Enzyme Inhibitors. XIII. The Synthesis of an Active-Site-Directed Irreversible Inhibitor of Adenosine Deaminase[1]

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Previous studies have suggested that there is a hydrophobic region on adenosine deaminase which is important for binding the alkyl group of some 9-alkyladenines. In an attempt to take advantage of this hydrophobic region of adenosine deaminase for the preparation of some inhibitors of this enzyme, a series of 9-(*para*-substituted benzyl)-6-substituted purines was prepared. In addition to studying reversible inhibition of adenosine deaminase, it was found that 9-(*p*-bromoacetamidobenzyl)adenine (XII) was an irreversible inhibitor of adenosine deaminase. On the basis of kinetic studies and because of the lack of inactivation of adenosine deaminase by iodoacetamide, it is suggested that the inactivation of the enzyme by XII occurs through an E–I complex and does not proceed through a random bimolecular process.

Previously it has been suggested that a hydrophobic region exists on adenosine deaminase in the region where the 9-substituent of some 9-alkyladenines bind.[2] In the hope of taking advantage of this hydrophobic region for the preparation of good reversible inhibitors of adenosine deaminase, we decided to prepare some 9-(*para*-substituted benzyl)-6-substituted purines. If the benzyl moiety of these purine derivatives were capable of binding to the hydrophobic region, it should be possible to utilize a substituent at the *para* position which is capable of forming a covalent bond with the enzyme.

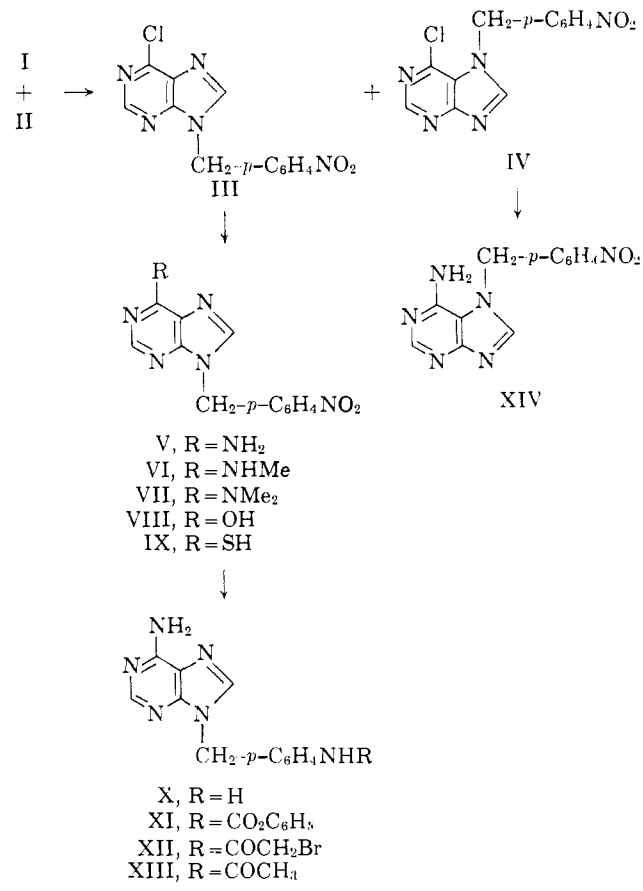
This paper describes the syntheses of some 9-(*para*-substituted benzyl)-6-substituted purines and their enzymic evaluation as reversible inhibitors of adenosine deaminase. In addition, a kinetic evaluation of 9-(*p*-bromoacetamidobenzyl)adenine as an irreversible inhibitor of adenosine deaminase is presented.

For the preparation of this series of inhibitors, we selected 9-(*p*-nitrobenzyl)-6-chloropurine as the key intermediate. The general method of synthesis is based on a modification of the procedure of Montgomery and Temple[3] and is outlined in Chart I. Condensation of 6-chloropurine (I) with *p*-nitrobenzyl bromide (II) gave a mixture of the 9- and 7-*p*-nitrobenzyl-6-chloropurines (III and IV) which was separated by chromatography on alumina. Treatment of III with ammonia, methylamine, dimethylamine, hydrochloric acid, or thiourea gave the corresponding 6-substituted isomers (V–IX). Catalytic reduction of V using a palladium-on-carbon catalyst gave the *p*-amino derivative (X) in good yield. When X was allowed to react with phenyl chloroformate, bromoacetyl bromide, or acetyl chloride the corresponding *para*-substituted benzyl derivatives (XI–XIII) were obtained. The assignment of structure to XI, XII, and XIII is based on the observation that N^6-acylation of adenine derivatives shifts the ultraviolet maxima to longer wavelengths.[4] For example, it has been found that N,$O^{3'}$-diacetyldeoxyadenylic 5′-acid exhibited an ultraviolet maximum at 273 mμ at pH 8,[4] whereas XI, XII, and XIII all exhibited ultraviolet maxima either at shorter wavelengths than X or at essentially the same wavelength as X. Furthermore, the ultraviolet spectra of XI, XII, and XIII did not shift to the spectrum of X when the compounds were heated at 80° at pH 13 for 30 min.

Finally, 7-*p*-nitrobenzyladenine (XIV) was prepared by allowing IV to react with liquid ammonia at 50° for 22 hr.

