ether layers were washed with water, dried (Na_2SO_4), and evaporated to give 18.1 g of colorless oil which was chromatographed on 270 g of silica gel (0.2–0.5 mm). Elution with ethyl acetate-benzene (1:4) and finally with pure ethyl acetate gave 16.1 g of colorless oil. Crystallization from ether–hexane gave 11.56 g of half-ester (XIIIc), mp 98–101.5°. An additional 2.51 g of product, mp 93–97.5°, was recovered from the mother liquors (total yield 73%). Crystallization from ether–hexane gave the analytical sample: mp 100.5–102.5°; $[\alpha]^{25}D$ +70.4° (c 1.0, C_2H_5OH); $\lambda_{max}^{C_2H_5OH}$ 219 mμ (ε 8470), 278 (2060), 287 (1950); $\lambda_{max}^{CHCl_3}$ 2.77 and 5.81 μ. This compound was described previously as an oil, $[\alpha]^{20}D$ +61 ± 4° (c 0.19, alcohol).[11]

Anal. Calcd for $C_{20}H_{26}O_5$: C, 69.34; H, 7.57. Found: C, 69.64; H, 7.69.

16-Azaestrone 3-Methyl Ether (XVa).—To a solution of 12.5 g (0.0362 mole) of XIIIc in 63 ml of benzene was added 12.5 ml of oxalyl chloride, and the resulting mixture was heated at 65° for 50 min. An additional 6.0 ml of oxalyl chloride was then added and heating was continued for 30 min. The reaction mixture was evaporated to dryness and the residue was crystallized from ether–hexane to give 11.68 g of crude acid chloride, mp 88.5–92°, $\lambda_{max}^{CHCl_3}$ 5.54 and 5.80 μ. This compound has been described previously as an oil.[11]

A solution of 9.0 g (0.138 mole) of NaN_3 in 35 ml of water was added over a 10-min period to a cold (0–5°) solution of 11.38 g of crude acid chloride in 228 ml of acetone. The mixture was allowed to stir in the ice bath for an additional 15 min and was then diluted with 1 l. of water. The mixture was extracted with ether and the ether extracts were washed with water, dried (Na_2SO_4), and evaporated to give the crude azide XIIId as an oil, $\lambda_{max}^{CHCl_3}$ 4.67 (sharp) and 5.81 μ. A solution of this azide in 400 ml of benzene was heated under reflux for 1 hr at which time the evolution of gas had ceased. The benzene was then removed under reduced pressure to give the crude isocyanate XIV as an oil, $\lambda_{max}^{CHCl_3}$ 4.40 (broad) and 5.81 μ.

A solution of 11.3 g (0.2 mole) of KOH in 11.5 ml of water was added to a solution of the crude isocyanate in 115 ml of methanol. The reaction mixture was heated under reflux for 1.5 hr and was then diluted with 500 ml of water. The resulting precipitate was filtered, washed with water, and dried to give 7.53 g (73% yield from the half-ester XIIIc) of the lactam XVa, mp 210.5–212.5° (vac). Crystallization from CH_2Cl_2–ether gave the analytical sample: mp 211–212° (vac); $[\alpha]^{25}D$ +78.4° (c 1.0, C_2H_5OH); $\lambda_{max}^{C_2H_5OH}$ 220 mμ (ε 8600), 279 (1980), 287 (1780); $\lambda_{max}^{CHCl_3}$ 2.92 and 5.92 μ; lit.[5] mp 202–205°, $[\alpha]D$ +70.5° ($CHCl_3$).

Anal. Calcd for $C_{18}H_{23}NO_2$: C, 75.75; H, 8.12; N, 4.91. Found: C, 75.56; H, 8.15; N, 5.08.

16-Azaestrone (XVb).—A mixture of 4.0 g (0.014 mole) of XVa and 80 g (0.79 mole) of pyridine hydrochloride was heated with stirring at 210° for 40 min in an atmosphere of nitrogen. The reaction mixture was then cooled and diluted with 600 ml of 2 N HCl. The resulting precipitate was filtered, washed with water, and dried to give 3.40 g of crude product, mp 362–364° (vac). Crystallization from chloroform–ethanol gave 1.88 g of XVb, mp 360–362.5° (vac). A further 0.89 g of XVb (mp 362–364°) was recovered from the mother liquors (total yield, 2.77 g, 73%). Crystallization from $CHCl_3$–ethanol gave the analytical sample: mp 362.5–364.5° (vac); $[\alpha]^{25}D$ +82.5° (c 0.1, C_2H_5OH); $\lambda_{max}^{C_2H_5OH}$ 220 mμ (ε 7500), 280 (2090), 288 (1810); λ_{max}^{KBr} 2.93, 5.95, and 6.01 μ.

