 6-hydroxyl group must form one or more H-bonds to charged groups on the enzyme (Kati & Wolfenden, 1989a); this conjecture has now been verified by X-ray diffraction from single crystals of **2** bound to adenosine deaminase (Wilson et al., 1991).

At the enzyme's active site, the equilibrium constant for hydration of nebularine is at least 10^8-fold more favorable than in free solution, and the effective concentration of water at the active site is approximately 10^{10} M (Jones et al., 1989). In free solution, the unfavorable position of this equilibrium arises mainly from a large, unfavorable entropy of hydration, –35 cal deg^{-1} mol^{-1} (Kati & Wolfenden, 1989b), similar to the entropy of activation (–37 cal deg^{-1} mol^{-1}) for the uncatalyzed hydrolysis of adenosine in water (Frick et al., 1987); this high thermodynamic hill is evidently leveled within the active site. If substrate water is assumed to be weakly bound, and if either water or **4** can be bound without greater steric hindrance than **2**, then the relative binding affinities of **2** and **4** suggest that entropic effects (Page & Jencks, 1971; Jencks, 1981) make a very large contribution to the binding affinity of **2** (Kati & Wolfenden, 1989b). The present paper describes an effort to evaluate the contribution of entropic effects in other ways, comparing the enzyme's affinity for the whole (compound **2**, or its stable analog 2′-deoxycoformycin) with the sum of its parts, obtained by cleaving the parent compound at various points.

In addition, it seemed desirable to prepare stable analogues of **2** that might take advantage of the H-bonding interactions mentioned above. We report here the synthesis and inhibitory properties of both isomers of 6-carboxy-1,6-dihydropurine ribonucleoside (**6**), prepared by photolysis of nebularine in ammonium formate. During our investigation of synthetic approaches to compound **6**, we discovered that the 1,6 carbon–nitrogen double bond of a protected derivative of nebularine was susceptible to addition of organolithium compounds at low temperatures, yielding various stable, 6-substituted-1,6-dihydropurine ribonucleosides, whose inhibitory properties were examined as a probe of the "leaving group" site.

EXPERIMENTAL PROCEDURES

Calf intestinal adenosine deaminase (type III), 1-methyl β-D-ribofuranoside, thymidine, and purine ribonucleoside were obtained from Sigma Chemical Co. Nucleoside 2-deoxyribosyltransferase II (EC 2.4.2.6) was a gift from Dr. Steven Short (Burroughs Wellcome Co.). Silica gel chromatography was performed using silica gel, grade 60 (Merck). Reverse-phase high-pressure liquid chromatography (RP-HPLC) was carried out using a Whatman Partisil 10 ODS-2 M9 column (9.4 × 500 mm). In all cases, isomer A refers to the C-6 isomer that was eluted earlier on RP-HPLC, relative to its diastereomer. ^{1}H-NMR spectra were recorded at 200, 250, or 400 mHz, and a positive chemical shift was used to denote a resonance at lower shielding with respect to tetramethylsilane. Fast-atom-bombardment mass spectra were obtained with a VG70-250SEQ tandem MS–MS system operated in the LSIMS mode by Dr. Dean Marbury.

*Synthesis of 6-Carboxy-1,6-dihydropurine Ribonucleoside (**6a** and **-b**).* An aqueous solution (22 mL) containing 7 M ammonium formate and purine ribonucleoside (200 mg, 0.80 mmol) was transferred under a nitrogen atmosphere to a cylindrical quartz reaction vessel (5 × 40 cm), which was mounted on a Buchi rotary evaporator and thoroughly flushed with nitrogen. The reaction vessel was placed in a horizontal position and allowed to rotate for 2 h at a speed of about 200 rpm under a steady stream of N_2. The solution was filtered through a 2-μm filter, and then 2-mL aliquots were purified by RP-HPLC (methanol in water: 0% methanol from 0 to 16 min followed by a gradient from 0% to 70% methanol over the next 20 min). Two early-eluting peaks were isolated and separately lyophilized to yield compounds **6a** and **6b**.

6a (23 mg, 8.9%): ^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 250 mHz) δ 7.39 (s, 1 H, H8), 6.93 (s, 1 H, H2), 5.47 (d, 1 H, $J_{1'-2'}$ = 6.4 Hz, H1′), 4.91 (s, 1 H, H6), 4.43 (m, 1 H, H2′), 4.00 (m, 1 H, H3′), 3.89 (m, 1 H, H4′), 3.58–3.40 (m, 2 H, 5′-CH_2); UV (0.05 M potassium phosphate buffer, pH 7.5) λ_{max} = 302, ε = 3500; λ_{max} = 244, ε = 2100; FAB-MS (M + H)$^+$ = 299 (free acid) and 317 (NH_4^+ salt).

6b (25 mg, 9.6%): ^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 250 mHz) δ 7.40 (s, 1 H, H8), 6.92 (s, 1 H, H2), 5.47 (d, 1 H, $J_{1'-2'}$ = 6.5 Hz, H1′), 4.88 (s, 1 H, H6), 4.44 (m, 1 H, H2′), 4.00 (m, 1 H, H3′), 3.89 (m, 1 H, H4′), 3.60–3.42 (m, 2 H, 5′-CH_2); UV (0.05 M potassium phosphate buffer, pH 7.5) λ_{max} = 302, ε = 3640; λ_{max} = 244, ε = 2550; FAB-MS (M + H)$^+$ = 299 (free acid) and 317 (NH_4^+ salt).

*Synthesis of 2′,3′,5′-Tris(tert-butyldimethylsilyl)purine Ribonucleoside (**7**).* Imidazole (6.8 g, 100 mmol) and purine ribonucleoside (2.0 g, 7.94 mmol) were dissolved in 8 mL of dry DMF in a dry 50-mL round-bottom flask at room temperature. *tert*-Butyldimethylsilyl chloride (7.3 g, 50 mmol) was added, and then the mixture was stirred at room temperature for 40 h. The precipitate was collected using Whatman No. 1 filter paper and a Buchner funnel and then washed with 5 × 60 mL water. The solid was air dried for 4 h, sublimed at 1 mmHg and 35 °C for 90 min, and then dried over P_2O_5 at reduced pressure to give **7** (4.33 g, 91%).

^{1}H-NMR ($CDCl_3$, 200 mHz) δ 9.11 (s, 1 H, H6), 8.94 (s, 1 H, H2), 8.80 (s, 1 H, H8), 6.11 (d, $J_{1'-2'}$ = 4.7 Hz, 1 H, H1′), 4.61 ("t", $J_{1'-2'}$ = 4.7 Hz, $J_{2'-3'}$ = 4.2 Hz, H2′), 4.31 ("t", $J_{2'-3'}$ = 4.2 Hz, $J_{3'-4'}$ = 3.7 H, H3′), 4.13 (m, 1 H, H4′), 4.02–3.75 (m, 2 H, 5′-CH_2), 0.94–0.75 (m, 27 H, 3 × *tert*-butyl-Si), 0.08 to –0.02 (m, 18 H, 3 × CH_3SiCH_3).

*Synthesis of 6-n-Butyl-1,6-dihydropurine Ribonucleoside (**8a** and **-b**).* Into a dry 15-mL three-necked flask was added **7** (597 mg, 1 mmol). The flask was placed under a nitrogen atmosphere, and then 4.0 mL of dry THF was added. The flask was immersed in a dry ice/acetone bath, and after cooling, 2.5 M *n*-butyllithium (1.70 mL, 4.25 mmol) was added over a period of about 30 s. After stirring for 15 min at –78 °C,

the burgundy reaction mixture was hydrolyzed with 0.30 mL of glacial acetic acid. The solution was stirred vigorously while warming to room temperature. The residue was dissolved in 30 mL of ether and extracted with 30 mL of saturated $NaHCO_3$. The ether layer was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation. Silica gel chromatography (2.5 × 60 cm) in 1:1 ether/$CHCl_3$ yielded two cleanly separated diastereomers.

Silylated **8a** (177 mg, 27.0%): ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.20 (s, 1 H, H8), 6.91 (d, J = 3.3 Hz, 1 H, H2), 6.00 (br s, 1 H, NH), 5.48 (d, 1 H, $J_{1'-2'}$ = 8.0 Hz, H1'), 4.95 (t, J = 5.1 Hz, 1 H, H6), 4.72 (d of d, $J_{1'-2'}$ = 8.0 Hz, $J_{2'-3'}$ = 4.6 Hz, 1 H, H2'), 4.17 (m, 1 H, H3'), 4.00 (m, 1 H, H4'), 3.78–3.50 (m, 2 H, 5'-CH_2), 1.70 (br m, 2 H, $CH_2CH_2CH_2CH_3$), 1.37 (br m, 4 H, $CH_2CH_2CH_2CH_3$), 0.90–0.75 (m, 31 H, $CH_2CH_2CH_2CH_3$ + 3 × *tert*-butyl-Si) 0.07 to –0.01 (m, 18 H, 3 × CH_3SiCH_3); mp 99–101 °C.

Silylated **8b** (184 mg, 28.1% as a syrup): ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.19 (s, 1 H, H8), 6.89 (d, J = 4.0 Hz, 1 H, H2), 5.94 (br s, 1 H, NH), 5.47 (d, 1 H, $J_{1'-2'}$ = 7.9 Hz, H1'), 4.92 (t, J = 5.6 Hz, 1 H, H6), 4.72 (d of d, $J_{1'-2'}$ = 7.9 Hz, $J_{2'-3}$ = 4.6 Hz, 1 H, H2'), 4.16 (m, 1 H, H3'), 4.01 (m, 1 H, H4'), 3.80–3.50 (m, 2 H, 5'-CH_2), 1.75 (br m, 2 H, $CH_2CH_2CH_2CH_3$), 1.34 (br m, 4 H, $CH_2CH_2CH_2CH_3$), 0.90–0.70 (m, 31 H, $CH_2CH_2CH_2CH_3$ + 3 × *tert*-butyl-Si), 0.01 to –0.04 (m, 18 H, 3 × CH_3SiCH_3).