That III and IV, and therefore compounds prepared from them, are the 9- and 7-substituted isomers, respectively, was established in the following manner. When adenine was allowed to react with *p*-nitrobenzyl bromide in the absence of an acid acceptor, a new

CHART I



(1) This investigation was supported by a Public Health Service research grant (CA-06388-03) and a research career program award (5-K3-CA-18718-04) from the National Cancer Institute, a training grant (5-T1-GM-555) from the Division of Medical Sciences, U. S. Public Health Services, Bethesda, Md., and by a grant (T-337) from the American Cancer Society.

(2) H. J. Schaeffer and D. Vogel, *J. Med. Chem.*, **8**, 507 (1965).

(3) J. A. Montgomery and C. Temple, Jr., *J. Am. Chem. Soc.*, **83**, 630 (1961).

(4) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

alkylated adenine (XV) was obtained. It has been reported that a similar alkylation of adenine with benzyl bromide produced 3-benzyladenine.[5] A comparison of the ultraviolet spectra and the pK_a' data of these isomerically alkylated adenines with certain related compounds is given in Table I. From these data it can be seen that V corresponds to the 9-substituted isomer, XIV to the 7-substituted isomer, and that XV can be assigned the structure, 3-(*p*-nitrobenzyl)adenine. These results are in agreement with the observation that the alkylation of 6-chloropurine in the presence of an acid acceptor produces a mixture of the 9- and 7-substituted 6-chloropurine.[3,6]

TABLE I

ULTRAVIOLET AND pK_a' DATA OF SOME SUBSTITUTED ADENINES

Compd (adenine deriv)	H^+ λ_{max}, mμ	H^+ $\epsilon \times 10^{-3}$	OH^- λ_{max}, mμ	OH^- $\epsilon \times 10^{-3}$	pK_a' (DMF–H_2O, 50:50)
1-Methyl[a]	259	11.7	270	14.4	6.95, 11.9
3-Methyl[a]	274	17.0	273	13.3	5.3
3-(*p*-Nitrobenzyl) (XV)	277	24.3	270	24.2	5.9[b]
7-Methyl[a]	272	15.0	270	10.5	3.6
7-(*p*-Nitrobenzyl) (XIV)	273	19.8	269	16.6	4.0[b]
9-Methyl[a]	260	14.2	260	14.7	3.25
9-(*p*-Nitrobenzyl) (V)[c]	264	22.1	264	22.1	4.0[b]

[a] Data taken from N. J. Leonard and J. H. Deyrup, *J. Am. Chem. Soc.*, **84**, 2148 (1962). [b] These data were determined by Dr. M. A. Schwartz of the State University of New York at Buffalo. [c] The ultraviolet maximum of V is at a slightly longer wavelength than a 9-alkyladenine because the *p*-nitrobenzyl moiety of V has an ultraviolet maximum at 273 mμ.

Experimental Section[7]

6-Chloro-9- and -7-*p*-nitrobenzylpurines (III and IV).—To a solution of 3.54 g (35.0 mmoles) of triethylamine in 60 ml of N,N-dimethylformamide was added 5.00 g (32.4 mmoles) of I and 7.56 g (35.0 mmoles) of II, and the reaction mixture was stirred at room temperature for 67 hr. After the mixture was poured into 180 ml of distilled water at 0°, the insoluble material was collected by filtration and gave 8.32 g (89.7%) of solid material. This material was dissolved in chloroform (200 ml) and introduced on a column (29.5-mm i.d.) of neutral alumina (230 g) in $CHCl_3$. The column was eluted with chloroform and 54 50-ml fractions were collected, whereupon a 10% solution of methanol in $CHCl_3$ was used to elute the column and 13 50-ml fractions were collected. Eluent fractions 9–31 were combined and evaporated *in vacuo*, which gave 5.80 g (62.5%) of III, mp 195–197°. Recrystallization from methanol gave the analytical sample: yield, 5.07 g (54.6%); mp 195–197°; ν (cm^{-1}) (KBr) 1585, 1560 and 1515 (C=C, C=N, and phenyl), 1515 and 1345 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] pH 1—268 (18.7), pH 7—268 (20.3), pH 13—268 (20.3).

Anal.[8] Calcd for $C_{12}H_8ClN_5O_2$: C, 49.75; H, 2.78; Cl, 12.24; N, 24.18. Found: C, 49.48; H, 2.59; Cl, 12.50; N, 24.40.

Eluent fractions 34–67 were combined and evaporated *in vacuo*, which gave 1.71 g (18.4%) of IV, mp 182–189°. This material was dissolved in methanol, decolorized with charcoal, and filtered through a Celite pad; recrystallization from methanol gave the analytical sample; yield, 1.15 g (12.4%); mp 194–195°, ν (cm^{-1}) (KBr) 1590, 1535, and 1510 (C=C, C=N, and phenyl), 1510 and 1340 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—268 (16.8), ethanol—268 (16.8), ethanol–1 *N* NaOH—263 (17.4).

Anal. Calcd for $C_{12}H_8ClN_5O_2$: C, 49.75; H, 2.78; Cl, 12.24; N, 24.18. Found: C, 50.00; H, 2.89; Cl, 12.46; N, 24.42.

