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Enzyme Inhibitors XIV

Syntheses of Some 9-(*m*-Substituted Benzyl)-6-substituted Purines and Their Evaluation as Inhibitors of Adenosine Deaminase

By HOWARD J. SCHAEFFER and R. N. JOHNSON

Recent studies have shown that 9-(*p*-bromoacetamidobenzyl)adenine is an irreversible inhibitor of adenosine deaminase. In order to study the effect of isomers on the inhibition of adenosine deaminase, a variety of reversible inhibitors of adenosine deaminase have been synthesized which are 9-(*m*-substituted benzyl)-6-substituted purines. In addition, it was found that 9-(*m*-bromoacetamidobenzyl)adenine is an irreversible inhibitor of adenosine deaminase, but the rate of irreversible inactivation by the *meta* derivative was lower than that by the corresponding *para* isomer. This decreased rate of irreversible inhibition by 9-(*m*-bromoacetamidobenzyl)-adenine may be rationalized by assuming that in the reversible E...I complex the alkylating group is not positioned as near a nucleophilic group on the enzyme as it is in the case of the corresponding *para* isomer or that the *meta* derivative alkylates a different amino acid on the enzyme than does 9-(*p*-bromoacetamidobenzyl)adenine.

In a recent study, it was found that 9-(*p*-bromoacetamidobenzyl)adenine was an irreversible inhibitor of adenosine deaminase, whereas iodoacetamide was not an irreversible inhibitor of this enzyme (1, 2). Kinetic analysis of the data indicated that the irreversible inhibition of adenosine deaminase by 9-(*p*-bromoacetamidobenzyl)adenine occurred only after the inhibitor had reversibly complexed with the enzyme. In this complex, then, the bromoacetamido moiety of the inhibitor is 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. Such inhibitors, which Baker has called active-site-directed irreversible inhibitors (3), should be quite specific in their irreversible inactivation of an enzyme. For example, when a comparison is made of isomers of some potential irreversible inhibitors, the environment on the enzyme in which the alkylating or acylating group of the inhibitor is held in the reversible E...I complex (enzyme–inhibitor) could be quite different. Thus, it is possible that the reactive group of one isomer could be held near an appropriate nucleophilic group on the enzyme, whereas the reactive group of an isomeric inhibitor could be held in the reversible E...I complex in such a position that it cannot form a covalent bond with the enzyme. In an attempt to determine the specificity of 9-(*p*-bromoacetamidobenzyl)adenine for adenosine deaminase, it was decided to investigate the possible reversible and irreversible inhibition of adenosine deaminase by some 9-(*m*-substituted benzyl)-6-substituted purines.

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DISCUSSION

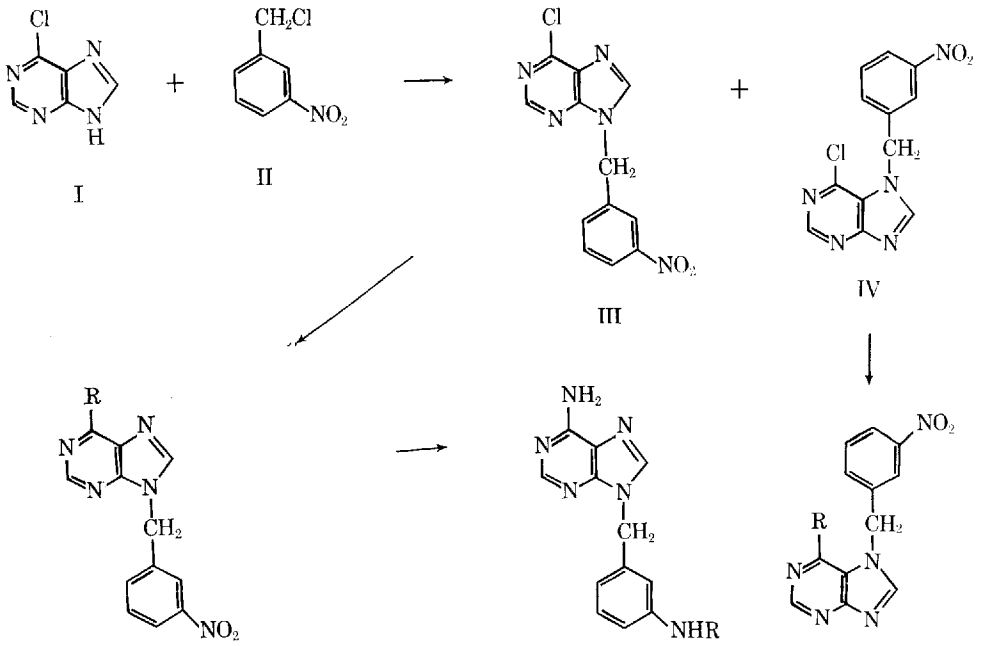
Chemistry.—Previous studies have shown that adenosine deaminase (calf intestinal mucosa) has a hydrophobic region to which the 9-alkyl group of some 9-alkyladenines can bind (4). Furthermore, it has been found that some 9-(*p*-substituted benzyl)-6-substituted purines were capable of inhibiting