Anal. Calcd for $C_{17}H_{21}NO_2$: C, 75.24; H, 7.80; N, 5.16. Found: C, 75.31; H, 7.78; N, 5.17.

16-Azaestra-1,3,5(10)-trien-3-ol 3-Methyl Ether Hydrochloride (XVI).—A solution of 1.38 g (4.84 mmoles) of XVa in 46 ml of dry dioxane was added rapidly to a boiling solution of 1.38 g (36.4 mmoles) of $LiAlH_4$ in 46 ml of dry dioxane and the resulting mixture was heated under reflux for 20 hr in a nitrogen atmosphere. Water (7.4 ml) was added slowly to the ice-cold reaction mixture which was then heated under reflux for 30 min. The hot mixture was filtered through a bed of Celite, the latter being washed with hot dioxane. The filtrate was evaporated to dryness and the resulting colorless oil was dissolved in benzene and passed through a short column of Florisil. Evaporation of the eluates gave an oil which was dissolved in CH_2Cl_2, washed three times with 2 N HCl, dried (Na_2SO_4), and evaporated. The residue was crystallized from CH_2Cl_2–ether to give 1.16 g (78%) of XVI, mp 298.5–301° (vac). Crystallization from ethanol gave the analytical sample: mp 298–301° (vac); $[\alpha]^{25}D$ +76° (c 0.5, ethanol); $\lambda_{max}^{C_2H_5OH}$ 219 mμ (ε 8050), 279 (2000), 287 (1810).

Anal. Calcd for $C_{18}H_{26}ClNO$: C, 70.22; H, 8.51; Cl, 11.52; N, 4.55. Found: C, 70.25; H, 8.28; Cl, 11.55; N, 4.53.

Acknowledgment.—We are indebted to Dr. Al Steyermark and his staff for the microanalyses and to Dr. V. Toome and Mr. S. Traiman for the ultraviolet and infrared spectra, respectively.

Enzyme Inhibitors. XV. A New Irreversible Inhibitor of Adenosine Deaminase[1]

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Previous studies have shown that 9-(*p*-bromoacetamidobenzyl)adenine (XVI) and 9-(*m*-bromoacetamidobenzyl)adenine (XVII) are both good reversible inhibitors of adenosine deaminase but that XVI causes an irreversible inactivation of this enzyme at a much higher rate than does XVII. In a continuation of studies on the effect of isomers on the inhibition of adenosine deaminase, a variety of 9-(*ortho*-substituted benzyl)-6-substituted purines have been synthesized. These compounds were weaker reversible inhibitors of adenosine deaminase than was 9-benzyladenine. However, 9-(*o*-bromoacetamidobenzyl)adenine, even though it was a weaker reversible inhibitor of this enzyme than XVI or XVII, was found to be a good irreversible inhibitor of adenosine deaminase. Evidence is presented that this irreversible inactivation of adenosine deaminase proceeds through an initial reversible enzyme-inhibitor complex and not by a random bimolecular reaction between the enzyme and the inhibitor.

Recent studies have shown that 9-(*p*-bromoacetamidobenzyl)adenine and 9-(*m*-bromoacetamidobenzyl)adenine are both good reversible inhibitors of adenosine deaminase obtained from calf intestinal mucosa.[2-4] However, when these compounds were evaluated as irreversible inhibitors of this enzyme, it was found that the *para* isomer was a good irreversible inhibitor,[3] whereas the *meta* isomer was a poor irreversible inhibitor of adenosine deaminase.[4] Since the chemical reactivity of the *meta* isomer is greater than that of the *para* isomer when 4-(*p*-nitrobenzyl)pyridine is used as the nucleophilic reagent,[4] it would appear that the differences in the rates at which these two compounds irreversibly inactivate adenosine deaminase is due to the difference in the environment on the enzyme in which the alkylating group of the inhibitor

(1) This investigation was supported by Grant T-337A from the American Cancer Society, by a Public Health Service research career program award 5-K3-CA 18718 from the National Cancer Institute and by Training Grant 5 T1 GM 55 from the Division of General Medical Sciences.

(2) H. J. Schaeffer and E. Odin, *J. Pharm. Sci.*, **54**, 1223 (1965).