6-Amino-9-*p*-nitrobenzylpurine (V).—A mixture of 600 mg (2.07 mmoles) of III in 50 ml of 20% methanolic NH_3 was heated in a stainless steel bomb at 73° for 45 hr. The material which precipitated was collected by filtration; recrystallization of the crude product from methanol gave the pure sample: yield, 396 mg (70.8%) of V; mp 256–257°; ν (cm^{-1}) (KBr) 3300 (NH_2), 1660 (NH_2), 1590, 1565, and 1500 (C=C, C=N, and phenyl), 1500 and 1335 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] pH 1—264 (22.1), pH 7—265 (21.1), pH 13—264 (22.1).

Anal. Calcd for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.42; H, 3.68; N, 31.29.

6-Methylamino-9-*p*-nitrobenzylpurine (VI).—A solution of 200 mg (0.690 mmole) of III in 12 ml of ethanol and 10 ml of methylamine in water (40%) was heated in a stainless steel bomb at 85° for 24 hr. The solid which precipitated was collected by filtration, and the filtrate was reduced to 5 ml *in vacuo*. The solid which precipitated was collected by filtration; the two crops were combined (135 mg, mp 248°) and recrystallized from methanol to give the pure sample (VI): yield, 102 mg (52.1%); mp 248°; ν (cm^{-1}) (KBr) 3380 (NH), 1640 (NH), 1620, 1590, and 1520 (C=C, C=N, and phenyl), 1520 and 1345 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] pH 1—268 (22.9), pH 7—271 (21.9), pH 13—271 (21.3).

Anal. Calcd for $C_{13}H_{12}N_6O_2$: C, 54.92; H, 4.26; N, 29.57. Found: C, 54.65; H, 4.50; N, 29.26.

6-Dimethylamino-9-*p*-nitrobenzylpurine (VII).—A solution of 40 mg (0.14 mmole) of III in 10 ml of ethanol and 2 ml of aqueous dimethylamine (25%) was heated in a stainless steel bomb at 78° for 21 hr. The solid material which precipitated was collected by filtration; recrystallization of the crude material from methanol gave the analytical sample (VII): yield, 27 mg (65.9%); mp 211°; ν (cm^{-1}) (KBr) 1585, 1565, and 1515 (C=C, C=N and phenyl), 1515 and 1340 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] pH 1—271 (27.9), pH 7—277 (27.9), pH 13—276 (28.2).

Anal. Calcd for $C_{14}H_{14}N_6O_2$: C, 56.37; H, 4.73; N, 28.18. Found: C, 56.14; H, 4.80; N, 28.23.

6-Hydroxy-9-*p*-nitrobenzylpurine (VIII).—A mixture of 210 mg (0.72 mmole) of III in 30 ml of 1 *N* HCl was heated under reflux for 22 hr, and then evaporated *in vacuo* to dryness. Two recrystallizations from ethanol gave 94.0 mg (48.2%), mp 284–286° dec, of desired product. The third recrystallization from ethanol gave the analytical sample (VIII): yield, 83.4 mg (42.8%); mp 285–287° dec; ν (cm^{-1}) (KBr) 3470 (OH), 1715 (C=O, enol), 1590, 1545, and 1520 (C=C, C=N, and phenyl), 1520 and 1345 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—252 (16.9), ethanol—252 (17.2), ethanol–1 *N* NaOH—260 (16.9).

Anal. Calcd for $C_{12}H_9N_5O_3$: C, 53.13; H, 3.34; N, 25.83. Found: C, 53.30; H, 3.45; N, 26.02.

6-Mercapto-9-*p*-nitrobenzylpurine (IX).—A solution of 200 mg (0.690 mmole) of III and 57 mg (0.75 mmole) of thiourea in 10 ml of *n*-propyl alcohol was heated under reflux for 45 min. After the reaction mixture was cooled in an ice bath, the precipitate which formed was collected by filtration. The crude product (166 mg, mp 288–292° dec) was dissolved in dilute NaOH, decolorized with charcoal, and filtered through a Celite pad Acidification of the filtrate at 0° with concentrated HCl gave the analytical sample (IX): yield, 121 mg (61.1%); mp 279–281° dec; ν (cm^{-1}) (KBr) 2800–2300 (acidic hydrogen), 1610, 1580, and 1520 (C=C, C=N, and phenyl), 1520 and 1343 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—327 (18.6), ethanol—327 (18.4), ethanol–1 *N* NaOH—323 (17.7).

Anal. Calcd for $C_{12}H_9N_5O_2S$: C, 50.18; H, 3.16; N, 24.39. Found: C, 49.96; H, 3.10; N, 24.51.

6-Amino-9-*p*-aminobenzylpurine (X).—A solution of 1.56 g (5.80 mmoles) of V in 200 ml of glacial acetic acid was added to 400 mg of Pd–C powder (5%), and the mixture was hydrogenated at room temperature in a Parr hydrogenator at an initial pressure of 4 kg/cm^2. After 25 min the catalyst was removed by filtration through a Celite pad, and the solvent was evaporated *in vacuo*. The residual solid was then dried *in vacuo* at 100° for 3 hr. Recrystallization of the crude material from methanol gave the analytical sample (X): yield, 1.05 g (75.3%); mp 273–275° dec; ν (cm^{-1}) (KBr) 3350 (NH_2), 1675 (NH_2), 1590. 1570, and 1510 (C=C, C=N, and phenyl); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] pH 1—259 (14.2), pH 7—258 (16.2), pH 13—259 (16.0).