adenosine deaminase (1, 2). In order to study the effect on inhibition of this enzyme by other 9-(substituted benzyl)-6-substituted purines, the preparation of some purines which contained at the 9-position a *meta*-substituted benzyl group was undertaken. The key compound for such a study is 9-(*m*-nitrobenzyl)-6-chloropurine, since this compound could be caused to undergo nucleophilic displacement at the 6-position to give a variety of products. In addition, the nitro group of appropriate inhibitors could be reduced to an amino group which could be converted into a potential alkylating or acylating group, such as a bromoacetamido or phenoxycarbonylamino group.

The general method which was employed for the synthesis of these compounds is a modification of a method that has previously been employed (5) and is outlined in Scheme I. Condensation of 6-chloropurine (I) with *m*-nitrobenzyl chloride (II) gave a mixture of 9- and 7-(*m*-nitrobenzyl)-6-chloropurines (III and IV) which was separated by chromatography on alumina. When III was allowed to react with ammonia, methylamine, dimethylamine, 1 *N* hydrochloric acid, or thiourea, the corresponding 6-substituted derivatives (V–IX) were obtained in good yields. Catalytic hydrogenation of V gave the corresponding *m*-aminobenzyl derivative (X) which on reaction with bromoacetic anhydride, phenyl chloroformate, and acetic anhydride gave XI, XII, and XIII, respectively. The assignment of structure to XI, XII, and XIII is based on the observation that N^6-acylation of adenine compounds shifts the ultraviolet maxima to longer wavelengths (6). Thus, it has been found (6) that *N*, $O^{3'}$-diacetyl deoxyadenylic 5′-acid exhibited an ultraviolet maximum at 273 mμ at pH 8, whereas XI, XII, and XIII exhibited ultraviolet maxima either at shorter wavelengths than X or at essentially the same wavelength as X.

In addition, it was found that when 7-(*m*-nitrobenzyl)-6-chloropurine (IV) was allowed to react with 20% methanolic ammonia, a mixture of the 6-amino and 6-methoxy derivatives (XIV and XV) was obtained. This unusual formation of a 6-methoxy derivative from the reaction of methanolic ammonia with a 7-substituted-6-chloropurine has been observed previously (7). However, treatment of IV with liquid ammonia at 65° gave, in moderate yield, 7-(*m*-nitrobenzyl)adenine (XIV). When IV was allowed to react with aqueous formic acid, a good yield of XVI was obtained.

Finally, the fact that III and IV, and therefore the compounds which were prepared from them, are the 9- and 7-substituted purine isomers, respectively, was established in the following manner. A 9-substituted adenine has a unique ultraviolet spectrum which allows it to be distinguished from a 1-,3-, or 7-substituted adenine (8, 9). Similarly, by ultraviolet spectroscopy a 1-substituted adenine may be distinguished from a 3-,7-, or 9-substituted



Scheme I

adenine. However, a 3- and a 7-substituted adenine have similar ultraviolet spectra but may be distinguished by their differences in pKa′ values (9).

A new alkylated adenine (XVII) was prepared by allowing adenine to react with *m*-nitrobenzyl chloride in the absence of an acid acceptor. In a similar alkylation of adenine with benzyl bromide, it was found that 3-benzyladenine was formed (10). A comparison of the ultraviolet spectra and pKa′ data of these isomerically alkylated adenines with certain known compounds is given in Table I. From this table, it can be seen that V corresponds to the 9-substituted isomer, XIV corresponds to the 7-substituted isomer, and that XVII corresponds to the 3-substituted isomer. These data constitute another example that the alkylation of 6-chloropurine in the presence of an acid acceptor produces a mixture of the 9- and 7-substituted-6-chloropurines (2, 5, 7).