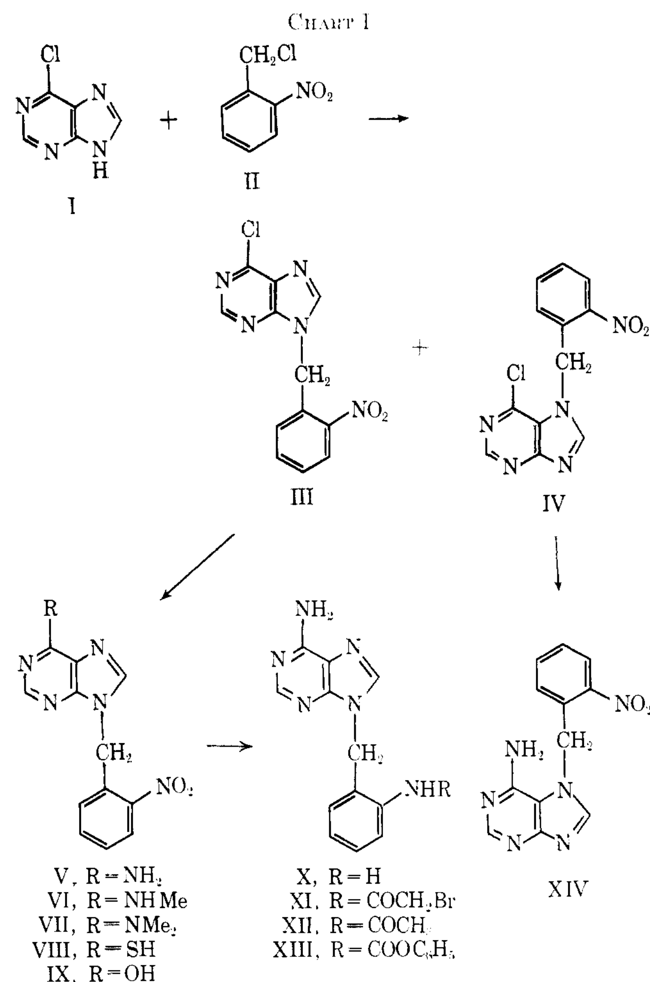
(3) H. J. Schaeffer and E. Odin, *J. Med. Chem.*, **9**, 576 (1966).

(4) H. J. Schaeffer and R. N. Johnson, *J. Pharm. Sci.*, **55**, 929 (1966).

is held during the initial reversible enzyme–inhibitor complex. In an attempt to study further the differences in the reversible and irreversible inhibition of adenosine deaminase, we decided to prepare some 9-(*ortho*-substituted benzyl)-6-substituted purines.

Chemistry.—The general procedure which was used for the synthesis of the required compounds is a modification of a method which has been employed previously[3,5] and is outlined in Chart I. Thus, condensa-

CHART I



tion of 6-chloropurine (I) with *o*-nitrobenzyl chloride (II) gave a mixture of 6-chloro-9- and -7-(*o*-nitrobenzyl)-purines (III and IV)[6] which was separated by chromatography on silica gel. When 6-chloro-9-(*o*-nitrobenzyl)purine (III) was allowed to react with ammonia, methylamine, dimethylamine, thiourea, or dilute hydrochloric acid, the corresponding 6-substituted derivatives (V–IX) were obtained. Catalytic hydrogenation of V gave, in good yield, 9-(*o*-aminobenzyl)-adenine (X) which on treatment with bromoacetyl bromide, acetic anhydride, or phenyl chloroformate gave the N-acylated derivatives (XI, XII, and XIII). The assignment of structure to XI, XII, and XIII is based on the observation that N,O$^{3'}$-diacetyldeoxyadenylic 5′-acid exhibited an ultraviolet maximum at 273 mμ at pH 8, whereas XI, XII, and XIII exhibited ultraviolet maxima in the range of an adenine derivative with an unsubstituted 6-amino group.[7]

Finally, treatment of IV with liquid ammonia at 45° gave the corresponding 7-(*o*-nitrobenzyl)adenine (XIV).

Experimental Section[8]

6-Chloro-9- and -7-*o*-nitrobenzylpurines (III and IV).—To a solution of 1.30 g (12.9 mmoles) of triethylamine in 30 ml of DMF was added 2.00 g (12.9 mmoles) of I and 2.40 g (14.0 mmoles) of II, and the reaction mixture was stirred at room temperature for 44 hr and then at 50° for an additional 22 hr. The mixture was then poured into 30 ml of H_2O at 0°, and the insoluble material was collected by filtration; yield, 2.98 g (79.7%). This material was dissolved in $CHCl_3$ and introduced on a column (29.5-mm inside diameter) of 150 g of silica gel (grade 950) in $CHCl_3$. The column was eluted with $CHCl_3$ and 87 100-ml fractions were collected. Eluent fractions 7–62 were combined and the solvent was evaporated *in vacuo* leaving a solid residue of III (see Table I for analyses[9] and physical constants).

Eluent fractions 74–87 were combined and the solvent was evaporated *in vacuo* leaving a solid residue of IV.