Into a 10-mL round-bottom flask containing silylated **8a** (168 mg, 0.256 mmol) was added 1 M tetrabutylammonium fluoride in THF (1.25 mL, 1.25 mmol), and the reaction mixture was stirred gently for 30 min. The reaction was quenched with 1 mL of water and filtered through a 2-μm filter, and aliquots were then purified by RP-HPLC (methanol in water: gradient from 10% to 40% methanol from 0 to 60 min, 40% methanol from 60 to 80 min). The desired fractions were pooled, rotary evaporated to a small volume, and lyophilized to yield **8a** as a fluffy white solid (61.5 mg, 77%).

^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 250 mHz) δ 7.42 (s, 1 H, H8), 6.90 (d, becomes s with D_2O exchange, 1 H, H2), 5.48 (d, 1 H, $J_{1'-2'}$ = 6.6 Hz, H1'), 4.80 (t, J = 5.2 Hz, 1 H, H6), 4.36 (d of d, $J_{1'-2'}$ = 6.6 Hz, $J_{2'-3'}$ = 5.2 Hz, 1 H, H2'), 3.99 (d of d, $J_{2'-3'}$ = 5.2 Hz, $J_{3'-4'}$ = 2.3 Hz, 1 H, H3'), 3.85 (m, 1 H, H4'), 3.59–3.40 (m, 2 H, 5'-CH_2), 1.58 (br m, 2 H, $CH_2CH_2CH_2CH_3$), 1.34 (br m, 4 H, $CH_2CH_2CH_2CH_3$), 0.85 (t, J = 6.9 Hz, 3 H, $CH_2CH_2CH_2CH_3$); UV (0.05 M potassium phosphate buffer, pH 7.5) λ_{max} = 298, ϵ = 4800; λ_{max} = 248, ϵ = 2100; FAB-MS $(M + H)^+$ = 311.

Silylated **8b** (164 mg, 0.25 mmol) was treated with 1 M tetrabutylammonium fluoride in THF (1.0 mL, 1 mmol) and the products were worked up in a manner analogous to that for silylated isomer A, to yield **8b** (45.9 mg, 60%) as a fluffy white solid.

^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 250 mHz) δ 7.43 (s, 1 H, H8), 6.90 (d, becomes s with D_2O exchange, 1 H, H2), 5.47 (d, 1 H, $J_{1'-2'}$ = 6.8 Hz, H1'), 4.81 (t, J = 5.0 Hz, 1 H, H6), 4.37 (d of d, $J_{1'-2'}$ = 6.8 Hz, $J_{2'-3'}$ = 5.0 Hz, 1 H, H2'), 3.98 (d of d, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 1.8 Hz, 1 H, H3'), 3.89 (m, 1 H, H4'), 3.63–3.41 (m, 2 H, 5'-CH_2), 1.60 (br m, 2 H, $CH_2CH_2CH_2CH_3$), 1.35 (br m, 4 H, $CH_2CH_2CH_2CH_3$), 0.88 (t, J = 6.8 Hz, 3 H, $CH_2CH_2CH_2CH_3$); UV (0.05 M potassium phosphate buffer, pH 7.5) λ_{max} = 298, ϵ = 4650; λ_{max} = 246, ϵ = 1850; FAB-MS $(M + H)^+$ = 311. Note: NMR spectroscopy also showed 10–20% contamination by tetrabutylammonium salts in final products of **8a** and **-b**. Analytical samples were further purified by RP-HPLC, which gave the above ^{1}H-NMR spectra.

*Synthesis of 6-[(Diethoxyphosphoryl)methyl]-1,6-dihydropurine Ribonucleoside (**9a** and **-b**).* A 15-mL 3-necked flask containing 3.7 mL of THF and diethyl methylphosphonate (0.40 mL, 2.7 mmol) under a dry, nitrogen atmosphere was placed into a –78 °C bath. After cooling, 2.5 M *n*-butyllithium (1.0 mL, 2.5 mmol) was added dropwise with stirring (Corey & Kwiatkowski, 1966). The solution was allowed to stir for 5 min at –78 °C, and then it was transferred via syringe to a 25-mL three-neck flask containing **7** (597 mg, 1 mmol) in 3.0 mL of THF under a dry, nitrogen atmosphere at –78 °C. The reaction mixture was allowed to stir for 15 min at –78 °C before it was hydrolyzed with 0.20 mL of glacial acetic acid. The solution was stirred vigorously while warming to room temperature, at which time it was diluted with 60 mL of ether and extracted with 30 mL of saturated $NaHCO_3$. The ether layer was dried with $MgSO_4$, filtered, and concentrated by rotary evaporation. Silica gel column chromatography (2.5 × 60 cm) with 2:1 $CHCl_3$/ether yielded two fractions as syrups. One fraction contained silylated **9a** in virtually pure form (183 mg, 24.5%) whereas the second fraction contained silylated **9a** (60.3 mg, 8.1%) and silylated **9b** (173 mg, 23.2%) as judged by ^{1}H-NMR.

Silylated **9a**: ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.31 (s, 1 H, H8), 6.79 (d, J = 3.7 Hz, 1 H, H2), 6.46 (d, J = 3.7 Hz, 1 H, NH), 5.53 (d, 1 H, $J_{1'-2'}$ = 6.0 Hz, H1'), 5.25 (m, 1 H, H6), 4.48 (d of d, $J_{1'-2'}$ = 6.0 Hz, $J_{2'-3'}$ = 4.4 Hz, 1 H, H2'), 4.10 (d of d, $J_{2'-3'}$ = 4.4 Hz, $J_{3'-4'}$ = 2.6 Hz, 1 H, H3'), 4.02–3.88 (m, 5 H, 2 × OCH_2CH_3 and H4'), 3.84–3.56 (m, 2 H, 5'-CH_2), 2.48–2.34 (m, 1 H, 6-C*H*P), 2.11–1.95 (m, 1 H, 6-C*H*P), 1.19 (m, 6 H, 2 × OCH_2CH_3), 0.80–0.69 (m, 27 H, 3 × *tert*-butyl-Si), –0.04 to –0.27 (m, 18 H, 3 × CH_3SiCH_3).

Silylated **9b**: ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.35 (s, 1 H, H8), 6.83 (d, J = 3.8 Hz, 1 H, H2), 6.49 (d, J = 3.8 Hz, 1 H, NH), 5.59 (d, 1 H, $J_{1'-2'}$ = 6.2 Hz, H1'), 5.30 (m, 1 H, H6), 4.50 (d of d, $J_{1'-2'}$ = 6.2 Hz, $J_{2'-3'}$ = 4.5 Hz, 1 H, H2'), 4.15 (d of d, $J_{2'-3'}$ = 4.5 Hz, $J_{3'-4'}$ = 2.3 Hz, 1 H, H3'), 4.10–3.92 (m, 5 H, 2 × OCH_2CH_3 and H4'), 3.88–3.62 (m, 2 H, 5'-CH_2), 2.53–2.39 (m, 1 H, 6-C*H*P), 2.18–2.07 (m, 1 H, 6-C*H*P), 1.25 (m, 6 H, 2 × OCH_2CH_3), 0.84–0.73 (m, 27 H, 3 × *tert*-butyl-Si), 0.01 to –0.24 (m, 18 H, 3 × CH_3SiCH_3).

Into a 10-mL flask containing silylated **9a** (183 mg, 0.245 mmol) was added 1 M tetrabutylammonium fluoride in THF (1.2 mL, 1.2 mmol). The reaction was allowed to stir for 30 min at room temperature. The reaction was quenched with 1 mL of 0.05 M sodium phosphate, pH 8.4, buffer and rotary evaporated to remove THF. The solution was then chromatographed on a 1- × 10-cm column of Bio-Rad AG50W-X8 (100–200 mesh, Na^+ form) using the same phosphate buffer for elution. Fractions were pooled and filtered through a 2-μm filter, and then aliquots were purified by RP-HPLC (methanol in water: gradient from 0% to 30% methanol from 0 to 60 min, 30% methanol from 60 to 70 min). The fraction containing the mixture of silylated **9a** and **9b** was treated with 1.4 mL of 1 M tetrabutylammonium fluoride in THF and worked up in an analogous manner except that the RP-HPLC methanol gradient was 0–30% from 0 to 80 min. Fractions containing **9a** or **9b** were pooled appropriately, concentrated by rotary evaporation, and lyophilized.

9a (84.6 mg, 64.5%): ^{1}H-NMR (D_2O, 400 mHz) δ 7.63 (s, 1 H, H8), 7.10 (s, 1 H, H2), 5.70 (d, 1 H, $J_{1'-2'}$ = 6.7 Hz, H1'), 5.45 (m, 1 H, H6), 4.61 (d of d, $J_{1'-2'}$ = 6.7 Hz, $J_{2'-3'}$ = 5.0 Hz, 1 H, H2'), 4.30 (d of d, $J_{2'-3'}$ = 5.0 Hz, $J_{3'-4'}$ = 2.0 Hz, 1 H, H3'), 4.21 (m, 1 H, H4'), 4.05–3.94 (m, 4 H, 2 × OCH_2CH_3), 3.84–3.71 (m, 2 H, 5'-CH_2), 2.43–2.29 (m, 2 H, 6-CH_2-P), 1.22 (m, 6 H, 2 × OCH_2CH_3); UV (0.05 M

potassium phosphate buffer, pH 7.5) $\lambda = 296$, $\epsilon = 3900$; $\lambda = 244$, $\epsilon = 1830$; FAB-MS $(M + H)^+ = 405$.