TABLE I.—ULTRAVIOLET AND pKa′ DATA OF SOME SUBSTITUTED ADENINES

Compd. (Adenine Derivative)	—H⁺— $\lambda_{max.}$ mμ	—H⁺— $\epsilon \times 10^{-3}$	—OH⁻— $\lambda_{max.}$ mμ	—OH⁻— $\epsilon \times 10^{-3}$	pKa′
1-Methyl[a]	259	11.7	270	14.4	
3-Methyl[a]	274	17.0	273	13.3	5.3[a,b]
3-(*m*-Nitrobenzyl) (XVII)	274	23.0	272	19.6	5.7[c]
7-Methyl[a]	272	15.0	270	10.5	3.6[a,b]
7-(*m*-Nitrobenzyl) (XIV)	270	19.5	267	16.6	3.6[c]
9-Methyl[a]	260	14.2	260	14.7	
9-(*m*-Nitrobenzyl) (V)	261	23.3	262	23.0	

[a] These data were taken from *Reference 9*. [b] Determined titrimetrically in 50% DMF in H_2O. [c] Determined spectrophotometrically in 4% DMF in H_2O.

EXPERIMENTAL[1]

9- and 7-(m-Nitrobenzyl)-6-chloropurine (III and IV).—A mixture of 1.53 Gm. (9.95 mmoles) of I, 1.80 Gm. (10.5 mmoles) of II, and 1.46 Gm. (10.5 mmoles) of anhydrous potassium carbonate in 20 ml. of dimethylformamide was stirred for 20 hr. at room temperature. The reaction mixture was poured into 150 ml. of ice cold water, which caused the precipitation of a yellow solid. This material was collected by filtration. A chloroform solution of the crude material was chromatographed on a column of neutral alumina (48.0 Gm). 9-(*m*-Nitrobenzyl)-6-chloropurine (III) was eluted with chloroform (180 ml.); yield, 1.97 Gm. (68.6%), m.p. 135°. The 9-isomer was recrystallized from methanol and gave 1.36 Gm. (47.5%) of the pure material, m.p. 141–142°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 265 (1.66); pH 7, 265 (1.73); pH 13, 265 (1.67). ν in cm.$^{-1}$ (KBr): 1580 and 1550 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.[2]—Calcd. for $C_{12}H_8ClN_5O_2$: C, 49.74; H, 2.78; N, 24.18. Found: C, 49.52; H, 2.81; N, 24.34.

7-(m-Nitrobenzyl)-6-chloropurine (IV).—This was eluted from the alumina column with chloroform containing 1% methanol (150 ml.). After removal of the solvent *in vacuo*, 529 mg. of the 7-substituted product was obtained; yield, 18.4%. The analytical sample was obtained by two recrystallizations of the crude material from ethanol. It melted at 191–192°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH, 1, 267 (1.53); pH 7, 267 (1.53); pH 13, 267 (1.53). ν in cm.$^{-1}$ (KBr): 1600 and 1570 (sh) (C=N and C=C); 1530 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_8ClN_5O_2$: C, 49.74; H, 2.78; N, 24.18. Found: C, 49.42; H, 2.88; N, 24.00.

9-(m-Nitrobenzyl)adenine (V).—A mixture of 1.00 (3.46 mmoles) of III and 50 ml. of 20% methanolic ammonia was heated in a steel bomb at 90° ± 2° for 20 hr. The white solid was collected by filtration, washed with water, and dried. One recrystallization of the crude material from methyl cellosolve gave 716 mg. (76.5%) of the pure product (V), m.p. 276–278° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 261 (2.33); pH 7, 262 (2.30); pH 13, 262 (2.30). ν in cm.$^{-1}$ (KBr): 3320, 3150, and 1660 (NH); 1590 and 1570 (C=N and C=C); 1530 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.13; H, 3.83; N, 30.94.

9-(m-Nitrobenzyl)-6-methylaminopurine (VI).—A solution of 145 mg. (0.50 mmole) of III in 5 ml. of ethanol and 8 ml. of aqueous methylamine (40%) was heated in a steel bomb at 98° ± 3° for 23 hr. The yellow product which precipitated was collected by filtration and washed with water. Addition of 20 ml. of water to the filtrate caused the precipitation of an additional amount of purine. Total yield, 101 mg. (71.1%). Two recrystallizations of the crude product from 1-propanol gave the analytical sample (VI); yield, 75 mg. (53%), m.p. 210–212°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 265 (2.68); pH 7, 268 (2.55); pH 13, 268 (2.49). ν in cm.$^{-1}$ (KBr): 3300 (NH); 1635 and 1570 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{13}H_{12}N_6O_2$: C, 54.92; H, 4.26; N, 29.57. Found: C, 55.03; H, 4.40; N, 29.73.