(5) J. A. Montgomery and H. J. Thomas, *J. Am. Chem. Soc.*, **85**, 2672 (1963).

(6) H. J. Schaeffer and R. Vince, *J. Med. Chem.*, **8**, 710 (1965).

(7) The infrared spectra were determined on a Perkin-Elmer Model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer Model 4000A 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 and are corrected.

(8) The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Anal. Calcd for $C_{12}H_{12}N_6$: C, 59.98; H, 5.03; N, 34.98. Found: C, 60.08; H, 5.10; N, 34.72.

6-Amino-9-*p*-phenoxycarbonylaminobenzylpurine (XI).—A cold solution of 72 mg (0.30 mmole) of X in 30 mg (0.30 mmole) of triethylamine and 75 ml of *p*-dioxane was added slowly with constant stirring to 50 μl (0.39 mmole) of phenyl chloroformate in an ice bath. After 15 min the ice bath was removed, and the reaction mixture was stirred for an additional 2.75 hr at room temperature. The mixture was filtered, and the solvent was reduced to 5 ml *in vacuo*. Cold water (100 ml) was added to the chilled filtrate with formation of a precipitate which was collected by filtration; recrystallization of the crude product from *p*-dioxane and water gave the pure sample (XI): yield, 56.4 mg (52.2%); mp 318–322° dec; ν (cm^{-1}) (KBr) 3350 (NH_2), 1720 (C=O), 1670 (NH_2), 1600, 1570, and 1540 (C=C, C=N, and phenyl); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—246 (26.3), ethanol—245 (25.3), ethanol–1 *N* NaOH—243 (26.6).

Anal. Calcd for $C_{19}H_{16}N_6O_2$: C, 63.32; H, 4.48; N, 23.32. Found: C, 63.16; H, 4.38; N, 23.14.

6-Amino-9-*p*-bromoacetamidobenzylpurine Hydrobromide (XII).—A solution of 72 mg (0.30 mmole) of X in 125 ml of 1,2-dimethoxyethane was added slowly with constant stirring to 35 μl (0.40 mmole) of bromoacetyl bromide in an ice bath. After 1 hr, the ice bath was removed, and the reaction mixture was stirred for an additional 2 hr at room temperature, and finally for an additional 3 hr in an ice bath. The precipitate which formed was collected by filtration and dissolved in warm methanol, filtered, and precipitated with ethyl ether. This process gave solid material which weighed 81.0 mg (75.0%) and showed one spot on thin layer chromatography. The melting point could not be determined since the material began to decompose at 225° but still had not melted at 380°. A second precipitation from methanol and ethyl ether gave the analytical product as the hydrobromide salt; yield, 66.5 mg (61.6%); ν (cm^{-1}) (KBr) 3340 (NH_2), 2750 (NH_3^+), 1690 (C=N–H), 1670 (C=O), 1610, 1545, and 1520 (C=C, C=N, and phenyl); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—260 (25.3), ethanol—260 (24.8), ethanol–1 *N* NaOH—261 (27.7).

Anal. Calcd for $C_{12}H_{14}Br_2N_6O$: C, 38.03; H, 3.19; Br, 30.15; N, 19.01. Found: C, 37.82; H, 3.04; Br, 36.23; N, 18.74.

6-Amino-9-*p*-acetamidobenzylpurine Hydrochloride (XIII).—A solution of 72 mg (0.30 mmole) of 6-amino-9-*p*-aminobenzylpurine in 125 ml of 1,2-dimethoxyethane was added slowly with constant stirring to 27 μl (0.38 mmole) of acetyl chloride in an ice bath. After 1 hr the ice bath was removed, and the reaction mixture was stirred for an additional 2 hr at room temperature. The fine white precipitate which formed was collected by filtration, dissolved in warm methanol, decolorized with charcoal, filtered through a Celite pad, and precipitated with ether. This process gave solid material which weighed 56 mg (59.0%). Recrystallization of this material from isopropyl alcohol, methanol, and hexane gave the analytical sample as the hydrochloride salt; yield, 42.8 mg (45.1%); mp decomposition starts at 230°, turns dark red at 263–265°, and evolves gas at 270–280°; ν (cm^{-1}) (KBr) 3350 (NH_2), 2550 (NH_3^+), 1695 (C=N–H), 1665 (C=O), 1595, 1530, and 1510 (C=C, C=N, and phenyl); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—254 (25.7), ethanol—254 (24.9), ethanol–1 *N* NaOH—254 (29.4).

Anal. Calcd for $C_{14}H_{15}ClN_6O$: C, 52.74; H, 4.74; Cl, 11.12; N, 26.37. Found: C, 52.95; H, 4.91; Cl, 10.90; N, 26.42.

6-Amino-7-*p*-nitrobenzylpurine Hydrochloride (XIV).—A mixture of 200 mg (0.690 mmole) of 6-chloro-7-*p*-nitrobenzylpurine in 15 ml of liquid NH_3 was heated in a stainless steel bomb at 50° for 22 hr. The ammonia was allowed to evaporate, and the dry residue was dissolved in 10% HCl with the subsequent evaporation of the volatile materials *in vacuo*. Recrystallization of the dry residue from methanol and ether gave the analytical sample as the hydrochloride salt; yield, 73 mg (34.6%); mp 257–259° dec; ν (cm^{-1}) (KBr) 3320 (NH_2), 2800–2200 (NH^+), 1660 (NH_2), 1580 (C=C and C=N), 1520 and 1345 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—273 (19.8), ethanol—269 (15.5), ethanol–1 *N* NaOH—269 (16.6).