9b (59.9 mg, 64.5%): ^{1}H-NMR (D_2O, 250 mHz) δ 7.66 (s, 1 H, H8), 7.12 (s, 1 H, H2), 5.71 (d, 1 H, $J_{1'-2'} = 6.8$ Hz, H1'), 5.45 (m, 1 H, H6), 4.59 (d of d, $J_{1'-2'} = 6.8$ Hz, $J_{2'-3'} = 5.2$ Hz, 1 H, H2'), 4.31 (d of d, $J_{2'-3'} = 5.2$ Hz, $J_{3'-4'} = 2.4$ Hz, 1 H, H3'), 4.20 (m, 1 H, H4'), 4.06–3.93 (m, 4 H, 2 × OCH_2CH_3), 3.85–3.70 (m, 2 H, 5'-CH_2), 2.45–2.33 (m, 2 H, 6-CH_2P), 1.23 (m, 6 H, 2 × OCH_2CH_3); UV (0.05 M potassium phosphate buffer, pH 7.5) $\lambda = 296$, $\epsilon = 4300$; $\lambda = 246$, $\epsilon = 1920$; FAB-MS $(M + H)^+ = 405$.

*Synthesis of 6-tert-Butyl-1,6-dihydropurine Ribonucleoside (**10a** and **-b**).* The synthesis and reaction mixture workup was conducted using the conditions described for the synthesis of **8a** and **-b** except that 1.7 M *tert*-butyllithium (1.5 mL, 2.55 mmol) was used instead of *n*-butyllithium. Silica gel chromatography (2.5 × 60 cm) using 2:1 ether/hexane yielded two completely resolved diastereomers as syrups.

Silylated **10a** (157 mg, 23.9%): ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.41 (s, 1 H, H8), 6.97 (d, $J = 3.0$ Hz, 1 H, H2), 5.70 (d, 1 H, $J_{1'-2'} = 6.3$ Hz, H1'), 5.64 (br s, 1 H, NH), 4.59 (d of d, $J_{1'-2'} = 6.3$ Hz, $J_{2'-3'} = 4.4$ Hz, 1 H, H2'), 4.53 (s, 1 H, H6), 4.20 (d of d, $J_{2'-3'} = 4.4$ Hz, $J_{3'-4'} = 2.2$ Hz, 1 H, H3'), 4.00 (m, 1 H, H4'), 3.92–3.66 (m, 2 H, 5'-CH_2), 0.90 (m, 36 H, 3 × *tert*-butyl-Si, 6-*tert*-butyl), 0.07 to –0.20 (m, 18 H, 3 × CH_3SiCH_3).

Silylated **10b** (70 mg, 10.7% as a syrup): ^{1}H-NMR ($CDCl_3$, 250 mHz) δ 7.44 (s, 1 H, H8), 7.01 (d, $J = 3.7$ Hz, 1 H, H2), 5.74 (d, 1 H, $J_{1'-2'} = 6.7$ Hz, H1'), 5.41 (br s, 1 H, NH), 4.56–4.50 (m, 2 H, H6, H2'), 4.19 (d of d, $J_{2'-3'} = 4.6$ Hz, $J_{3'-4'} = 2.1$ Hz, 1 H, H3'), 4.01 (m, 1 H, H4'), 3.86–3.68 (m, 2 H, 5'-CH_2), 0.94–0.80 (m, 36 H, 3 × *tert*-butyl-Si, 6-*tert*-butyl), 0.08 to –0.16 (m, 18 H, 3 × CH_3SiCH_3).

Silylated **10a** and **10b** were treated with 1.02 and 0.49 of mL of 1 M tetrabutylammonium fluoride, respectively, for 30 min at room temperature. Reactions were quenched with 1 mL of 0.05 M sodium phosphate, pH 8.4 buffer, concentrated by rotary evaporation, and subjected to ion-exchange chromatography as described for **9a** and **-b**. RP-HPLC (methanol in water: gradient from 10% to 50% methanol from 0 to 60 min) was used for the purification of the deprotected nucleosides. Active fractions were concentrated and lyophilized to yield **10a** and **10b** as fluffy white powders.

10a (53.7 mg, 72.4%): ^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 200 mHz) δ 7.46 (s, 1 H, H8), 6.97 (d, becomes s with D_2O exchange, 1 H, H2), 5.50 (d, 1 H, $J_{1'-2'} = 6.7$ Hz, H1'), 4.44 (s and d of d partially overlapped, 2 H, H6, H2'), 4.00 (d of d, $J_{2'-3'} = 4.8$ Hz, $J_{3'-4'} = 2.0$ Hz, 1 H, H3'), 3.87 (m, 1 H, H4'), 3.59–3.41 (m, 2 H, 5'-CH_2), 0.84 (s, 9 H, 6-*tert*-butyl); UV (0.05 M potassium phosphate buffer, pH 7.5): $\lambda = 296$, $\epsilon = 4040$; $\lambda = 244$, $\epsilon = 1800$; FAB-MS $(M + H)^+ = 311$.

10b (22.0 mg, 61%): ^{1}H-NMR (Me_2SO-d_6, D_2O exchange, 250 mHz) δ 7.44 (s, 1 H, H8), 6.97 (d, becomes s with D_2O exchange, 1 H, H2), 5.50 (d, 1 H, $J_{1'-2'} = 6.7$ Hz, H1'), 4.42–4.33 (s and d of d partially overlapped, 2 H, H6, H2'), 3.99 (m, 1 H, H3'), 3.88 (m, 1 H, H4'), 3.62–3.41 (m, 2 H, 5'-CH_2), 0.84 (s, 9 H, 6-*tert*-butyl); UV (0.05 M potassium phosphate buffer, pH 7.5): $\lambda_{max} = 296$, $\epsilon = 3720$; $\lambda = 245$, $\epsilon = 1720$; FAB-MS $(M + H)^+ = 311$.

*Synthesis of 4-(Hydroxymethyl)-1-(2'-deoxy-β-D-ribofuranosyl)imidazole (**11**).* A solution (1.0 mL) containing 4-(hydroxymethyl)imidazole hydrochloride (20 μmol), thymidine (20 μmol), nucleoside deoxyribosyltransferase II (2.8 mg), and potassium phosphate (100 mM, pH 6.0) was allowed to stand for 2 h at 25 °C. Ethanol (95%, 4 mL) as added to the solution, and the denatured protein was removed by centrifugation. The solution was then reduced to a volume of 3 mL, and the nucleoside was isolated from unreacted starting materials and thymine by semipreparative RP-HPLC (50 mM potassium phosphate, pH 6.0 buffer containing 5% methanol at a flow rate of 4.0 mL/min). The yield, based on the extinction coefficient of 4-(hydroxymethyl)imidazole ($\epsilon = 5840$ at $\lambda = 213$ nm and pH 7.0) was 7.9 μmol (40%). This yield was consistent with the concentration determined independently by comparison of integrated intensities of ^{1}H-NMR signals of the nucleoside, using acetonitrile as an internal standard. The imidazole ring was judged to be 1,4-disubstituted rather than 1,5-disubstituted on the basis of comparisons of the value of the value of the cross-ring coupling constant with those from a series of 1,4- and 1,5-disubstituted imidazoles (Rapaport & Matthews, 1973). Moreover, nucleoside deoxyribosyltransferase would not be expected to form a 1,5-disubstituted product, because the enzyme does not tolerate purine substrates with substituents at the 3 position (Cardinaud & Holguin, 1975).

^{1}H-NMR (D_2O, 200 mHz) δ 7.90 (s, 1 H, im-H), 7.25 (s, 1 H, im-H), 6.05 (t, 1 H, H1'), 4.40 (m, 1 H, H3'; overlapping s, 2 H, CH_2), 3.95 (m, 1 H, H4'), 3.60 (m, 2 H, H5'), 2.43 (m, 2 H, H2').

^{1}H-NMR (Me_2SO-d_6) δ 7.68 (d, 1 H, H5, $J = 1.21$ Hz), 7.11 (s, 1 H, H2), 5.93 (t, 1 H, H1'), 5.20 (br s, 1 H, O*H*), 4.80 (br s, 2 H, 2 × O*H*), 4.25 (m, 1 H, H3'; overlapping s, 2 H, CH_2), 3.72 (m, 1 H, H4'), 3.43 (d, 2 H, H5'), 2.20 (m, 2 H, H2').

Synthesis of 4-Formyl-1-(2'-deoxy-β-D-ribofuranosyl)imidazole. A solution (1.0 mL) containing 4-formylimidazole hydrochloride (20 μmol), prepared by the procedure of Papadopoulos et al. (1966), thymidine (20 μmol), nucleoside deoxyribosyltransferase II (2.8 mg), and potassium phosphate (100 mM, pH 6.0) was allowed to stand for 2 h at 25 °C. Ethanol (95%, 4 mL) was added to the solution, and the denatured protein was removed by centrifugation. The solution was reduced to a volume of 3 mL, and the nucleoside was isolated from unreacted starting materials and thymine by semipreparative RP-HPLC, using 50 mM potassium phosphate, pH 6.0, with a linear gradient from 0% to 5% methanol at a flow rate of 4.0 mL/min. The yield, based on the extinction coefficient of 4-formylimidazole ($\epsilon = 11\,700$ at $\lambda = 256$ nm in 95% ethanol) was 14 μmol (70%).

^{1}H-NMR (D_2O, 200 mHz) δ 2.38 (m, 2 H, 2'-H, 2''-H), 3.53 (m, 2 H, 5'- and 5''-H), 3.90 (m, 1 H, 4'-H), 4.32 (q, 1 H, 3'-H), 6.13 (t, 1 H, 1'-H), 7.85 (s, 1 H, im), 8.03 (s, 1 H, im), 9.45 (s, 1 H, aldehyde).