General Method 1. Preparation of V–VII and XIV.—A mixture of 200 mg (0.690 mmole) of III in methanolic NH_3, aqueous methylamine, or aqueous dimethylamine was heated in a stainless steel bomb at 85–100° for 20–24 hr. After the reaction mixture was cooled, the material which precipitated was collected by filtration to give V–VII, respectively.

The same general procedure was used for XIV except liquid NH_3 was employed at 45°.

6-Mercapto-9-*o*-nitrobenzylpurine (VIII).—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 1 hr. The insoluble material which precipitated from the cooled mixture was collected by filtration (194 mg, 98.5%), mp 291–292° dec (evolution of gas).

9-(*o*-Aminobenzyl)adenine (X).—A solution of 346 mg (1.28 mmoles) of V in 100 ml of glacial acetic acid containing 100 mg of 5% Pd–C was hydrogenated on a Parr hydrogenator at an initial pressure of 3.96 kg/cm². After 30 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 1.5 hr which gave 294 mg (95.8%) of material, mp 212–215° dec.

9-(*o*-Bromoacetamidobenzyl)adenine Hydrobromide (XI).—A solution of 266 mg (1.11 mmoles) of X in 225 ml of 1,2-dimethoxyethane was added slowly with constant stirring to 323 mg (1.60 mmoles) of bromoacetyl bromide in an ice bath, and the cold reaction mixture was stirred for 5.5 hr. The insoluble material which precipitated (450 mg, 91.8%) was collected by filtration.

9-(*o*-Acetamidobenzyl)adenine (XII).—To a cold solution of 150 mg (0.625 mmole) of X in 5 ml of THF and 0.6 ml of 10% acetic acid was added dropwise 462 mg (4.56 mmoles) of acetic anhydride in 4 ml of THF, and the solution was stirred at room temperature for 1.5 hr. Upon cooling the solution in an ice bath, a precipitate formed which was collected by filtration.

9-(*o*-Phenoxycarbonylaminobenzyl)adenine (XIII).—A solution of 144 mg (0.600 mmole) of X and 60 mg (0.600 mmole) of triethylamine in 200 ml of *p*-dioxane was added slowly with constant stirring to 153 mg (0.98 mmole) of phenyl chloroformate. After 22 hr of stirring, the insoluble material was removed by filtration, and the solvent was reduced *in vacuo* to 10 ml. Cold water (100 ml) was added, and the mixture was cooled in an ice bath. The fine precipitate which formed was collected by filtration.

Chemical Reactivity of the Alkylating Agents.—These experiments were performed by a modification of a procedure de-

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

(6) The structures of 6-chloro-9- and 7-(*o*-nitrobenzyl)purines (III and IV) were assigned by analogy to similar alkylations.

(7) F. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

(8) The ultraviolet spectra were determined on a Perkin-Elmer Model 202 spectrophotometer; the enzyme studies were done on a Gilford Model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp and are corrected.

(9) The analyses reported in this paper were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and Micro-Analysis, Inc., Wilmington, Del.

TABLE I

9-(*ortho*-SUBSTITUTED BENZYL)-6-SUBSTITUTED PURINES

Compd	Recrystn solvent[a]	Mp, °C	Yield, %	Ultraviolet λ_{max}, mμ ($\epsilon \times 10^{-3}$): Ethanol–1 N HCl	Ethanol	Ethanol–1 N NaOH	Formula	Carbon, %: Calcd	Found	Hydrogen, %: Calcd	Found	Nitrogen, %: Calcd	Found	Other, %[b]: Calcd	Found
III	A	178–179.5	32.1	264 (13.8)	264 (13.6)	257 (14.6)	$C_{12}H_8ClN_5O_2$	49.75	49.60	2.78	2.90	24.18	23.96	12.24[b]	11.99[b]
IV	A	194.5–197	4.6	266 (18.4)	266 (18.4)	263 (18.8)	$C_{12}H_8ClN_5O_2$	49.75	49.95	2.78	2.91	24.18	24.02	12.24[b]	12.12[b]
V[c]	A	281–282[d]	61.3	259 (18.8)	259 (18.8)	260 (20.9)	$C_{12}H_{10}N_6O_2$	53.33	53.03	3.73	3.85	31.10	31.39		
VI[c]	B	245–247[d]	45.9	262 (23.6)	264 (21.7)	263 (27.3)	$C_{13}H_{12}N_6O_2$	54.92	55.30	4.26	4.31	29.57	29.65		
VII[c]	A	169–171	61.5	268 (21.9)	273 (21.7)	273 (26.8)	$C_{14}H_{14}N_6O_2$	56.37	56.45	4.73	4.93	28.18	28.06		
VIII	C	292–294[d]	82.3	323 (23.7)	322 (23.7)	317 (27.9)	$C_{12}H_9N_5O_2S$	50.18	49.86	3.16	3.45	24.39	24.04	11.16[f]	11.24[f]
IX	B	268–270[d]	54.4	250 (16.6)	250 (15.1)	255 (17.4)	$C_{12}H_9N_5O_3$	53.13	52.95	3.34	3.00	25.83	25.70		
X	D	217–218[d]	87.6	259 (13.9)	261 (13.5)	261 (16.0)	$C_{12}H_{12}N_6$	59.98	60.26	5.03	5.26	34.98	34.76		
XI	E	248–250[d]	67.8	259 (16.8)	260 (17.1)	265 (25.8)	$C_{14}H_{14}Br_2N_6O$	38.03	38.08	3.19	3.20	19.01	18.93	36.15[g]	35.93[g]
XII	F	222–223	52.2	260 (14.7)	261 (14.6)	262 (15.0)	$C_{14}H_{14}N_6O$	59.56	59.26[e]	5.00	5.12	29.77	29.81		
XIII	G	131–132[d]	18.1	260 (14.1)	262 (14.6)	237 (28.6), 260 (20.8)	$C_{19}H_{16}N_6O_2 \cdot 0.25H_2O$	62.52	62.85	4.56	4.60	23.03	22.92	9.86[h]	9.73[h]
XIV[c]	B	257–258	37.1	264 (24.8)	264 (24.6)	263 (25.4)	$C_{12}H_{10}N_6O_2$	53.33	53.66	3.73	3.94	31.10	31.30		