Anal. Calcd for $C_{12}H_{11}ClN_6O_2$: C, 46.98; H, 3.62; Cl, 11.56; N, 27.40. Found: C, 47.08; H, 3.61; Cl, 11.47; N, 27.22.

3-(*p*-Nitrobenzyl)adenine Hydrobromide (XV).—A mixture of 1.71 g (10.0 mmoles) of adenine dihydrate and 6.81 g (31.5 mmoles) of II in 35 ml of dimethylacetamide was heated at 113° for 21 hr at which time the solvent was evaporated *in vacuo*. The crude residue was washed with 50 ml of boiling ethanol and then dissolved in boiling methanol, decolorized with charcoal, filtered through a Celite pad, and allowed to crystallize. Another recrystallization of this material from methanol and ether gave the analytical sample: yield, 1.38 g (39.3%); mp 282–283° dec; ν (cm^{-1}) (KBr) 3300 (NH_2), 1665 (NH_2), 1620, 1590 (shoulder), and 1510 (C=C, C=N, and phenyl), 1510 and 1340 (NO_2); λ_{max} [mμ ($\epsilon \times 10^{-3}$)] ethanol–1 *N* HCl—277 (24.3), ethanol—274 (18.4), ethanol–1 *N* NaOH—270 (24.2).

Anal. Calcd for $C_{12}H_{11}BrN_6O_2$: C, 41.10; H, 3.14; Br, 22.80; N, 23.90. Found: C, 41.15; H, 3.30; Br, 22.95; N, 23.96.

Chemical Reactivity of the Alkylating Agents.—These experiments were performed by the procedure described in the literature[9] with the modification that 1 ml of triethylamine was used in place of the KOH solution to generate the dye and that the optical densities were determined at 573 mμ.

Reagents and Assay Procedure.—Adenosine and adenosine deaminase (type I) were purchased from the Sigma Chemical Co. The assay procedure for the reversible inhibitors has been described previously[10] and is a modification of the general procedure described by Kaplan.[11] The measurements of the rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6. The stock solution of the enzyme, substrate, and inhibitors were prepared in 0.05 *M* phosphate buffer at pH 7.6. Those inhibitors which were only slightly soluble in phosphate buffer were dissolved in phosphate buffer containing 10% dimethyl sulfoxide. The addition of dimethyl sulfoxide caused a slight decrease in the initial rate of the enzyme reaction. Consequently, in those experiments where it was necessary to employ dimethyl sulfoxide to dissolve the inhibitor in phosphate buffer, the stock solutions of enzyme, substrate, and inhibitors were all prepared in phosphate buffer containing 10% dimethyl sulfoxide. In this way, a constant 10% concentration of dimethyl sulfoxide was maintained during the determination of the velocities of the enzymic reactions. In order to determine that the dimethyl sulfoxide did not cause variation in the index of inhibition, the following experiment was performed. For an inhibitor which was readily soluble, the index of inhibition was determined in one set of experiments where all reagents were dissolved in 0.05 *M* phosphate buffer and in another set of experiments where the enzyme, substrate, and inhibitor were dissolved in 0.05 *M* phosphate buffer containing 10% dimethyl sulfoxide. In this way, it was found that the index of inhibition did not vary in the two different determinations.

The adenosine deaminase inactivation procedure is a modification of an elegant method described in the literature.[12] A solution of the enzyme was prepared such that a 50-μl aliquot when diluted in phosphate buffer to 3.1 ml which was 0.065 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 or solutions of the irreversible inhibitor in phosphate buffer containing 10% dimethyl sulfoxide. 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 27° in triplicate experiments by using a 100-μl sample of each aliquot. It was shown that the irreversible inhibitor did not inactivate the enzyme during the time it was kept at 0°. Each point on the plots is an average of three determinations; each inactivation experiment was repeated at least twice.

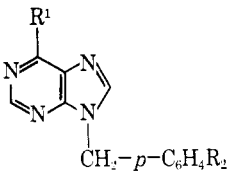
Results and Discussion

As the first phase of this study, it was necessary to determine which, if any, of the 6-substituted purines that contained a 9-(*para*-substituted benzyl) group were capable of forming reversible complexes with adenosine deaminase. An examination of Table II reveals that a number of these compounds were capable of forming reversible complexes with the enzyme. Those compounds which were substituted at the 6 position of the purine nucleus by an amino or methylamino group were inhibitors of adenosine deaminase. In agreement with our previous finding, those compounds which were

(9) B. R. Baker and J. H. Joshel, *J. Heterocyclic Chem.*, **2**, 21 (1965).
(10) H. J. Schaeffer and P. S. Bhargava, *Biochemistry*, **4**, 71 (1965).
(11) N. O. Kaplan, *Methods Enzymol.*, **2**, 473 (1955).
(12) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