9-(m-Nitrobenzyl)-6-dimethylaminopurine (VII).—A solution of 96 mg. (0.33 mmole) of III in 5 ml. of ethanol and 5 ml. of aqueous dimethylamine (25%) was heated in a steel bomb at 96° ± 1° for 16 hr. The yellow solid which precipitated from the reaction mixture was collected by filtration and washed with water. To the filtrate was added an additional 20 ml. of water which caused the precipitation of a second crop of product. Total yield, 86 mg. (88%). One recrystallization of the crude material from ethanol–water gave the pure product (VII); yield, 64 mg. (65%), m.p. 156–157°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 269 (2.55); pH 7, 275 (2.51); pH 13, 275 (2.47). ν in cm.$^{-1}$ (KBr): 1590 and 1560 (sh) (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{14}H_{14}N_6O_2$: C, 56.36; H, 4.73; N, 28.18. Found: C, 56.10; H, 4.90; N, 28.01.

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 4000 A spectrophotometer; the enzyme studies were done on a Gilford instrument, model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected. All analytical samples exhibited only one spot on thin-layer chromatography.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

9-(m-Nitrobenzyl)-6-hydroxypurine (VIII).—A solution of III (873 mg.; 3.08 mmoles) in 90 ml. of 1 *N* hydrochloric acid was refluxed for 1 hr. Evaporation of the volatile materials *in vacuo* and two recrystallizations of the crude material from ethanol gave the pure product (VIII); yield, 587 mg. (72.5%), m.p. 271–271.5°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 252 (1.72); pH 7, 251 (1.85); pH 13, 257 (1.92). ν in cm.$^{-1}$ (KBr): 3500 (OH); 2800–2300 (acidic hydrogen); 1690 (C=O); 1590 and 1550 (sh) (C=N and C=C); 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_3$: C, 53.13; H, 3.34; N, 25.83. Found: C, 53.12; H, 3.31; N, 25.63.

9-(m-Nitrobenzyl)-6-mercaptopurine (IX).—A mixture of 146 mg. (0.504 mmole) of III, 41 mg. (0.54 mmole) of thiourea in 3.0 ml. of 1-propanol was refluxed for 0.75 hr. The product which precipitated from the reaction mixture was collected by filtration and dried; yield, 140 mg. The analytical sample was obtained by dissolving the purine in aqueous sodium hydroxide and precipitating it by addition of 5% aqueous hydrochloric acid; yield, 126 mg. (81.3%), m.p. 294–294.5° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/15% DMSO 325 (2.45); H_2O/15% DMSO 322 (2.20); 0.1 *N* NaOH/15% DMSO 314 (2.26). ν in cm.$^{-1}$ (KBr): 2800–2650 (acidic hydrogen); 1590 and 1570 (sh) (C=N and C=C): 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_2S$: C, 50.18; H, 3.16; N, 24.39. Found: C, 50.35; H, 3.30; N, 24.32.

9-(m-Aminobenzyl)adenine (X).—A solution of 210 mg. (0.778 mmole) of V in 50 ml. of acetic acid containing 66 mg. of 5% palladium-on-charcoal catalyst was hydrogenated for 1.75 hr. under an initial pressure of 60 p.s.i. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. An ethanolic solution of the residual solid was heated with decolorizing carbon, filtered, and allowed to crystallize. The product was collected by filtration and after drying gave 111 mg. (59.7%) of the desired product (X), m.p. 230–231°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 259 (1.53); pH 7, 262 (1.65); pH 13, 262 (1.66). ν in cm.$^{-1}$ (KBr): 3300, 3150, and 1660 (NH); 1600 and 1550 (sh) (C=N and C=C).

Anal.—Calcd. for $C_{12}H_{12}N_6$: C, 59.99; H, 5.04; N, 34.98. Found: C, 59.73; H, 4.83; N, 34.76.

9-(m-Bromoacetamidobenzyl)adenine (XI).—To a cold solution of 200 mg. (0.832 mmole) of X in 5 ml. of tetrahydrofuran and 0.8 ml. of 10% aqueous acetic acid was added a solution of 351 mg. (1.97 mmoles) of bromoacetic anhydride in 2 ml. of tetrahydrofuran. The solution was stirred for 1.5 hr. at 0°, then for 0.5 hr. at room temperature. After filtration, 15 ml. of chloroform was added to the filtrate to cause precipitation of the crude product which was collected by filtration; yield, 271 mg. (81.5%). Two precipitations from tetrahydrofuran–hexane gave 118 mg. (39.4%) of the analytical sample (XI), which did not melt below 400°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 257 (2.28); H_2O/10% EtOH, 259 (2.30); 0.1 *N* NaOH/10% EtOH, 259 (2.24). ν in cm.$^{-1}$ (KBr): 3310, 3160 (NH); 1700 (amide I); 1655 (NH) and 1530 (amide II); 1590 and 1570 (C=N and C=C).

Anal.—Calcd. for $C_{14}H_{13}BrN_6O$: C, 46.55; H, 3.62; Br, 22.12; N, 23.26. Found: C, 46.57; H, 3.81; Br, 21.91; N, 23.52.

