acetate as described above and then was refluxed with a mixture of 1500 ml. of peroxide-free 1,2-dimethoxyethane and 425 ml. of 3.56 *N* hydrogen chloride (1.51 moles) in the same solvent. After 2 hr. an insoluble oily phase became solid. The suspension was filtered hot and insoluble hydrazine hydrochloride was extracted with three 1-l. portions of fresh solvent. The filtrate and extracts deposited a total of 204 g. of fairly pure **18** on cooling and an additional 26 g. was obtained by partial concentration of the filtrate (85% yield). The product was recrystallized from peroxide-free 1,2-dimethoxyethane (33 ml./g.).

The bisacylhydrazines **19** and **20** were prepared from the corresponding mercaptopropionhydrazides in the same way.

When the aqueous or alcoholic solutions of these substances were warmed with a little acid, rapid conversion to the corresponding mercapto acid or its ester occurred.

A cyclic (or polymeric) disulfide derivative of **18** was prepared by dissolving 15 g. (0.075 mole) in 300 ml. of hot water and stirring in aqueous iodine–potassium iodide until a persistent yellow color formed. The product, which is quite insoluble in water, precipitated as it formed and was washed with dilute sodium thiosulfate to remove entrapped iodine. There was obtained 9.6 g. (63%), m.p. 236–237° dec.

Anal. Calcd. for $C_4H_6N_2O_2S_2$: C, 27.0; H, 3.37; N, 15.7; S, 35.9. Found: C, 26.9; H, 3.36; N, 15.3; S, 35.9.

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Enzyme Inhibitors. VI. Studies on the Bulk Tolerance of Adenosine Deaminase for 6-Substituted Amino-9-(3-hydroxypropyl)purines[1]

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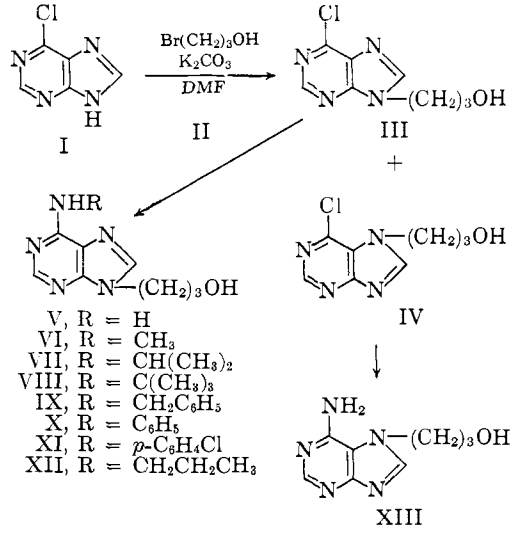
In order to study the ability of the enzyme adenosine deaminase to tolerate bulk at the 6-position of certain purine nucleoside analogs, several 6-substituted amino-9-(3-hydroxypropyl)purines were prepared. These compounds were synthesized by allowing 6-chloro-9-(3-hydroxypropyl)purine to react with the appropriate amines. Enzymatic evaluation of these compounds revealed that increasing the size of the substituent on the 6-amino group decreased the inhibitory property of the compound. These results establish that adenosine deaminase has little bulk tolerance for substituents on the 6-amino group of the purine nucleus.

In several previous studies on the enzyme adenosine deaminase, it has been found that the formation of a complex with the enzymes by a purine is dependent on several factors. For example, the purine must be substituted at the 9-position since it was observed that adenine itself was not an inhibitor.[2] It has also been determined that the substituent at the 6-position of a 9-substituted purine is critical for binding, and for compounds that are exclusively inhibitors, it has been found that a basic or neutral group at the 6-position of the purine nucleus is essential for inhibition.[3] In general, a 9-substituted purine with an amino group at the 6-position is a more effective inhibitor than the corresponding compound with a 6-methylamino group. Furthermore, the corresponding purine with a 6-dimethylamino group is either only weakly inhibitory or noninhibitory when evaluated at concentrations 2–3 times that of substrate.[2,3] Thus, it appeared that steric factors play an important role in the formation of a complex between a 9-substituted 6-aminopurine and adenosine deaminase, although it is possible to suggest that other factors such as electronic effects of the 6-substituent are the determinate factors in binding to the enzyme. The present paper describes the synthesis and enzymatic evaluation of some 6-substituted 9-(3-hydroxypropyl)purines so that the tolerance of adenosine deaminase for bulky substituents at the 6-position of the purine nucleus could be measured.

Chemistry.—Because 6-amino-9-(3-hydroxypropyl)-purine (V) has been found to be a good inhibitor of adenosine deaminase,[4] we decided to prepare some derivatives of V which were substituted at the 6-amino group by a variety of alkyl and aryl groups in order to study the ability of the enzyme to tolerate bulk at that position. The compounds which we selected for synthesis were the methyl, isopropyl, *t*-butyl, benzyl, phenyl, *p*-chlorophenyl, and *n*-propyl analogs of V. For the preparation of the 6-substituted analogs of V, it appeared that 6-chloro-9-(3-hydroxypropyl)purine (III) would be an ideal intermediate. We have previously employed this intermediate (III) which was syn-

CHART I



(1) This investigation was supported by Public Health Service Research Grant CA-06388-03 from the National Cancer Institute, 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-555 from the Division of General Medical Sciences.