TABLE II

INHIBITION INDEX OF SOME 6-SUBSTITUTED 9-(*para*-SUBSTITUTED BENZYL)PURINES WITH ADENOSINE DEAMINASE



Compd[a]	R_1	R_2	mM conc for 50% inhib[b]	$[I]/[S]_{0.5}$	$K_i \times 10^5$ M
V	NH_2	NO_2	0.24 ± 0.01[c]	3.6 ± 0.1[c]	
VI	NHMe	NO_2	0.27 ± 0.01	4.1 ± 0.2	
X	NH_2	NH_2	0.14 ± 0.01	2.2 ± 0.1	
XI	NH_2	$NHCOOC_6H_5$	0.030 ± 0.003	0.45 ± 0.04	2.7 ± 0.3
XII	NH_2	$NHCOCH_2Br$	0.018 ± 0.003	0.27 ± 0.05	1.3 ± 0.1
XIII	NH_2	$NHCOCH_3$	0.031 ± 0.001	0.47 ± 0.003	
XVI	NH_2	H	0.10 ± 0.01	1.5 ± 0.2	

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*. In no experiment did the concentration of inhibitor exceed 0.12 m*M*. In those cases where a higher concentration is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/V *vs.* [I], where V_0 = initial velocity of the uninhibited enzymatic reaction, V = initial velocity of the inhibited enzymatic reaction at various inhibitor concentrations, and [I] = the various concentrations of inhibitor. [c] Average deviation.

substituted at the 6 position of the purine nucleus by a chloro, dimethylamino, hydroxy, or mercapto group (III, VII, VIII, or IX) were either noninhibitory or at best, very weakly inhibitory relative to the corresponding adenine derivative. In addition, 7-*p*-nitrobenzyladenine (XIV) was esentially noninhibitory, whereas XV exhibited an $[I]/[S]_{0.5}$ of 5.3 ± 0.1.

Regarding the *para* substituent on the 9-benzyl group of the 6-aminopurines, it was found that the inhibitory power of these compounds decreases in the following order: $NHCOCH_2Br$ (XII) > $NHCOOC_6H_5$ (XI) > $NHCOCH_3$(XIII) > H (XVI) > NH_2 (X) > NO_2 (V). The K_i of the two potential irreversible inhibitors (XI and XII) was determined by the double reciprocal plot method.[13] These compounds were competitive inhibitors of adenosine deaminase with K_i values that differed by a factor of approximately 2. At the present time, it is not possible to rationalize the order of inhibition which is influenced by the *para* substituent of the benzyl group but further studies are planned from which it is hoped an understanding of this phenomenon will be obtained.

When the two potential irreversible inhibitors (XI and XII) were incubated with adenosine deaminase at 37°, it was found that the phenoxycarbonylamino derivative (XI) was not an irreversible inhibitor, whereas the bromoacetamido derivative (XII) was an irreversible inhibitor. The inactivation of the enzyme by XII could not be reversed by dialysis; since the bromoacetamido moiety of XII is a good alkylating agent for nucleophilic groups, we believe that the irreversible inactivation of adenosine deaminase is caused by the formation of a covalent bond between XII and a nucleophilic group on the enzyme. Similar observations have recently been made on chymotrypsin[14,15] and lactic and glutamic dehydrogenase.[16,17] An examination of Figure 1 shows the apparent first-order loss of enzyme

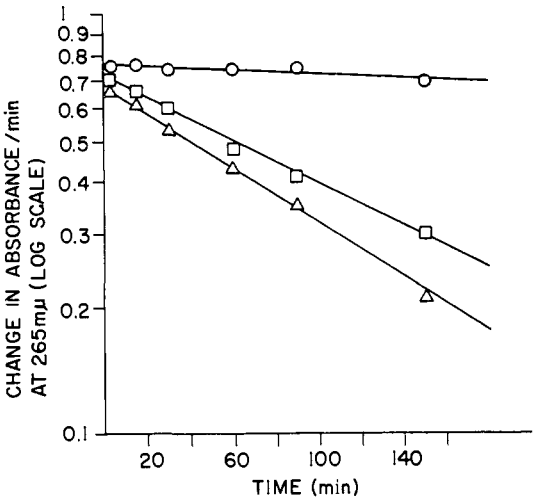


Figure 1.—Comparison of the irreversible inhibition of adenosine deaminase by 9-*p*-bromoacetamidobenzyladenine: ○, enzyme control; □, 0.015 m*M*; △ 0.030 m*M*.

during incubation with two different concentrations of XII.[18] In addition, it has also been found that iodoacetamide did not cause inactivation of the enzyme, even at concentrations much higher than that of XII. Comparative chemical reactivities of iodoacetamide and XII were determined using 4-(*p*-nitrobenzyl)pyridine as the nucleophilic reagent,[9,19,20] and it was found that XII is almost three times more reactive than iodoacetamide (see Figure 2). Since iodoacetamide was employed in the enzyme inactivation study at concentrations 6–12 times greater than XII and still did not cause inactivation, it follows that the irreversible inhibition of adenosine deaminase by XII is not caused by a random bimolecular process. Indeed, we believe that this experiment offers strong evidence that the irreversible inhibition of adenosine deaminase by XII

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(18) It has been found that XII is relatively stable when incubated in phosphate buffer, *i.e.*, less than 10% of XII is lost during a 4-hr incubation with phosphate buffer.