[a] Recrystallization solvents: A, MeOH; B, absolute EtOH; C, solution of NaOH precipitate with 6 N HCl; D, xylene; E, MeOH–Et_2O; F, H_2O; G, MeOH–H_2O. [b] Chlorine. [c] Prepared by general method 1. [d] Melts with decomposition. [e] Analyzed for the 0.75 hydrate and then for the anhydrous compound after drying *in vacuo* at 100°. [f] Sulfur. [g] Bromine. [h] Oxygen.

scribed in the literature.[10–12] Equal volumes (10 ml) of the following preheated (37°) solutions were mixed: (a) 5% 4-(*p*-nitrobenzyl)pyridine in 2-methoxyethanol, (b) 0.05 *M* phthalate buffer (pH 4.2) in water, and then (c) 0.81 m*M* solution of the alkylating agent in 2-methoxyethanol. The reaction mixture was incubated at 37° and at appropriate time intervals, a 3-ml aliquot was removed and cooled briefly in an ice bath. Then 1 ml of triethylamine was added to generate the colored quinoid-like free base, and the absorbance was immediately determined at 573 mμ against a blank which had been treated in an identical manner and contained all of the reagents except the alkylating agent. A comparison of the initial rates of reaction are given in Figure 2.

Reagents and Assay Procedure.—Adenosine deaminase (Type I, calf intestinal mucosa) was purchased from the Sigma Chemical Co. The assay procedure for the reversible inhibitors has been described previously[3] and is a modification of the procedure developed by Kalckar[13] and by Kaplan.[14] The measurement of the initial rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6. Those inhibitors which were only slightly soluble in phosphate buffer were evaluated in solutions of phosphate buffer containing 10% dimethyl sulfoxide as previously described.[3]

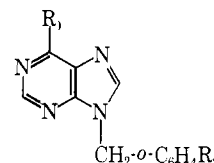
The method used to study the irreversible inactivation of adenosine deaminase at 37° has been described earlier[3,4] and is a modification of a published procedure.[15]

Results and Discussion

When the 9-(*ortho*-substituted benzyl)-6-substituted purines were evaluated as reversible inhibitors of adenosine deaminase, it was found that they were weaker reversible inhibitors than 9-benzyl adenine (see Table II). This weaker reversible inhibition of adenosine deaminase by the 9-(*ortho*-substituted benzyl)adenines suggests that adenosine deaminase has limited bulk

TABLE II

REVERSIBLE INHIBITION OF ADENOSINE DEAMINASE BY SOME 6-SUBSTITUTED 9-(*ortho*-SUBSTITUTED BENZYL)PURINES



Compd[a]	R_1	R_2	m*M* concn for 50% inhib[b]	$([I]/[S])_{0.5}$
V	NH_2	NO_2	0.13 ± 0.01[c]	1.9 ± 0.1[c]
VI	NHMe	NO_2	0.31 ± 0.01	4.7 ± 0.1
X	NH_2	NH_2	0.27 ± 0.01	4.1 ± 0.1
XI[d]	NH_2	$NHCOCH_2Br$	0.41 ± 0.01	6.2 ± 0.1
XII	NH_2	$NHCOCH_3$	0.53 ± 0.03	8.0 ± 0.4
XIII	NH_2	$NHCOOC_6H_5$	0.35 ± 0.02	5.3 ± 0.3
XV	NH_2	H	0.10 ± 0.01	1.5 ± 0.2[e]

[a] None of these compounds served as a substrate of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*. In no experiment of reversible inhibition 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 reaction, V = initial velocity of the inhibited reaction at various inhibitor concentrations, and [I] = the various concentrations of inhibitor. [c] Average deviation. [d] $K_i = 44 \times 10^{-5}$ *M*, competitive inhibitor as shown by the double reciprocal plot method. [e] Data taken from ref 3.