9 - (m - Phenoxycarbonylaminobenzyl)adenine (XII).—To a solution of 95 mg. (0.40 mmole) of X and 50 mg. (0.50 mmole) of triethylamine in 25 ml. of *p*-dioxane was added 62 mg. (0.40 mmole) of phenyl chloroformate. The mixture was stirred at room temperature for 45 min., filtered, and the filtrate evaporated *in vacuo* at room temperature. One recrystallization of the crude material from methanol–water gave 60 mg. (42%) of pure material (XII), decomposition point about 310°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 239 (2.08); 259 (1.58); H_2O/10% EtOH, 240 (2.04); 259 (1.58); 0.1 *N* NaOH/10% EtOH 239 (2.54); 259 (1.70). ν in cm.$^{-1}$ (KBr): 3310 and 3140 (NH); 1700 (C=O and NH); 1530 (amide II); 1610, 1590, and 1570 (C=N and C=C); 1200 (C—O—C, ester).

Anal.—Calcd. for $C_{19}H_{16}N_6O_2$: C, 63.32; H, 4.48; N, 23.32. Found: C, 63.17; H, 4.74; N, 23.51.

9-(m-Acetamidobenzyl)adenine (XIII).—To a cold solution of 150 mg. (0.652 mmole) of X in 5 ml. of tetrahydrofuran and 0.60 ml. of 10% aqueous acetic acid was added 154 mg. (1.51 mmoles) of acetic anhydride in 2 ml. of tetrahydrofuran over a period of 7 min. The solution was stirred at 0° for 2 hr., and the resulting precipitate was collected by filtration to give 108 mg. (58.6%) of product melting at 234–238°. Recrystallization from ethanol–water gave 77 mg. (42%) of the analytical sample (XIII), m.p. 237–239°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 255 (1.84); H_2O/10% EtOH, 254 (1.90); 0.1 *N* NaOH/10% EtOH, 254 (1.99). ν in cm.$^{-1}$ (KBr): 3340, 3180 (NH); 1670 (amide I); 1650 (sh) (NH) and 1540 (amide II); 1600 and 1570 (C=N and C=C).

Anal.—Calcd. for $C_{14}H_{14}N_6O$: C, 59.56; H, 5.00; N, 29.77. Found: C, 59.67; H, 5.01; N, 29.95.

7-(m-Nitrobenzyl)adenine (XIV) and 7-(m-Nitrobenzyl)-6-methoxypurine (XV).—A mixture of 0.29 Gm. (1.0 mmole) of IV and 21 ml. of 20% methanolic ammonia was heated in a steel bomb at 106° ± 3° for 3 hr. The solution was filtered to give 36 mg. (13%) of the 6-methoxypurine derivative (XV), m.p. 192–193.5°. One recrystallization from water gave 23 mg. (8%) of the analytical material, m.p. 195–196°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 260 (1.66); pH 7, 261 (1.49); pH 13, 262 (1.45). ν in cm.$^{-1}$ (KBr): 1610 and 1550 (C=N and C=C); 1520 and 1350 (NO_2); 1250 and 1070 (=C—O—CH_3).

Anal.—Calcd. for $C_{13}H_{11}N_5O_3$: C, 54.73; H, 3.89; N, 24.55. Found: C, 55.00; H, 4.03; N, 24.75.

Evaporation of the methanolic ammonia solution from which the 6-methoxypurine was obtained, produced a yellow solid which was extracted with hot methanol[3] (2 × 1 ml.). The residue (57 mg., 21%) which did not dissolve melted at 254–259°.

One recrystallization from methanol gave 35 mg. (13%) of the pure 7-(*m*-nitrobenzyl)adenine (XIV), m.p. 265–267° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 270 (1.95); pH 7, 268 (1.67); pH 13, 267 (1.66). ν in cm.$^{-1}$ (KBr): 3380, 3150, and 1660

[3] By thin-layer chromatography, this extract was shown to be a mixture of 7-(*m*-nitrobenzyl)-6-methoxypurine and 7-(*m*-nitrobenzyl)adenine. Since the desired 6-aminopurine was prepared by an alternate method, no attempts were made to further separate this mixture.

(NH); 1600 and 1540 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.26; H, 3.90; N, 30.90.

7-(m-Nitrobenzyl)adenine (XIV).—A mixture of 290 mg. (1.00 mmole) of IV in about 20 ml. of liquid ammonia was heated in a steel bomb at 65° ± 5° for 19 hr. After evaporation of the ammonia, the crude product was recrystallized from water; yield, 169 mg. (62.6%), m.p. 255–259° dec. An additional recrystallization from methanol gave 130 mg. (48.1%) of the pure product (XIV), m.p. 264–265° dec. ν in cm.$^{-1}$ (KBr): 3390, 3150, and 1660 (NH); 1600 and 1540 (C=N and C=C); 1520 and 1340 (NO_2).