^{1}H-NMR (Me_2SO-d_6) δ 2.30 (m, 2 H, 2'-H, 2''-H), 3.52 (m, 2 H, 5'- and 5''-H), 3.82 (m, 1 H, 4'-H), 4.30 (t, 1 H, 3'-H), 6.10 (t, 1 H, 1'-H), 8.08 (d, 1 H, im, $J = 1.3$ Hz), 8.25 (d, 1 H, im, $J = 1.3$ Hz), 9.70 (s, 1 H, aldehyde).

Enzyme Inhibition. Initial velocities of the enzymatic deamination of adenosine in 0.05 M potassium phosphate, pH 7.5 buffer were measured spectrophotometrically at 266 nm using a 1-cm path length cuvette. In order to determine values for the inhibitor dissociation constants, initial velocities from a minimum of four different substrate concentrations at each of four different inhibitor concentrations were measured. Slopes of double-reciprocal plots were then replotted as a function of changing inhibitor concentration, and K_i values were obtained by linear regression analysis (Segel, 1975). Initial velocities were measured from 7–10 inhibitor concentrations at a single, low substrate concentration for investi-

Table I: Substrates and Inhibitors of Calf Intestinal Adenosine Deaminase, at pH 7 and 25 °C[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_m	ref
adenosine	35	190	1.00	this work
2′-deoxyadenosine	25	167	1.24	Haertle, 1988
3′-deoxyadenosine	25	99	0.7	Zielke & Suelter, 1971
2′,3′-dideoxyadenosine	100	234	0.43	Haertle, 1988
5′-deoxyadenosine	450	0.051	2.0×10^{-5}	this work
adenine	150	0.0027	3.3×10^{-6}	Wolfenden et al., 1969

inhibitor	K_i (μM)		
inosine	160		Wolfenden, 1969
nebularine[b] (**1**)	≫2.6		Kurz & Frieden, 1987
nebularine 1,6-hydrate[c] (**2**)	3×10^{-7}		Jones et al., 1989
2′-deoxynebularine[b] (**1′**)	≫2.7		this work
2′-deoxynebularine 1,6-hydrate[c] (**2′**)	3.1×10^{-7}		this work
1,6-dihydronebularine (**4**)	5.4		Kati & Wolfenden, 1989a
coformycin (**5**)	1×10^{-5}		Nakamura et al., 1976
deoxycoformycin (**5′**)	2.5×10^{-6}		Agarwal et al., 1977
coformycin aglycon (**14**)	33		Hanvey, 1983
4-(hydroxymethyl)-1-(2′-deoxyribofuranosyl)imidazole (**11**)	21		this work
4-formyl-1-(2′-deoxyribofuranosyl)imidazole[d]	27		this work
4-(hydroxyethyl)-5-methyl-1-(2′-deoxyribofuranosyl)imidazole (**13**)	60		Montgomery et al., 1985
6-carboxy-1,6-dihydropurine ribonucleoside (**6**)	90	isomer A[f]	this work
	84 (9.3)[e]	isomer B	this work
6-*n*-butyl-1,6-dihydropurine ribonucleoside (**8**)	1360	isomer A	this work
	48	isomer B	this work
6-*tert*-butyl-1,6-dihydropurine ribonucleoside (**10**)	850	isomer A	this work
	107	isomer B	this work
6-[(diethoxyphosphoryl)methyl]-1,6-dihydropurine ribonucleoside (**9**)	1160	isomer A	this work
	830	isomer B	this work
6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside[g] (**16**)	0.76		Evans & Wolfenden, 1970
methanol	1.3×10^6		Ronca & Ronca-Testoni, 1969
imidazole	2.5×10^4		Ronca & Ronca-Testoni, 1969
1-methyl β-D-ribofuranoside	1.0×10^5		this work
D-ribose	4.5×10^4		this work

[a] Compound numbers (boldface) refer to structures in Figure 1. [b] Minimal value for K_i, since the unhydrated form is the major species in solution, but spectra indicate that this compound is bound almost entirely as the rare 1,6-hydrate. [c] Based on the K_i value observed for the major unhydrated species, multiplied by the equilibrium constant for 1,6-hydration (Jones et al., 1989). X-ray crystallography shows that nebularine 1,6-hydrate is bound in the 6*R* configuration (Wilson et al., 1991). [d] May be bound either intact or as the *gem*-diol formed by hydration of the aldehydic group. [e] Values determined from initial and, in parentheses, steady-state velocities (see text). [f] For each pair of isomers, "A" was the isomer eluted first by reverse-phase HPLC. [g] The absolute configuration of this isomer has been established as 6*S* by X-ray crystallography (Shimazaki et al., 1983).

gations of the inhibition by compounds **8a**, **9a** and **-b**, **10a**, 1-methyl β-D-ribofuranoside, and D-ribose. Reciprocal velocities were plotted as a function of inhibitor concentration, and K_i values were then determined by the method of Dixon (1953) assuming competitive inhibition.

All compounds were tested for time-dependent inhibition by preincubating enzyme and inhibitor for 20–60 min prior to starting the reactions by the addition of adenosine. Only inhibitor **6b** showed evidence of time-dependent inhibition. The overall K_i value for the time-dependent inhibitor **6b** was determined using the protocol described above, except that steady-state velocities were measured after the enzyme and inhibitor had been preincubated for 20 min. Progress curves started by the addition of enzyme to solutions of adenosine and **6b** were fitted to eq 1, using the nonlinear regression program of Duggleby (1984). The individual rate and equilibrium constants were calculated as described under Results.

RESULTS

Inhibition of Adenosine Deaminase by Components of Nebularine 1,6-Hydrate (2) or 2′-Deoxycoformycin (5). The results of the present investigation are shown in Table I, along with some rate and inhibition constants from the literature. In Figure 1A, the binding affinities of several pairs of compounds are compared with those of parent compounds **2** and **5**. When the binding determinants of a ligand are present in two separate molecules, the sum of the free energies of binding of these two molecules, at some standard state, is not expected to be equivalent to the free energy of binding of the ligand itself, because of differences in translational and rotational entropy (Jencks, 1981). In the column to the right of Figure 1A, ΔG_S shows the difference in kcal/mol between the free energy of binding of the parent compound, ΔG_{AB}, and the sum of the free energies of binding of its constituent parts, ΔG_A and ΔG_B, as shown in the equation:

$$\Delta G_{A\text{-}B} = \Delta G_A + \Delta G_B - \Delta G_S \quad (1)$$

The first entries in Figure 1 compare the binding affinity of nebularine 1,6-hydrate (**2**) with those of 1,6-dihydropurine ribonucleoside (**4**) and methanol (or a water molecule). Methanol, which should be able to gain access to any site that normally binds the CHOH group of **2**, was bound very weakly, with a K_i value in the neighborhood of 1.3 M (Bauer et al. 1966; Ronca & Ronca-Testoni, 1969). This comparison suggests that the component parts fall short of the binding affinity for **2** by approximately 10 kcal/mol.[1] Assuming a K_d

[1] The location of the binding site is not known for small molecules such as methanol or imidazole. Thus, inhibitory methanol could well be bound at the site that is normally occupied by the 5′-hydroxyl group, rather than at the location normally occupied by the C-6 hydroxyl group of **2**. Likewise, imidazole could be bound at the site that normally binds the imidazole portion of the purine ring, rather than at the site that binds the N1–N3 section of the purine ring. If the observed K_i values reflect binding at these alternative sites, then binding at the locations of interest must presumably be weaker, in which case the value for ΔG_S will have been underestimated.

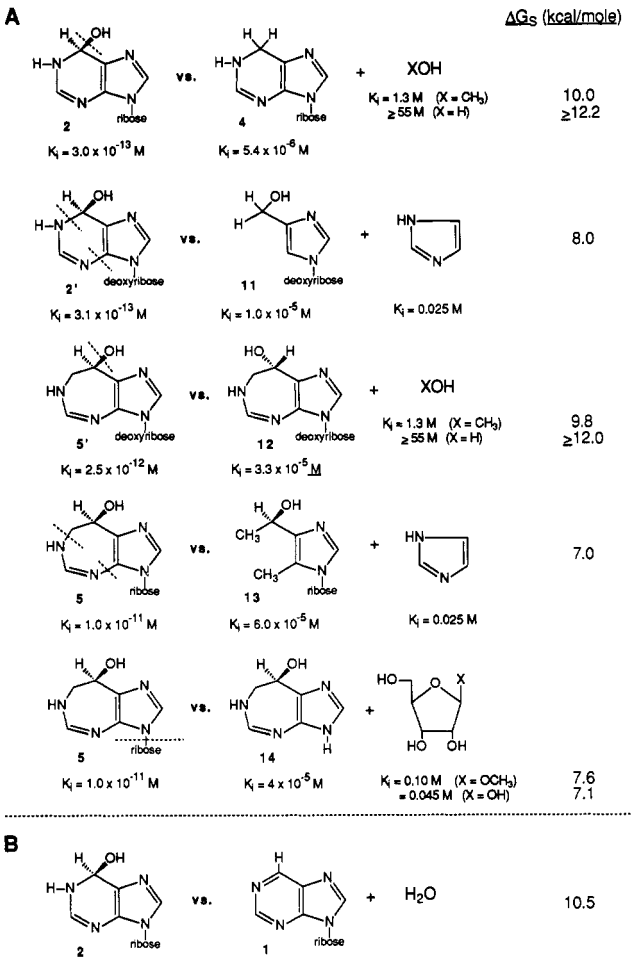


FIGURE 1: Estimation of entropic contributions to the binding of 6-hydroxy-1,6-dihydropurine ribonucleoside (**2**) and the stable analog 2′-deoxycoformycin (**5**) by adenosine deaminase. (A) Entropic contributions (ΔG_S) to the binding of **2** or **5** by adenosine deaminase were estimated by separating the binding determinants of **2** or **5** into two separate molecules, and comparing the sum of the binding free energies of the two parts relative to **2** or **5** according to eq 1. (B) Approximate entropic contribution ($T\Delta S$) to the binding of **2**, as compared with the substrate purine ribonucleoside (**1**) and a water molecule, inferred from the observation that hydration is unfavorable in solution (ΔS = −35 cal deg^{-1} mol^{-1}), but favorable at the active site (Kati & Wolfenden, 1989b).

value for water equivalent to its K_m value of at least 55 M (Dzingeleski and Wolfenden, in preparation), the sum of the free energies of binding of these constituents parts fell short of the binding affinity observed for **2** by at least 12 kcal/mol (Kati & Wolfenden, 1989a).