(10) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955).
(11) T. J. Bardos, N. Datta-Gupta, P. Hebborn, and D. J. Triggle, *J. Med. Chem.*, **8**, 167 (1965).
(12) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **2**, 21 (1965).
(13) H. M. Kalckar, *J. Biol. Chem.*, **167**, 461 (1947).
(14) N. O. Kaplan, *Methods Enzymol.*, **2**, 473 (1955).
(15) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

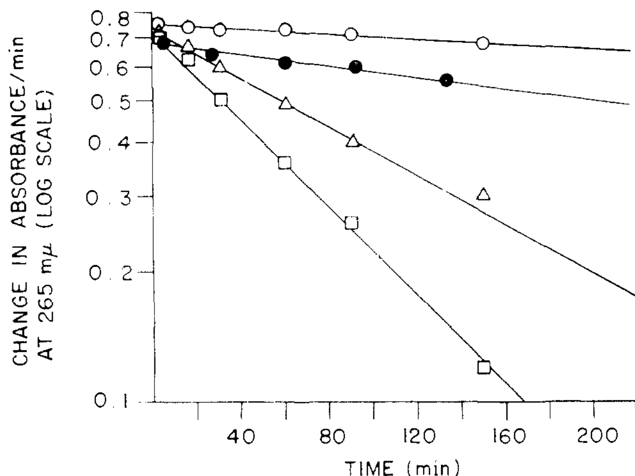
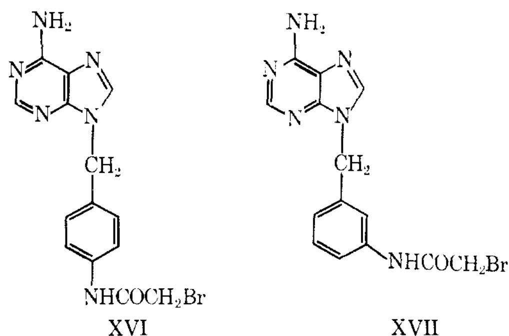


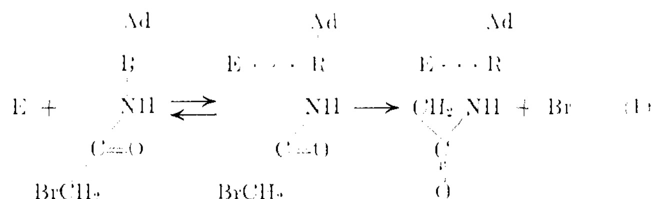
Figure 1.—Irreversible inhibition of adenosine deaminase by 9-(*o*-bromoacetamidobenzyl)adenine (XI) and the protective effect on irreversible inhibition by 9-(2-hydroxypropyl)adenine: O, enzyme control: △, 0.04 mM XI: □, 0.08 mM XI: ●, 0.08 mM XI and 0.10 mM 9-(2-hydroxypropyl)adenine

tolerance for substituents on the *ortho* position of the benzyl group. Electronic factors are also undoubtedly involved but it appears that the main factor causing a weakening of reversible inhibition is of steric origin. In addition, the 6-dimethylamino, 6-mercapto, and the 6-hydroxy derivatives (VII–IX) either did not inhibit or, at best, weakly inhibited adenosine deaminase when the compounds were evaluated at a concentration of 0.12 mM. Finally, 7-(*o*-nitrobenzyl)adenine (XIV) was found to be a reversible inhibitor of adenosine deaminase with an $([I]/[S])_{0.5}$ of 3.7 ± 0.2. We have previously observed that 7-substituted adenines were either weakly or noninhibitory.[3,4,16] That XIV is inhibitory may reflect a different mode of binding by this compound to this enzyme relative to the other 7-substituted adenines.