7-(m-Nitrobenzyl)-6-hydroxypurine (XVI).—A solution of 205 mg. (0.710 mmole) of IV in 10 ml. of 88% aqueous formic acid was heated under reflux for 20 min. The yellow solution was evaporated *in vacuo*, and the residue was recrystallized from water to yield 142 mg. (73.2%) of the product, m.p. 254–256°. An additional recrystallization from water gave the analytical sample, 110 mg. (56.7%), m.p. 255–256°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 257 (1.48); pH 7, 260 (1.62); pH 13, 265 (1.63). ν in cm.$^{-1}$ (KBr): 1690 (C=O); 1630 and 1580 (C=N and C=C); 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_3$: C, 53.13; H, 3.34; N, 25.83. Found: C, 53.11; H, 3.35; N, 26.07.

7-(m-Nitrobenzyl)-6-hydroxypurine (XVI).—Dry hydrogen chloride was passed into a solution of 3.2 mg. (0.011 mmole) of XV in 1.5 ml. of methanol. The solution was evaporated *in vacuo*, and the residue recrystallized from water to give 2.6 mg. (72%) of the hydroxypurine (XVI), m.p. 255–255.5°. $\lambda_{max.}$: pH 1, 257; pH 7, 260; pH 13, 265. ν in cm.$^{-1}$ (KBr): 1690 (C=O); 1630 (sh) and 1580 (C=N and C=C); 1530 and 1340 (NO_2). This sample was identical with a sample prepared by the previous procedure.

3-(m-Nitrobenzyl)adenine Hydrochloride (XVII).—A mixture of 6.33 Gm. (37.0 mmoles) of adenine dihydrate and 19.0 (111 mmoles) of II in 100 ml. of *N,N*-dimethylacetamide was heated at 115° for 20 hr. The solution was evaporated *in vacuo* to a yellow solid which was triturated with water and then with ethanol. The residue, 9.16 Gm. (80.5%), m.p. 264–267° dec., was recrystallized from water to give 6.79 Gm. (59.5%) of the analytical material, m.p. 264–266° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/4% EtOH, 274 (2.30); H_2O/4% EtOH, 272 (1.82); 0.1 *N* NaOH/4% EtOH, 272 (1.96). ν in cm.$^{-1}$ (KBr): 3090 (NH); 1670 (C=NH^+); 1610, 1580, and 1520 (C=C and C=N); 1540 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_{11}ClN_6O_2$: C, 46.98; H, 3.62; Cl, 11.56; N, 27.40. Found: C, 46.86; H, 3.64; Cl, 11.45; N, 27.26.

CHEMICAL REACTIVITY OF THE ALKYLATING AGENTS

These experiments were performed by a modification of a procedure described in the literature (11–13). 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 is given in Fig. 2.

REAGENTS AND ASSAY PROCEDURE

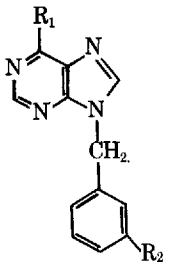
Adenosine and adenosine deaminase (type I) from calf intestinal mucosa were purchased from the Sigma Chemical Co. The assay procedure for the reversible inhibitors has been described by Kaplan (14). The measurements of the rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6. The stock solutions 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% dimethylsulfoxide. The addition of dimethylsulfoxide caused a slight decrease in the initial rate of the enzyme reaction. Consequently, in those experiments where it was necessary to employ dimethylsulfoxide to dissolve the inhibitor in phosphate buffer, the stock solutions of enzyme, substrate, and inhibitors were all prepared in phosphate buffer containing 10% dimethylsulfoxide. In this way, a constant 10% concentration of dimethylsulfoxide is maintained during the determination of the velocities of the enzymic reactions. In order to determine that the dimethylsulfoxide 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% dimethylsulfoxide. In this way, it was found that the index of inhibition did not vary in the two different determinations.

The method employed to study the irreversible inactivation at 37° of adenosine deaminase has been described previously (2) and is a modification of a published procedure (15).