In the operation described by the next entry in Figure 1A, the binding determinants of **2** were separated by cleaving the pyrimidine ring at the 1–2 and 3–4 positions, to yield compound **11** and formamidine. Unlike **2**, formamidine is not conjugated and showed very weak inhibition at pH values where the enzyme is active. Imidazole, which is conjugated in way that resembles the "missing piece" of compound **11**, appears to serve as a competitive inhibitor at pH 7, with a K_i value of 0.05 M, yielding stronger inhibition at higher pH (Ronca & Ronca-Testoni, 1969). Since the pK_a value of imidazole is 7.0, the neutral form of imidazole appears to be bound by adenosine deaminase with a K_i value of approximately 0.025 M. This result, combined with a K_i value of 2.1 × 10^{-5} M for compound **11**, suggests a value of 8.0 kcal/mol for the contribution of ΔG_S to the binding of **2**.

The naturally occurring 8*R* isomer of 2′-deoxycoformycin (compound **5′**, Table I) inhibits adenosine deaminase with a

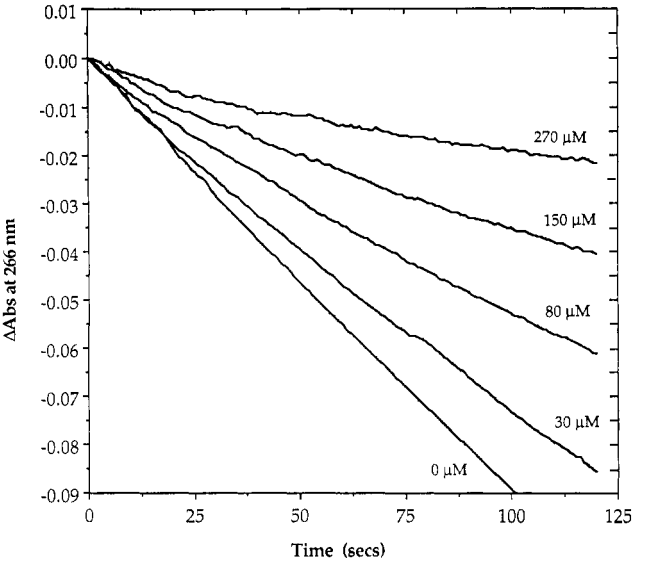


FIGURE 2: Progress curves for the hydrolysis of adenosine catalyzed by adenosine deaminse in the presence and absence of compound **6b**, as monitored spectrophotometrically at λ = 266 nm. Assays were initiated by the addition of adenosine deaminase to reaction mixtures which contained 50 μM adenosine in 0.05 M potassium phosphate buffer at pH 7.5. The final concentration of compound **6b** for each reaction is shown.

K_i value of 2.5 × 10^{-12} M (Agarwal et al., 1977). This potent inhibition is presumably a consequence of its structural similarity of **2**, so that **5′** might also be expected to exhibit large values for ΔG_S if its binding determinants were separated. The 8*R* isomer of 2′-deoxycoformycin (**5**) is a potent inhibitor of adenosine deaminase, but the 8*S* isomer (**12**) exhibits a K_i value of only 3.3 × 10^{-5} M (Schramm & Baker, 1985). Steric hindrance does not appear to offer a satisfactory explanation of the weak binding observed for **12**, because the 8(*S*)-hydroxyl group presumably occupies the position that would be occupied by the leaving group during substrate hydrolysis. Thus, the enzyme serves as an efficient catalyst for hydrolytic removal of several leaving groups (e.g., $NHCH_3$, NH_2NH_2) that are larger than the hydroxyl group (Chassy & Suhadolnik, 1967), and further evidence that the leaving group site is permissive is provided by the binding affinities of the new inhibitors to be discussed below. Accordingly, the behavior of compound **12** may be taken as a rough representation of the behavior to be expected of compound **5′**, with a hydrogen atom in place of the critical hydroxyl group. Comparison of the binding affinity of **5′** with the affinities of its component parts, as represented by compound **12** and methanol, suggests that connecting binding determinants by a covalent bond contributes 9.8 kcal/mol to the binding affinity of **5′**. If one assumes a K_d value for water of at least 55 M (Dzingeleski and Wolfenden, in preparation), then ΔG_S is at least 12.0 kcal/mol.

The next analysis, in which the binding determinants of coformycin are separated to form the truncated nucleoside **13** (Montgomery et al., 1985) and imidazole, suggests an entropic advantage of 7.0 kcal/mol.

Finally, cleaving the glycosidic bond of coformycin to form the aglycon **14** (Hanvey, 1987) and a sugar moiety, represented by D-ribose or 1-methyl β-D-ribofuranoside, suggests an entropic contribution of 7.1 and 7.6 kcal/mol, respectively, to

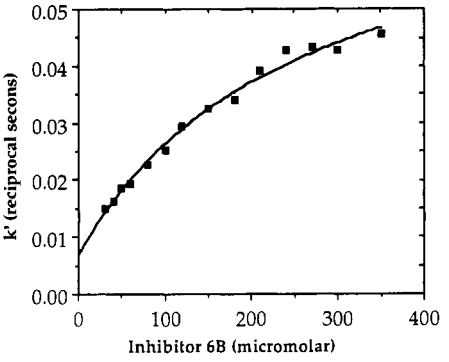


FIGURE 3: Effect of inhibitor **6b** concentration on the rate of approach to steady state (k'). Data from progress curves were fit to equation X to obtain values for k'. The solid line represents the best fit of these data to equation Y using [S] = 50 μM and K_m = 25 μM.

Scheme II: Alternative Kinetic Mechanisms for Time-Dependent Inhibition of Adenosine Deaminase by Compound **6b**

Mechanism A

$$E + I \underset{K_i}{\rightleftharpoons} EI \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} EI^*$$

Mechanism B

$$E + I \underset{K_i^*\ (slow)}{\rightleftharpoons} EI^*$$

$$K_i \uparrow\downarrow$$

EI

the binding of **5**.[2]

These results indicate that the entropic advantage of connecting the binding determinants of **2** or **5'** by a covalent bond amounts to at least 7–12 kcal/mol, regardless of where the connection is broken.

Photolysis of Purine Ribonucleoside in Ammonium Formate, and Kinetics of Inhibition of Adenosine Deaminase by 6-Carboxy-1,6-dihydropurine Ribonucleoside. Photolysis of purine ribonucleoside in a 7 M aqueous solution of ammonium formate resulted in addition of the elements of formate across the 1,6 carbon–nitrogen double bond to give both isomers of 6-carboxy-1,6-dihydropurine ribonucleoside (**6a** and **-b**). Purine ribonucleoside did not react in aqueous solutions containing ammonium formate at concentrations less than 0.5 M. RP-HPLC analysis showed that isomers **6a** and **-b**, individually, comprised about 15% of the total composition of the reaction mixture. Other components included unreacted purine ribonucleoside (~10%), unknown (~10%), and 1,6-dihydropurine ribonucleoside (~50%). The yield of 1,6-dihydropurine ribonucleoside was comparable with that obtained by reduction of purine ribonucleoside (Kati & Wolfenden, 1989b) or 6-chloropurine ribonucleoside (Kati & Wolfenden, 1991) with sodium borohydride and UV light. Light-dependent reduction of heteroaromatic compounds (e.g., flavins) in solutions containing electron-rich buffers has been reported previously (Frisell et al., 1958; Heelis et al., 1987).

The isomer that was eluted first from the RP-HPLC column (**6a**) proved to be a linear competitive inhibitor of adenosine deaminase with a K_i value of 90 μM, and no evidence of time-dependent inhibition was observed. In contrast, progress curves obtained when adenosine deaminase was added to mixtures containing the other isomer (**6b**) showed inhibition that became stronger with the passage of time (Figure 2). Progress curves were fit by nonlinear regression (Duggleby, 1984) to the equation:

$$A = A_0 + v_s t + (v_0 - v_s)[1 - \exp(-k't)]/k' \qquad (2)$$

where the parameters A_0 and A represent absorbance readings at time zero and time t, respectively; and v_0 and v_s represent initial and final steady-state velocities. The parameter k' represents a first-order rate constant for the transition from v_0 to v_s. A hyperbolic relationship, concave in the downward direction, was observed when values of k' were plotted as a function of inhibitor concentration as shown in Figure 3. This relationship was consistent with mechanisms A and B in Scheme II, which involve formation of two distinct enzyme–inhibitor complexes (Cha, 1975a,b; 1976; Duggleby et al., 1982). The relationship between k' and inhibitor concentration for mechanism A is shown in eq 3 (Morrison & Stone, 1985):

$$k' = k_{off} + \frac{k_{on}([I]/K_i)}{1 + [S]/K_m + [I]/K_i} \qquad (3)$$

in which [S] and K_m refer to adenosine concentration and the apparent Michaelis constant, [I] refers to inhibitor concentration, and K_i refers to the dissociation constant for the loosely formed EI complex. For mechanism A, the overall inhibitor dissociation constant, K_i^*, is a function of rate and equilibrium constants as shown in the equation:

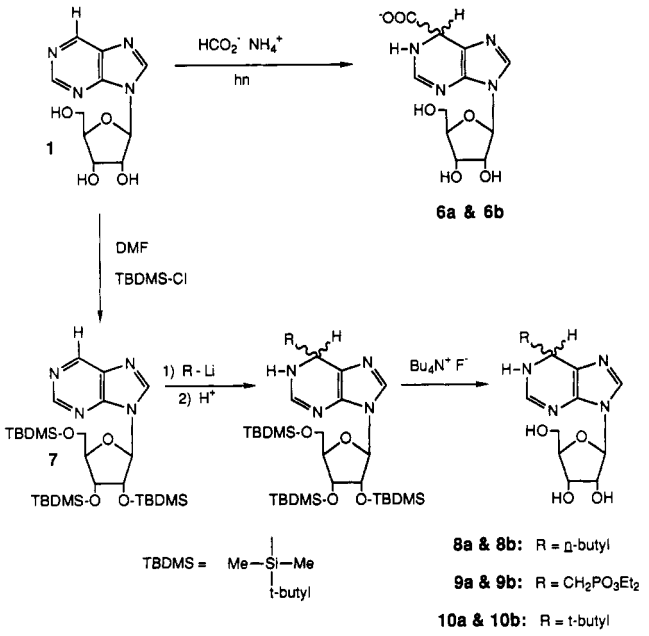
$$K_i^* = K_i[k_{off}/(k_{on} + k_{off})] \qquad (4)$$

The data in Figure 3 were fit to eq 3 by nonlinear regression (Duggleby, 1984), yielding best fit values for k_{on} = 0.0689 ± 0.0059 s^{-1}, k_{off} = 0.00690 ± 0.0018 s^{-1}, and K_i = 85 ± 21 μM. A value of K_i^* = 7.7 μM was calculated from eq 4 using these values.

Reaction velocities, measured at four substrate concentrations at each of four inhibitor concentrations, were examined by double-reciprocal plots, and values for the inhibitor dissociation constant were determined by the method of Segel (1975). In one experiment, initial velocities were measured from reactions that were started by the addition of enzyme. These results yielded a value for K_i = 84 μM. In a second experiment, enzyme and inhibitor were preincubated for 20 min prior to starting the reaction with adenosine. The steady-state velocities that were measured after a lag phase resulted in a value for K_i^* = 9.3 μM. Inhibition was found to be competitive with respect to the substrate adenosine in both the initial and steady-state velocity investigations. Values for K_i and K_i^* determined from this set of experiments were in good agreement with those estimated from progress curves using Figures 2 and 3 and eq 3 and 4 which apply to mechanism A. If the inhibition followed mechanism B, then values estimated for K_i and K_i^* would reflect the dissociation constants for the weakly and strongly formed enzyme–inhibitor complexes, respectively. In either case, these results show that the inhibitor **6b** was more strongly bound in the EI* complex than in the EI complex, by a factor of 9.5- to 11-fold.

[2] The K_i value of the β-anomer of D-ribose was estimated as 0.045 M, based on an observed K_i value of 0.25 M and the assumption that the β-anomer of ribofuranose, constituting 18% of the total ribose in aqueous solution (Angyal, 1969), is the form that is bound.

Scheme III: Synthetic Routes for the Preparation of 6-Substituted-1,6-dihydropurine Ribonucleosides via Photochemical Addition of Formate, or Low-Temperature (–78 °C) Addition of Organolithium Reagents



1,6-Dihydropurine Nucleosides Obtained by Low-Temperature Addition of Organolithium Compounds to 2′,3′,5′-Tris(tert-butyldimethylsilyl)purine Ribonucleoside (7). Protection of the three hydroxyl groups of purine ribonucleoside with *tert*-butyldimethylsilyl chloride by the method of Corey and Venkateswarlu (1972) resulted in high yields of 2′,3′,5′-tris(*tert*-butyldimethylsilyl)purine ribonucleoside (7), which was found to undergo addition of organolithium reagents at –78 °C in THF (Scheme III), to give a series of compounds in which alkyl groups replaced one of the 6-hydrogen atoms of 1,6-dihydropurine ribonucleoside, furnishing an opportunity to explore the bulk tolerance of the active site. Acid hydrolysis, workup, and silica gel chromatography yielded both isomers from the addition which were completely separated in some cases. Isolated yields were 35–56%. The reaction was fairly general (with the notable exception of methyllithium), primary as well as tertiary organolithium reagents adding across the 1,6 double bond of 7. Less than 20% of the starting material reacted at –78 °C when up to 15 mequiv of methyllithium was added. Protecting groups were removed with tetrabutylammonium fluoride (Corey & Venketeswarlu, 1972), and the deprotected nucleosides were purified by ion-exchange chromatography to remove tetrabutylammonium salts, followed by RP-HPLC. Solutions of these compounds were slowly oxidized by air over a period of several days, but after lyophilization, these nucleosides were found to be stable to air for periods of weeks or months. Addition at the 1,6 position was indicated by the close similarity of the UV and ^{1}H-NMR spectra of the products to those of 1,6-dihydropurine derivatives, prepared by the addition of alcohols and molecular hydrogen to purine ribonucleoside, ^{1}H-NMR spectral assignments of these reference compounds having been established earlier by deuterium labeling experiments (Connolly & Linschitz, 1968; Kati & Wolfenden, 1989a).

After deprotection, these nucleosides were tested as inhibitors of adenosine deaminase, with the results reported in Table I. All appeared to serve as simple competitive inhibitors, and none showed evidence of time-dependent inhibition.

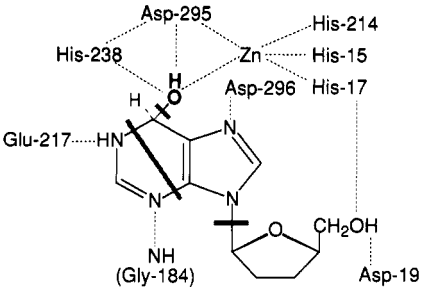


FIGURE 4: Schematic view [based on Wilson et al. (1991)] of some enzyme interactions with nebularine hydrate. The dark lines show positions of cleavage of ligands considered in the present work.

DISCUSSION

Nonadditivity of Substituent Contributions to the Binding of Nebularine 1,6-Hydrate, Coformycin, and the Transition State for Adenosine Deamination. To investigate the forces that are responsible for the strong binding of nebularine 1,6-hydrate or 2′-deoxycoformycin, we measured the binding affinities of several compounds from which various structural features had been deleted. This approach yielded results which might, at first glance, appear to be contradictory. Binding of the unstable transition-state analog nebularine hydrate was rendered 9.9 kcal/mol less favorable by removal of the 6-hydroxyl group, and 10.2 kcal/mol less favorable by removal of atoms 1–3 of the pyrimidine ring. Binding of the stable transition-state analog inhibitor 2′-deoxycoformycin was rendered 9.8 kcal/mol less favorable by removal of substituent ribose, 9.7 kcal/mol less favorable by inversion of the 8-hydroxyl substituent of the diazepine ring, and 10.0 kcal/mol less favorable by removal of atoms 4–6 of the diazepine ring. Similarly, the free energy of stabilization of the transition state for hydrolysis of adenosine, expressed as k_{cat}/K_m, was rendered 6.4 kcal/mol less favorable by removal of the 5′-hydroxyl group, substituent ribose itself having been shown earlier to make a very large contribution (7.5 kcal/mol) to transition-state stabilization without having much effect on the inherent reactivity of adenine (Frick et al., 1987).

Qualitatively, the importance of each of these interactions seems understandable in terms of the observed crystal structure of the inhibitory complex formed between nebularine hydrate and adenosine deaminase, shown schematically in Figure 4. The apparent contributions of 9.7, 10.0, and 9.8 kcal/mol by the 8-hydroxyl, N4-C5-N6, and ribose constituents of deoxycoformycin, respectively, indicate the intrinsic free energies of binding of these constituent groups. Any single one of these comparisons, taken by itself, might seem to suggest that the high enzyme affinity of nebularine, 1,6-hydrate or deoxycoformycin is mainly due to recognition of that particular constituent of the inhibitor by adenosine deaminase. The combined results indicate, however, that strong binding requires that *all* binding determinants be present and that their effects are synergistic.

This synergism can be made explicit by comparing the binding affinities of these transition-state analogs with the binding affinities of their component parts, as indicated in Figure 1A. In principle, the binding of region A of ligand A-B permits binding of region B to occur in an intramolecular reaction with little additional loss of entropy. In the absence of compensating effects, much more entropy may be lost when A and B are bound by a protein than when the single molecule A-B is bound: it has been estimated that, under favorable circumstances, ΔG_S in eq 1 may reach a magnitude of approximately 11 kcal/mol, lower values being expected if A