However, our main interest in the preparation of this series of compounds was to determine the effect of structure on the irreversible inhibition of calf intestinal mucosa adenosine deaminase. We have previously described the preparation and enzymic evaluation of 9-(*p*-bromoacetamidobenzyl)adenine (XVI) and 9-(*m*-bromoacetamidobenzyl)adenine (XVII). It



was found that the *para* isomer (XVI) was a slightly better reversible inhibitor and a much better irreversible inhibitor of adenosine deaminase than was XVII.[3,4] A comparison of the present data reveals that 9-(*o*-bromoacetamidobenzyl)adenine (XI) is an even weaker reversible inhibitor of adenosine deaminase than is the corresponding *meta* derivative (XVII). For example, for XVI the $K_i = 1.3 \times 10^{-5}$ M and the $([I]/[S])_{0.5} = 0.27$ and for XVII the $K_i = 3.6 \times 10^{-5}$ M and the $([I]/[S])_{0.5} = 0.85$. However, when the *ortho* derivative XI was evaluated for its ability to cause irreversible inactivation of adenosine deaminase, it was found to be a good irreversible inhibitor. The irreversible inactivation of adenosine deaminase by XI proceeded to greater than 95%, and the inactivation was not reversed by dialysis. Furthermore, the enzyme was not irreversibly inhibited by 9-(*o*-acetamidobenzyl)-adenine (XII). Since a major difference between XI and XII is the alkylating ability of XI, we believe that the irreversible inhibition caused by XI is a result of the alkylation of the enzyme by the bromoacetamido moiety with the formation of a stable covalent bond between the inhibitor and the enzyme. A third potential irreversible inhibitor of this enzyme is XIII which could inactivate adenosine deaminase by an acylation reaction. When XIII was incubated with the enzyme, irreversible inactivation did not occur; thus, in common with the *para* and *meta* series, the irreversible inactivation occurs by an alkylation process. Other structural features than mere alkylating ability in the inhibitor are necessary for the irreversible inactivation of adenosine deaminase. For example when a 1.6 mM concentration of iodoacetamide was incubated with the enzyme, no detectable irreversible inhibition occurred, but when 0.04 mM and 0.08 mM concentrations of XI were incubated at 37° with adenosine deaminase, a rapid irreversible inhibition occurred (see Figure 1). Thus, even when iodoacetamide was employed in a concentration 20 to 40 times greater than XI, no detectable irreversible inhibition was evident. On the basis of these results, we suggest that the irreversible inhibition of adenosine deaminase by XI is not a random bimolecular process but occurs through an initial reversible enzyme–inhibitor complex as shown in eq 1. This mechanism would explain why



iodoacetamide is inactive at 1.6 mM, whereas XI is active at much lower concentrations. According to eq 1, the irreversible step occurs only after an initial reversible complex has occurred. Since XI is a reversible inhibitor of the enzyme, whereas iodoacetamide at 1.6 mM is not, it is apparent that the bromoacetamido moiety of XI is positioned in the reversible E···I complex near a nucleophilic group on the enzyme and then a reaction related to a neighboring-group reaction occurs resulting in a rapid irreversible inactivation of the enzyme. Whereas it is theoretically possible for iodoacetamide to react with the same nucleophilic group on the enzyme, this reaction does not occur because there is little or no reversible complex to direct the alkylating agent to the nucleophilic group on the enzyme. If the irreversible inactivation of adenosine deaminase by XI occurs through a reversible E···I complex, the pseudo-first-order loss of enzyme activity should be proportional to the con-

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

centration of the reversible E···I complex. Baker[17] has derived and employed eq 2 to calculate the concentration of the E···I complex in terms of total enzyme, E_t. In eq 2, K_i is the dissociation constant of

$$[E\cdots I] = [E_t]/(K_i/[I] + 1) \quad (2)$$

the [E···I] complex and [I] is the concentration of the inhibitor. In the case of XI, when [I] = 0.04 m*M*, [E···I] = 0.083[E_t] and when [I] = 0.08 m*M*, [E···I] = 0.15[E_t]. Therefore, increasing the concentration of XI results in an increase of the [E···I] complex by 0.15[E_t]/0.083[E_t] or 1.8 times. Since the rate of irreversible inactivation is dependent on the concentration of the [E···I] complex, it follows that by increasing the concentration of XI from 0.04 to 0.08 m*M*, the rate of inactivation should increase by a factor of 1.8. An examination of Figure 1 reveals that the ratio of the rates of inactivation is, in fact, 1.8 in excellent agreement with the calculated ratio. We believe that these data offer strong support to the suggestion that the irreversible inactivation of adenosine deaminase proceeds through an initial reversible E···I complex through which covalent bond formation occurs as outlined in eq 1.