RESULTS AND DISCUSSION

The results of the enzymic evaluation of these compounds are given in Table II. For those compounds which were substituted at the 9-position of the purine nucleus by a *m*-nitrobenzyl group, it was found that the 6-amino and 6-methylamino analogs (V and VI) were significantly inhibitory; the 6-amino compound (V) being approximately three times more effective than the 6-methylamino derivative (VI). Those compounds which were substituted at the 6-position of the purine nucleus by a chloro, dimethylamino, hydroxy, or mercapto group (III, VII, VIII, or IX) when tested at 0.12 m*M* concentration were either noninhibitory or at

TABLE II.—INHIBITION INDEX OF SOME 9-(*m*-SUBSTITUTED BENZYL)-6-SUBSTITUTED PURINES WITH ADENOSINE DEAMINASE



Compd.[a]	R_1	R_2	m*M* Concn. for 50% Inhibition[b]	$[I/S]_{0.5}$[b]	$K_i \times 10^5$ *M*
V	NH_2	NO_2	0.016 ± 0.004[c]	0.30 ± 0.03[c]	
VI	NHMe	NO_2	0.063 ± 0.001	0.96 ± 0.02	
X	NH_2	NH_2	0.20 ± 0.02	3.0 ± 0.3	
XI	NH_2	$NHCOCH_2Br$	0.056 ± 0.003	0.85 ± 0.05	3.6 ± 0.9
XII	NH_2	$NHCOOC_6H_5$	0.038 ± 0.004	0.58 ± 0.05	
XIII	NH_2	$NHCOCH_3$	0.095 ± 0.004	1.4 ± 0.1	
XVII	NH_2	H	0.10 ± 0.01	1.5 ± 0.2[d]	

[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 of reversible inhibition did the concentration of inhibitor exceed 0.12 m*M*. In that case 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 inhibitors. $[I/S]_{0.5}$ = the ratio of the m*M* concentration of the inhibitor for 50% inhibition to the m*M* concentration of the substrate. [c] Average deviation. [d] Data taken from *Reference 2*.

best, very weakly inhibitory relative to the corresponding adenine derivative (V). Thus, it would appear that the 6-amino group of V makes a contribution, either directly or indirectly through the purine nucleus, to the binding of the inhibitor to adenosine deaminase. In addition, when the 7-substituted isomers (IV, XIV, XV, and XVI) were evaluated as inhibitors of adenosine deaminase at 0.12 m*M* concentrations, they were essentially noninhibitory or at best, very weakly inhibitory relative to V. In the case of XIV, one could rationalize this result by assuming that the enzyme has little bulk tolerance for a group at the 7-position of the purine nucleus of an inhibitor or that an important binding group at the 9-position of the inhibitor is absent. Similar results have been obtained earlier (7).

With regard to the *meta* substituent on the 9-benzyl group of the 6-aminopurines, it was found that the reversible inhibitory power of the compounds decreases in the following order: $-NO_2$ (V)> $-NHCOOC_6H_5$(XII)> $-NHCOCH_2Br$ (XI)> $-NHCOCH_3$(XIII)≥ H (XVII)> NH_2(X). It was observed by the double reciprocal plot method (16) that XI is a competitive inhibitor of adenosine deaminase with a K_i of 3.6×10^{-5} *M*. This order of decreasing inhibition is different from that obtained with the corresponding *para* derivatives (2). As one example, the $[I/S]_{0.5}$ for the *m*-nitro derivative (V) is 0.3, whereas the $[I/S]_{0.5}$ of 9-(*p*-nitrobenzyl)adenine is 3.6 (2). Thus, of the adenine derivatives prepared in the *meta* series, the *m*-nitro derivative (V) is the most potent reversible inhibitor of adenosine deaminase whereas in the corresponding 9-(*p*-substituted benzyl)adenines, the *p*-nitro derivative is the weakest reversible inhibitor of the series. The reasons for the differences in the order of reversible inhibition in the two series is not yet clear. Further studies on this problem are planned and will be the subject of a future paper.

Finally, when the two potential irreversible inhibitors (XI and XII) were incubated with adenosine deaminase, it was found that the *m*-phenoxycarbonylamino derivative (XII) did not cause irreversible inactivation of the enzyme whereas the *m*-bromoacetamido derivative (XI) irreversibly inhibited the enzyme but at a rate which was very low relative to 9-(*p*-bromoacetamidobenzyl)adenine. It was found, however, that both XI and 9-(*p*-bromoacetamidobenzyl)adenine were capable of causing more than 90% irreversible inhibition of adenosine deaminase. The inactivation of the enzyme by these compounds was not reversed by dialysis. A comparison of the apparent first-order loss of enzyme activity caused by XI and 9-(*p*-bromoacetamidobenzyl)adenine is given in Fig. 1. Because the reaction of XI with adenosine deaminase is slow, these enzyme inactivation experiments have larger experimental errors than in the case of 9-(*p*-bromoacetamidobenzyl)adenine. Since the irreversible inhibitors have limited solubility in phosphate buffer, the reactions were performed in phosphate buffer containing 10% dimethylsulfoxide but even with the addition of dimethylsulfoxide, a concentration of 0.20 m*M* was near the upper range of solubility of XI. However, an examination of Fig. 1 reveals that XI irreversibly inhibited adenosine deaminase at concentrations of 0.10 and 0.20 m*M* at a much lower rate than did 9-(*p*-bromoacetamidobenzyl)adenine at a concentration of 0.03 m*M*. This significant difference in the rate of irreversible inactivation of the enzyme is not caused by a lack of chemical reactivity of the *meta* isomer since the