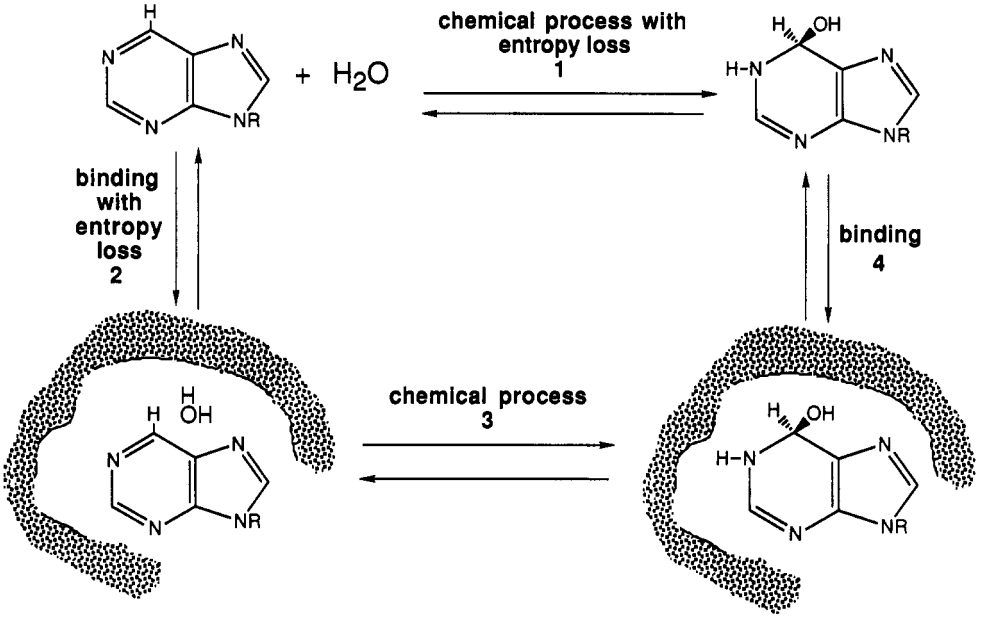


FIGURE 5: An "entropy-trap" mechanism for hydration of purine ribonucleoside at the active site of adenosine deaminase. By binding the two substrates, purine ribonucleoside (**1**) and a water molecule, in the proper orientation and position for reaction at the active site (step 2), adenosine deaminase overcomes the unfavorable entropic component of the reaction. The chemical combination of the two substrates at the active site (step 3) can occur in an intramolecular reaction, with no further loss of entropy, so that the equilibrium constant for the hydration reaction at the active site (step 3) will be more favorable than the equilibrium constant for the nonenzymatic reaction (step 1) by a factor that corresponds to the net entropy loss for the reaction ($T\Delta S$). Note that this thermodynamic cycle requires that if step 3 is more favored than step 1 by a factor corresponding to $T\Delta S$, then this same $T\Delta S$ factor must be manifested as a more favorable binding of product (**2**) in step 4 relative to the binding of the two substrates in step 2.

and B retain significant freedom of relative movement when they are connected in ligand A-B (Jencks, 1981). For ligands in which many rotors must be frozen for optimal interaction with a binding site, observed affinities are also expected to fall short of these optima [see, for example, Williams et al. (1991)]. The results reported in Figure 1A imply that ΔG_S contributions attain values in the neighborhood of 7–12 kcal/mol. It seems likely that when nebularine hydrate or coformycin is bound by adenosine deaminase, little rotational entropy needs to be lost. Even the glycosidic linkage, a site of apparent flexibility, probably favors the anti configuration as in other purine nucleosides and is also bound in this form.

These structural arguments are approximate, in the sense that it is not rigorously possible to divide a ligand A-B into two parts, A and B, without further structural modifications such as the addition of hydrogen atoms or conversion to a cyclic molecule such as imidazole. Nevertheless, the ΔG_S values recorded in Figure 1 are *similarly* large, regardless of where these transition-state analogs are cleaved, and their recurring magnitude suggests a common origin for these effects. Because ΔG_S is a property of the molecule as a whole and is not confined to a particular constituent, deletion of any of several structural constituents may result in the loss of the large contribution of the ΔG_S term to binding affinity, so that *every one of these constituents can be considered critically important for binding.*

Implications of Catalysis and Inhibitor Design. If nebularine 1,6-hydrate is formed by hydration of nebularine within the active site, and if major entropic effects are manifested in the binding of this transition-state analog, it is of interest to consider some possible implications for catalysis by adenosine deaminase.

An enzyme that condenses two substrates to form a single molecule in the transition state can, at least in principle, produce a very large rate enhancement by acting as an entropy trap (Page & Jencks, 1971; Jencks, 1975). In the thermodynamic cycle shown in Figure 5, the free energy change that accompanies nonenzymic hydration of purine ribonucleoside (step 1) is pictured as including the change in enthalpy that accompanies hydration and the unfavorable change in entropy that results when two molecules are combined into one. The first step of the enzyme reaction (step 2) can formally be considered to be the binding of the two substrates, purine ribonucleoside and a water molecule;[3] part of the intrinsic binding energy of these molecules would presumably be used to overcome their unfavorable entropy of binding, resulting in weaker binding than would otherwise have been observed. Once these two compounds are bound in the proper orientation and position at the active site, hydration could occur (step 3) with little further loss of entropy. As a result, the hydration reaction at the active site (step 3) would be more favorable than the reaction in free solution (step 1) by a factor that corresponds to the entropy loss for the nonenzymatic reaction. The observed binding affinity of nebularine 1,6-hydrate (step 4) wold then be stronger than the sum of the observed binding affinities of the two substrates (step 2) by this same entropic factor. Thus, the *equilibrium* advantage of a unimolecular reaction at the active site, relative to the bimolecular nonenzymatic reaction, is manifested as a *binding* advantage for nebularine 1,6-hydrate, compared with the binding of compounds in which the nucleoside and hydroxyl group binding determinants are present in two separate molecules.

The possibility that adenosine deaminase uses such a strategy in catalysis would be strengthened if an actual entropic advantage could be demonstrated for enzyme binding of nebularine 1,6-hydrate, compared with enzyme binding of the combination of nebularine and a water molecule. It is therefore

[3] There is no direct evidence as yet for the existence of a discrete binding site for solvent water in adenosine deaminase. Thus, substrate water might attack from a bound site on the enzyme (presumably the active site zinc atom), or directly from bulk solvent, its affinity for the enzyme increasing with progress toward the transition state.

of special interest that, for the nonenzymatic hydration of nebularine, the entropic component ($\Delta S = -35$ cal deg^{-1} mol^{-1}; $T\Delta S = -10.5$ kcal/mol) is much larger than the enthalpic component ($\Delta H = +1.2$ kcal/mol) of the free energy of reaction, +11.7 kcal/mol (Kati & Wolfenden, 1989b). If this entropic cost were "paid" during the initial binding of the two substrates in the enzymatic reaction, then entropic effects might be expected to allow nebularine 1,6-hydrate to be bound more strongly than the combination of nebularine and a water molecule by approximately −10.5 kcal/mol (Figure 1B). Since the K_d value of unhydrated nebularine must be larger than 10^{-5} M (Kati & Wolfenden, 1989b), and the K_m value of water appears to be at least 55 M (Dzingeleski and Wolfenden, in preparation), nebularine 1,6-hydrate appears to be bound more strongly than these parts by at least −12.8 kcal/mol in free energy. From the present results, the part of this increased binding affinity that results from the entropic advantage of joining two substrates in a single molecule can be estimated as being between −7 and −12 kcal/mol. This is comparable with the above-mentioned value of −10.5 kcal/mol for $T\Delta S$, measured for nebularine hydration and expected for adenosine deamination by an entropy trap mechanism, suggesting that adenosine deaminase promotes the equilibrium of hydration by 8 orders of magnitude by converting the bimolecular hydration reaction to a unimolecular reaction at its active site. Since the entropy of activation for nonenzymatic deamination of adenosine is −37 cal deg^{-1} mol^{-1}, it seems likely that these entropic effects contribute a similar factor to the stabilization of the transition state of enzymatic deamination of adenosine.[4]

The ΔG_S values recorded in Figure 1A are very large, regardless of where these transition-state analogs are cleaved; i.e., binding affinity is greatly reduced by any one of a number of structural modifications.[5] The extreme losses of binding affinity that result from any of these structural modifications are of practical interest from the standpoint of enzyme inhibitor design. If efforts had been made to design these inhibitors in the laboratory, using an approach based on successive approximation, these efforts might have resulted in early disappointment. Evidently the binding affinity of an enzyme inhibitor may suffer greatly from any of a number of apparently minor defects, but conversely, an inhibitor of modest attainments may gain enormously in potency from the introduction of a single substituent at the proper position.

6-Substituted-1,6-dihydropurine Ribonucleosides. Because nebularine 1,6-hydrate is thermodynamically unstable, it seemed desirable to explore the possibility of preparing an analog of nebularine 1,6-hydrate that was stable in aqueous solution but with the potential to form all of the interactions with adenosine deaminase required for strong binding. 6-Carboxy-1,6-dihydropurine ribonucleoside was chosen as a target compound because the 6-carboxyl group would not be susceptible to 1,6-elimination, and because the hydrogen bond(s) that the 6-carboxyl group might form with the enzyme could be stronger, in principle, than those between the enzyme and the 6-hydroxyl group. The latter notion is based on comparisons of enthalpies of formation in the gas phase (Meotnir & Sieck, 1986) and in protein–ligand interactions in solution (Fersht et al., 1985) which show that hydrogen bonds in which one or both partners are charged are stronger than hydrogen bonds between neutral partners. We found that both isomers of 6-carboxy-1,6-dihydropurine ribonucleoside (**6a** and **-b**) could be synthesized by photolysis of purine ribonucleoside in the presence of ammonium formate in a reaction analogous to the 1,6-photoaddition of alcohols (Linschitz & Connolly, 1968; Evans & Wolfenden, 1970). Compounds **6a** and **-b** were found to serve as only modest inhibitors of adenosine deaminase (Table I), resulting in a loss of more than 10^8-fold in binding affinity when the carboxylate functional group was substituted for the hydroxyl group. The 6-carboxylate group is much larger than the 6-hydroxyl group, so that the 6-carboxylate group may be sterically hindered from occupying the site which normally accommodates the 6-hydroxyl group of **2**. Alternatively, the 6-carboxylate may fit into this binding site, but fail to make all three bonds that the 6-hydroxyl group of **2** appears to make with the enzyme (Wilson et al., 1991).