Additional evidence supporting the mechanism of irreversible inactivation of adenosine deaminase by XI was obtained in the following manner. When adenosine deaminase was incubated with a mixture of XI and 9-(2-hydroxypropyl)adenine, a reversible inhibitor of this enzyme, the rate of irreversible inactivation was lower (Figure 1). This protection of the enzyme from irreversible inactivation may be rationalized by assuming that the effect of the reversible inhibitor, 9-(2-hydroxypropyl)adenine, is to lower the concentration of the reversible E···I complex between the enzyme and XI which in turn would result in a reduction of the rate of irreversible inhibition. It has been suggested that such protection from irreversible inhibition indicates that the active site of the enzyme is involved.[17,18]

Finally, the interesting observation has been made that for reversible inhibition of adenosine deaminase, the activity decreases in the following order: XVI-(*para*) > XVII(*meta*) > XI(*ortho*). However, for irreversible inhibition of adenosine deaminase it has been found that the *ortho* and *para* compounds (XI and XVI) are much more effective than is the *meta* derivative (XVII). Comparative chemical reactivities using 4-(*p*-nitrobenzyl)pyridine[11-12] as the nucleophilic reagent reveal that the order of reactivity is XI = 1.9, XVII = 1.4, and XVI = 1.0 (Figure 2). Thus, even though the chemical reactivities of the three compounds with 4-(*p*-nitrobenzyl)pyridine are not dramatically different, the rates of irreversible inactivation of adenosine deaminase are quite different.

(17) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).
(18) J. A. Thoma and D. E. Koshland, Jr., *J. Mol. Biol.*, **2**, 169 (1960).

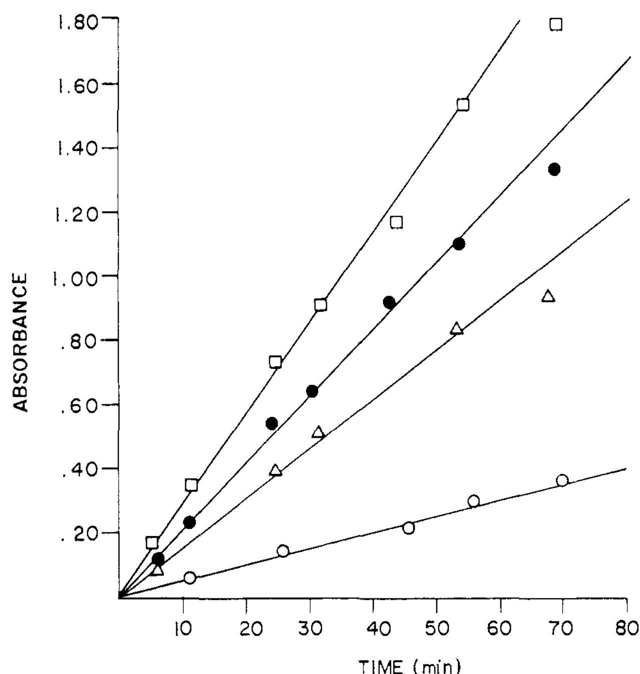


Figure 2.—Comparison of the initial rates of reaction of some alkylating agents with 4-(*p*-nitrobenzyl)pyridine at pH 4.2: ○, 0.27 m*M* iodoacetamide; △, 0.27 m*M* XVI; ●, 0.27 m*M* XVII; □, 0.27 m*M* XI

We have previously calculated that the amount of the total enzyme E_t in the initial reversible E···I complex when XI is employed at 0.08 m*M* concentration is 0.15-[E_t]. When XVI is employed at 0.03 m*M* concentration, the amount of E_t in the initial reversible complex is 0.70[E_t][3] and when XVII is employed at 0.10 m*M* concentration, the amount of E_t in the initial reversible E···I complex is 0.73[E_t].[4] Under these conditions the half-life period for the irreversible inactivation of adenosine deaminase is 58 min for XI, 94 min for XVI,[3] and >600 min for XVII.[4] Therefore, even though the concentration of the initial reversible E···I complex is lower with XI than with XVI or XVII, the half-life period of irreversible inactivation is shorter. These data establish that the alkylation reaction between XI and the enzyme in the E···I complex must be faster than the same step for XVI and much faster than the same step for XVII. Because of these differences in the rates of alkylation of adenosine deaminase, it is possible that XI and XVI are alkylating different amino acids on the enzyme.[19] The *meta* derivative (XVII) in the reversible E···I complex is not held as close to a nucleophilic group on the enzyme and therefore has a much lower rate of irreversible inactivation. A kinetic analysis of the irreversible inhibition of adenosine deaminase will be the subject of a future paper.

(19) It is also possible that XI and XVI are alkylating the same amino acid on the enzyme but that the alkylating group of XI is positioned in the E···I complex closer to the amino acid than it is with XVI. Therefore, the alkylation step is more rapid with XI than with XVI. Experiments are in progress to differentiate these two ideas.