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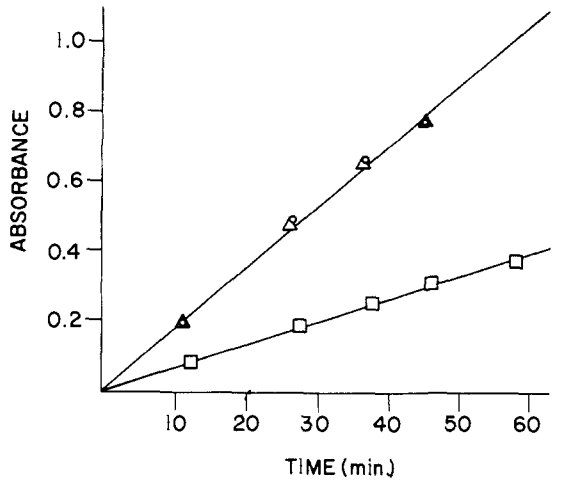

Figure 2.—Comparative chemical reactivities of some alkylating agents with 4-(*p*-nitrobenzyl)pyridine at pH 4.2: □, 0.27 m*M* iodoacetamide; △, 0.27 m*M* XII; ○, 0.27 m*M* XII and 2.7 m*M* 9-(3-hydroxypropyl)adenine.

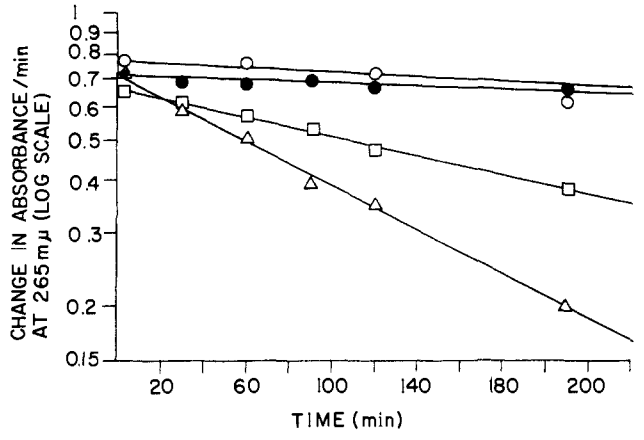

Figure 3.—Protection of adenosine deaminase from irreversible inhibition: ○, enzyme control; ●, 0.12 m*M* 9-(3-hydroxypropyl)-adenine; □, 0.12 m*M* 9-(3-hydroxypropyl)adenine and 0.015 m*M* 9-(*p*-bromoacetamidobenzyl)adenine; △, 0.015 m*M* 9-(*p*-bromoacetamidobenzyl)adenine.

proceeds as shown in eq 1. Thus, the inhibitor and enzyme form a reversible complex, and it is through this complex that the irreversible inactivation occurs.

$$E + I \rightleftharpoons E\text{-}I \longrightarrow EI \qquad (1)$$

It is postulated that when the inhibitor is complexed with the enzyme, the bromoacetamido group of XII is then held near a nucleophilic group on the enzyme, and a reaction related to a neighboring-group reaction occurs with the formation of a covalent bond. The lack of inactivation of adenosine deaminase by iodoacetamide is, then, readily explained since iodoacetamide does not form a complex with this enzyme, and consequently, is not held in the proper position for reaction with the nucleophilic group on the enzyme.

Additional evidence that the irreversible inhibition of adenosine deaminase proceeds through a reversible E-I complex can be obtained by a comparison of the rates of inactivation caused by two different concentrations of XII. Baker has derived and employed eq 2 to calculate a theoretical ratio of the rate of inactivation of an enzyme by two different concentrations of an irreversible inhibitor.[17] In eq 2, [EI] is the concentration of the reversible E-I complex, $[E_t]$ is the total enzyme concentration, K_i is the enzyme–inhibitor dissociation constant, and [I] is the concentration of the inhibitor employed in the enzyme inactivation experiments.

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad (2)$$

When [I] = 0.015 m*M*, [EI] = 0.54$[E_t]$, and when [I] = 0.030 m*M*, [EI] = 0.70$[E_t]$. Thus, increasing the concentration of XII from 0.015 m*M* to 0.030 m*M* results in an increase of [EI] by 0.70/0.54 or 1.30 times. If the rate of inactivation of the enzyme proceeds through an E-I complex, it follows that the ratio of the apparent first-order rates of inactivation by two different concentrations of XII should equal the calculated ratio of the concentration of the E-I complex. It has been found that increasing the concentration of XII from 0.015 m*M* to 0.030 m*M* increased the rate of inactivation by a factor of 1.27 (see Figure 1), in excellent agreement with the calculated value of 1.30.

One might argue that XII is an irreversible inhibitor of adenosine deaminase, whereas iodoacetamide is not if one assumes that the benzyladenine moiety of XII causes a conformational change of the enzyme exposing a nucleophilic group. If this were true, it should be possible to irreversibly inhibit the enzyme with a mixture of iodoacetamide and 9-benzyladenine.[21] However, when an equimolar mixture of iodoacetamide and 9-benzyladenine was incubated with adenosine deaminase, it did not cause an irreversible inhibition of the enzyme. Consequently, the irreversible inhibition of the enzyme by XII appears to be specifically related to the E-I complex, with or without a conformational change of the enzyme, in which the irreversible process occurs.