The time-dependent inhibition of adenosine deaminase by compound **6b** was shown to be consistent with the kinetic mechanisms shown in Scheme II, both of which suggest initial formation of a weak EI complex and a slower formation of a second complex (EI*) in which the inhibitor is bound approximately 10-fold more strongly. We have not further characterized the two EI complexes and can only speculate on the differences between the two complexes. If compound **6b** were the 6*S* isomer, then the more stable EI complex may reflect a subtle rearrangement of the weaker EI complex to allow the 6(*S*)-carboxylate group to rotate in such a way as to take advantage of additional interactions with those active site residues that normally bind the 6(*R*)-hydroxyl group of hydrated intermediates. The potent inhibition observed for the 6*S* isomer of 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside (**16**) appears to result from these additional interactions (Evans & Wolfenden, 1970; Shimazaki et al., 1983). Compound **16** also shows time-dependent inhibition (Frieden et al., 1980; Kurz & Frieden, 1983), and protein fluorescence measurements are consistent with the possibility

[4] It should be recognized that the active site may have difficulty in accommodating the substrates adenosine (or nebularine) and water simultaneously, without nonbonded interactions that interfere with their binding. Although we have no evidence that this is occurring, such a mechanism, resulting in ground-state destabilization, might well contribute to the difference in binding affinities between the hydrated reaction intermediate and the two substrates. The above analysis suggests that entropic differences may contribute 7–12 kcal/mol to the greater than 12.8 kcal/mol binding difference between nebularine hydrate and the two substrates: nebularine and a water molecule. Accordingly, there is room for substantial contributions by other mechanisms for relative stabilization of the hydrate. It is of interest to consider the possibility that destabilizing interactions might also contribute to the ΔG_S terms comparing nebularine hydrate with its parts, shown in Figure 1A. In some of these comparisons, the binding affinity of each part was measured individually, in the absence of its partner, eliminating the possibility of nonbonded interactions between the partners at the active site. In other cases, such as the measurement of the dissociation constant of reduced nebularine (**4**), it is not known whether a water molecule, the second binding partner, is also present at the active site. However, if the observed dissociation constant of **4**, for example, reflects interactions of **4** with the active site *with water bound at the same time*, then *binding of 4 without water would presumably be weaker than the observed K_i value suggests.* Otherwise the latter mode of binding, yielding the more stable complex, would have been observed (Kati & Wolfenden, 1989b).

[5] Substituent ribose, although it has little effect on the nonenzymatic reactivity of adenine, is distant from the site of bond making and breaking in substrates, and is connected to the purine ring only by a single bond, nevertheless exerts a profound effect on V_{max} and on the affinities of transition-state analog inhibitors. To produce such a large effect, it seems probable that the substituent in question would need to be rather rigidly connected to the site of bond making and breaking. Although precise thermodynamic information is not available, purine nucleosides are known to favor a trans arrangement at the glycosidic linkage, and this arrangement is also observed in nebularine 1,6-hydrate at the active site of adenosine deaminase (Wilson et al., 1991). Thus, the major binding determinants are probably restricted in their relative positions, with free rotation occurring only at the C–O bonds that link the critical OH groups to the carbon skeleton.

of a protein conformational change when compound **16** binds to adenosine deaminase (Kurz & Frieden, 1983; Kurz et al., 1985). We cannot, however, rule out the possibility that the slow "on" rate for the binding of **6b** to the more stable EI complex may reflect slow release of active site water molecules, a possibility that has been suggested for inhibitor binding by thermolysin (Bartlett & Marlowe, 1987; Holden et al., 1987).

During the course of this investigation, we found that organolithium reagents would add across the 1,6 carbon–nitrogen double bond of a protected derivative of purine ribonucleoside (**7**) at low temperatures to yield stable 6-substituted-1,6-dihydropurine ribonucleosides, which were found to be modest inhibitors of adenosine deaminase (Table I). Comparison of the K_i values for the two *n*-butyl isomers (**8a** and **-b**), and also comparison of K_i values for the two *tert*-butyl isomers (**10a** and **-b**), suggests that one isomer can be accommodated by adenosine deaminase, but not the isomer of opposite configuration. These findings are consistent with an earlier suggestion (Frick et al., 1986) that adenosine deaminase contains a large, nonspecific cavity that accommodates a variety of leaving groups, as indicated by the efficient binding and hydrolysis of a number of bulky 6-substituted purine ribonucleoside substrates (Chassy & Suhadolnik, 1967). The relatively weak binding of both isomers of the (diethoxyphosphoryl)methyl derivative, containing a substituent that is longer than an *n*-butyl group and is branched at the phosphorus atom, suggests that there is a limit to the size of substituents that can be accommodated at the leaving group site.

The occurrence of 1,6-addition of primary as well as tertiary organolithium reagents to compound **7** suggests that organolithium reagent addition may have broader uses in preparing 6-substituted-1,6-dihydropurine derivatives, since a wide range of organic compounds can be converted to their corresponding organolithium derivatives. The products are easily converted to the corresponding fully heteroaromatic 6-substituted purine ribonucleosides by simple oxidation procedures (Shimazaki et al., 1983; Kati & Wolfenden, 1989b). Apart from their effectiveness as inhibitors of adenosine deaminase, the biological activities of both classes of compounds remain to be investigated.

REFERENCES

Agarwal, R. P., Spector, T., & Parks, R. E., Jr. (1977) *Biochem. Pharmacol. 26*, 359.

Angyal, S. J. (1969) *Angew. Chem. 8*, 157.

Bartlett, P. A., & Marlowe, C. K. (1987) *Biochemistry 26*, 8553.

Bauer, C., Ronca, G., & Rossi, C. A. (1966) *Ital. J. Biochem. 15*, 357.

Cardinaud, J., & Holguin, R. (1975) *Eur. J. Biochem. 54*, 515.

Cha, S. (1975a) *Biochem. Pharmacol. 24*, 2177.

Cha, S. (1975b) *Biochem. Pharmacol. 25*, 1561.

Chassy, B. M., & Suhadolnik, R. J. (1967) *J. Biol. Chem. 242*, 3655.

Connolly, J. S., & Linschitz, H. (1968) *Photochem. Photobiol. 7*, 791.

Corey, E. J., & Kwiatkowski, G. J. (1966) *J. Am. Chem. Soc. 88*, 5654.

Corey, E. J., & Venkateswarlu, A. (1972) *J. Am. Chem. Soc. 94*, 6190.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Duggleby, R. G. (1984) *Comput. Biol. Med. 14*, 447.

Duggleby, R. G., Atwood, P. V., Wallace, J. C., & Keech, D. B. (1982) *Biochemistry 21*, 3354.

Evans, B. E., & Wolfenden, R. (1970) *J. Am. Chem. Soc. 92*, 4751.

Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235.

Frick, L., Wolfenden, R. V., Smal, E., & Baker, D. C. (1986) *Biochemistry 25*, 1616.

Frick, L., MacNeela, J. P., & Wolfenden, R. (1987) *Bioorg. Chem. 15*, 100.

Frieden, C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry 19*, 5303.

Frisell, W. R., Chung, S. W., & Mackenzie, C. G. (1958) *J. Biol. Chem. 234*, 1297.

Hanvey, J. C. (1987) Ph.D. dissertation, University of Alabama.

Heelis, P. F., Payne, G., & Sancar, A. (1987) *Biochemistry 26*, 4634.

Holden, H. M., Tronrud, D. E., Monzingo, A. F., Weaver, L. H., & Matthews, B. M. (1987) *Biochemistry 26*, 8542.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219.

Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046.

Jones, W., & Wolfenden, R. (1986) *J. Am. Chem. Soc. 108*, 7444.

Jones, W., Kurz, L., & Wolfenden, R. (1989) *Biochemistry 28*, 1242.

Kati, W. M., & Wolfenden, R. (1989a) *Science 243*, 1591.

Kati, W. M., & Wolfenden, R. (1989b) *Biochemistry 28*, 7919.

Kati, W. M., & Wolfenden, R. (1991) *Int. J. Pur. Pyr. Res. 2*, 27.

Kurz, L. C., & Frieden, C. (1983) *Biochemistry 22*, 382.

Kurz, L., & Frieden, C. (1987) *Biochemistry 26*, 8450.

Kurz, L. C., LaZard, D., & Frieden, C. (1985) *Biochemistry 24*, 1342.

Linschitz, H., & Connolly, J. S. (1968) *J. Am. Chem. Soc. 90*, 2979.

Meot-Nir, M., & Sieck, L. W. (1986) *J. Am. Chem. Soc. 108*, 7525.

Montgomery, J. A., Thomas, H. J., Zell, A. L., Einspahr, H. M., & Bugg, C. E. (1985) *J. Med. Chem. 28*, 1751.

Morrison, J. F., & Stone, S. R. (1985) *Comments Mol. Cell. Biophys. 2*, 347.

Page, M. I., & Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.

Papadopoulos, E. P., Jarrar, A., & Issidorides, C. H. (1966), *J. Org. Chem. 31*, 615.

Rapaport, H., & Matthews, H. R. (1973) *J. Am. Chem. Soc. 95*, 2297.

Ronca, G., & Ronca-Testoni, S. (1969) *Biochim. Biophys. Acta 178*, 577.

Schramm, V. L., & Baker, D. C. (1985) *Biochemistry 24*, 641.

Segel, I. H. (1975) in *Enzyme Kinetics*, pp 106–111, Wiley-Interscience, New York.

Shimazaki, M., Nakamura, H., Iitaka, Y., & Ohno, M. (1983) *Chem. Pharm. Bull. 31*, 3104.

Williams, D. H., Cox, J. P. L., Doig, A. J., Gardner, M., Gerhard, U., Kaye, P. T., Lal, A. R., Nicholls, I. A., Salter, C. J., & Mitchell, R. C. (1991) *J. Am. Chem. Soc. 113*, 7020.

Wilson, D. K., Rudolph, F. B., & Quiocho, F. A. (1991) *Science 252*, 1278.

Wolfenden, R., & Frick, L. (1987) in *Enzyme Mechanisms* (Page, M. I., & Williams, A., Eds.) pp 97–122, Royal Society of Chemistry, London.