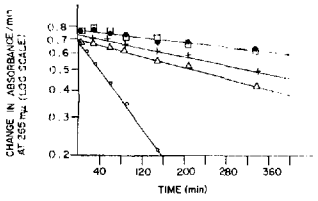


Fig. 1.—Irreversible inactivation of adenosine deaminase. Key: ●, enzyme control; □, iodoacetamide (1.0 mM); +, 9-*m*-bromoacetamidobenzyladenine (0.10 mM); △, 9-*m*-bromoacetamidobenzyladenine (0.20 mM); ○, 9-*p*-bromoacetamidobenzyladenine (0.030 mM).

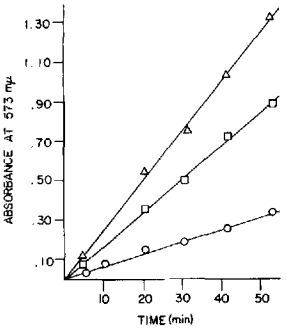


Fig. 2.—Comparative chemical reactivities of some alkylating agents with 4-(*p*-nitrobenzyl)-pyridine at pH 4.2. Key: ○, iodoacetamide (0.27 mM); □, 9-(*p*-bromoacetamidobenzyl)adenine (0.27 mM); △, 9-(*m*-bromoacetamidobenzyl)-adenine (0.27 mM).

meta isomer (XI) is actually more reactive than the *para* isomer when 4-(*p*-nitrobenzyl)pyridine (11–13) was used as the nucleophilic reagent (Fig. 2). In addition, the differences in the rates of irreversible inactivation of adenosine deaminase by XI and 9-(*p*-bromoacetamidobenzyl)adenine cannot be explained by the suggestion that XI is rapidly destroyed by the phosphate buffer solution. This suggestion was excluded when it was found that less than 10% of the alkylating ability of XI is lost during a 4-hr. incubation of XI with phosphate buffer containing 10% dimethylsulfoxide at 37°. Because iodoacetamide does not significantly irreversibly inhibit adenosine deaminase even at concentration of 1.6 mM, the authors believe that the irreversible inactivation which is caused by XI occurs mainly through a reversible E . . . I complex and not significantly by a random biomolecular process. In addition, XIII did not cause an irreversible inactivation of the enzyme. Thus, the irreversible inactivation of the enzyme by XI appears to be specifically related to its alkylating ability through an E . . . I complex.

When a comparison of the irreversible inactivation of the enzyme is made on the basis of the amount of the enzyme in the reversible E . . . I complex, it is found that 9-(*p*-bromoacetamidobenzyl)adenine is still much more reactive than the corresponding *meta* derivative (XI). Baker (17) has derived Eq. 1 for the calculation of the fraction of the total enzyme that is in the E . . . I complex.

$$[E\ldots I] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

In the case where a 0.10 mM solution of XI is incubated with the enzyme, the amount of the total enzyme (E_t) in the reversible E . . . I complex is 0.73 E_t, and the half-life of the inactivation is > 600 min. When a 0.030 mM solution of 9-(*p*-bromoacetamidobenzyl)adenine is employed under identical conditions with identical total enzyme concentration, the amount of the total enzyme in the reversible E . . . I complex is 0.70 E_t, and the half-life for the inactivation is 94 min. Thus, even when the concentration of the reversible E . . . I complex for the two inhibitors is nearly equal, the rate of inactivation by the *meta* derivative is much lower than that of the *para* isomer. The authors believe that these results offer evidence that in the initial reversible E . . . I complex between XI and adenosine deaminase, the alkylating agent of XI is not positioned as close to a nucleophilic group on the enzyme as occurs when 9-(*p*-bromoacetamidobenzyl)adenine is employed; consequently, the rate of alkylation by XI in the E . . . I complex is lower. However, it is possible that when the alkylating group is in the *meta* position of 9-benzyladenine as in XI, it is alkylating a different amino acid of the enzyme than when the alkylating group is in the *para* position of 9-benzyladenine. Further studies are necessary to differentiate these two suggestions.

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