When adenosine deaminase was incubated with a mixture of XII and 9-(3-hydroxypropyl)adenine, a reversible inhibitor of the enzyme, the rate of irreversible inactivation was lower (Figure 3). Such protection from irreversible inhibition by a reversible inhibitor has been taken as evidence that the irreversible inhibition involves the active site.[17,22] It might be suggested that the protection of the enzyme by 9-(3-hydroxypropyl)adenine might be due to a chemical interaction between the reversible inhibitor and XII. However, it is doubtful that such an interaction occurs because it has been found that the rates of alkylation of 4-(*p*-nitrobenzyl)pyridine by XII and by a mixture of XII and 9-(3-hydroxypropyl)adenine were essentially equal (see Figure 2). These data, in conjunction with the enzyme inactivation experiments, support the concept that XII inactivates the enzyme by means of an E-I complex.

Finally, it has been found that 9-(*p*-acetamidobenzyl)adenine (XIII) is a reversible, but not an irreversible inhibitor of adenosine deaminase. Since the structures of XII and XIII are very closely related, the major difference is that XII is an alkylating agent, it is reasonable to assume that the irreversible inactivation of the enzyme by XII occurs by alkylation and not

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by some nonspecific poisoning effect of its gross structure.

In summary, we believe that these data indicate that the irreversible inhibition of adenosine deaminase by XII is not a random bimolecular process but proceeds through an initial reversible E–I complex which is dependent on the gross structure of the inhibitor. The enzyme is then irreversibly inhibited by alkylation within the E–I complex by the bromoacetamido moiety of XII with the resultant formation of a relatively stable covalent bond between the enzyme and inhibitor. The inhibitor which is covalently bound to the enzyme may then exert its inhibitory effect on the enzymic reaction by any one of a number of processes, *e.g.*, the enzyme-bound inhibitor may prevent, for steric reasons, the approach of the substrate to the enzyme, or the enzyme-bound inhibitor may cause a conformational change in the enzyme with the result that there no longer exists an attraction of the substrate to the enzyme.

Boron Hydride Anions. I. Nitrogen Mustards[1,2]

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Reactions of carbonyl-containing boron hydrides, 1,12-$B_{12}H_{10}(CO)_2$ and 1,6-$(CH_3)_2SB_{10}H_8CO$, with amines, in work directed toward the synthesis of boron cage compounds containing a nitrogen mustard moiety, have shown the high polarizability of the carbonyl group. This is demonstrated by infrared spectra, the nucleophilicity of the carbonyl oxygen, and the facile hydrolysis of amides and esters. Synthesis of a nitrogen mustard containing boron hydride was accomplished by acylation of the decahydrodecaborate anion.

Use of boron-10 neutron-capture therapy in the treatment of brain tumors has been unsuccessful to date.[3] Failure to a great extent has resulted from an inability to incorporate a boron compound into the tumor without concomitantly high levels in the surrounding tissues, such as normal brain, muscle, and more especially blood. To overcome these difficulties, the concept of developing compounds which have two moieties: one, a "handle" for incorporation into tumors, and two, a neutron absorber, was tried,[4] but was without success.[5] This approach has become more pertinent with the recently described synthesis of stable boron hydride anions, $B_{12}H_{12}^{2-}$ and $B_{10}H_{10}^{2-}$,[6] and the carboranes.[7] These structures possess high boron percentages and, in view of their chemical stability, it is highly possible that they will not be extensively destroyed by the normal biological processes.[8]

Nitrogen mustards, $RN(CH_2CH_2Cl)_2$, have a profound effect on tumors and there is evidence of incorporation of certain mustards into brain tumors.[9] On this basis the synthesis of a nitrogen mustard containing boron hydride anion was undertaken with the idea that such a compound might concentrate preferentially in brain tumor relative to other adjacent tissues. The reaction[10] of the carbonyl derivatives of these boron hydride cage compounds with primary and secondary amines and with ammonia itself occurs with the formation of ammonium salts of the corresponding carboxamide derivatives of the cage anions, *e.g.*, $(NH_4)_2B_{12}H_{10}(CONH_2)_2$. We have sought to apply this reaction to bis(2-chloroethyl)amine with the thought of incorporating the mustard moiety into a boron hydride cage compound.

Results and Discussion

B_{12}-Cage Compounds.—As a model for this reaction, 1,12-$B_{12}H_{10}(CO)_2$[10] was treated with diethylamine in an acetonitrile solution. The expected amide derivative, $[(C_2H_5)_2NH_2]_2B_{12}H_{10}[CON(C_2H_5)_2]_2$ (I), was obtained. Such structures appeared to have a low order of stability as shown by the cleavage of the amide linkage by both cold aqueous sodium hydroxide and refluxing ethanol. In the former case the sodium salt of the carboxylic acid derivative of the cage anion was obtained, and this was characterized as the triethylammonium, tetramethylammonium, and methyltriethylphosphonium salts of the $B_{12}H_{10}(COOH)_2^{2-}$ anion (IIa–c). In the latter case the carbethoxy derivative of the cage anion was obtained, $B_{12}H_{10}(COOC_2H_5)_2^{2-}$, characterized as the diethylammonium, triethylammonium, and tetramethylammonium salts (IIIa–c).

In contrast with the extreme lability of the amide linkage, the product obtained by the reaction of $B_{12}H_{10}(CO)_2$ with excess bis(2-chloroethyl)amine was surprisingly stable. Instead of the expected 4 moles of

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