(2) H. J. Schaeffer, S. Marathe, and V. Alks, *J. Pharm. Sci.*, in press.

(3) R. H. Shah, H. J. Schaeffer, and D. H. Murray, *ibid.*, in press.

(4) H. J. Schaeffer and P. S. Bhargava, *Biochemistry*, in press.

thesized by a modification of the method of Ikehara, *et al.*,[5] in which 5-amino-4,6-dichloropyrimidine was condensed with 3-aminopropanol to give the substituted pyrimidine which was cyclized to III with ethyl orthoformate and acetic anhydride. Because we were also interested in determining if 6-amino-7-(3-hydroxypropyl)purine would be an inhibitor of adenosine deaminase, we elected to use a modification of an alternate synthesis developed by Montgomery and Temple[6] for 6-chloropurines in which a mixture of the 7- and 9-substituted compounds would be obtained. Thus, when 6-chloropurine was allowed to react with 3-bromopropanol in dimethylformamide in the presence of potassium carbonate, a mixture of products was obtained from which III and IV were isolated in 61 and 14% yields, respectively. When III was allowed to react with an appropriate amino compound, good yields of the corresponding 6-substituted analogs (V–XII) were obtained. Finally, the reaction of IV with methanolic ammonia proceeded smoothly, and the desired product (XIII) was isolated as its hydrochloride salt in a moderate yield.

Experimental[7]

9- and 7-(3-Hydroxypropyl)-6-chloropurine (III and IV).—A mixture of 5.00 g. (32.2 mmoles) of 6-chloropurine, 4.67 g. (33.8 mmoles) of anhydrous potassium carbonate, and 4.71 g. (33.8 mmoles) of 3-bromo-1-propanol in 30.0 ml. of dimethylformamide was stirred at room temperature for 3.5 hr. and at 50° for 30 min. The insoluble material was removed by filtration to the filtrate, and the mixture was extracted with five 50-ml. portions of chloroform. The chloroform extract was dried ($MgSO_4$), and the volatile materials were removed *in vacuo*. A solution of the crude material in chloroform was chromatographed on a neutral alumina column (20.0 g.) using chloroform as the eluent, and 50-ml. fractions were collected. The desired product III was collected from the first four fractions. The crude material was recrystallized from chloroform–hexane and gave an analytical sample; yield, 3.83 g. (61.5%); m.p. 120°. The infrared spectrum, melting point, and ultraviolet spectrum were identical with those of an authentic sample prepared by a different procedure.[4]

The alumina column was eluted with methanol which removed a yellow material. Removal of the methanol *in vacuo* gave 0.902 g. (14.5%) of a yellow solid which on recrystallization from chloroform–hexane gave IV as white needles, m.p. 136°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 268 (8.46); pH 7, 269 (8.80); pH 13, 269 (8.73); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3300 (OH), 1600, and 1535 (C=N and C=C).

Anal.[8] Calcd. for $C_8H_9ClN_4O$: C, 45.05; H, 4.28; N, 26.40. Found: C, 45.19; H, 4.33; N, 26.60.

9-(3-Hydroxypropyl)-6-aminopurine (V) and 9-(3-Hydroxypropyl)-6-methylaminopurine (VI).—These compounds were prepared as previously described.[4]

9-(3-Hydroxypropyl)-6-isopropylaminopurine Dihydrochloride.—A solution of 213 mg. (1.00 mmole) of III in 20.0 ml. of isopropylamine was heated in a steel bomb at 80° for 19 hr. Removal of the volatile materials *in vacuo* gave a clear oil. Ethyl acetate (5.0 ml.) was added and the isopropylamine hydrochloride was removed by filtration. Evaporation of the filtrate gave an oil which did not crystallize on standing. The oil was dissolved in methanol and ether (1:2) and converted to the dihydrochloride salt by passing HCl through the solution. The salt was collected by filtration and recrystallized from methanol and ether saturated with HCl; yield, 177 mg. (57.5%); m.p. 174–176° (oil bath); λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 264 (4.79); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3400 (OH), 2700 (NH_2^+), 1600 (C=N$^+$H), 1590 (C=C).

Anal. Calcd. for $C_{11}H_{19}Cl_2N_5O$: C, 42.99; H, 6.23; Cl, 22.77; N, 22.78. Found: C, 42.93; H, 6.50; Cl, 22.65; N, 22.96.

9-(3-Hydroxypropyl)-6-*t*-butylaminopurine (VIII).—A solution of 300 mg. (1.41 mmoles) of III in *t*-butylamine was heated in a steel bomb at 80° for 48 hr. *t*-Butylamine hydrochloride was removed from the reaction mixture by filtration and washed with chloroform. The filtrate was evaporated and gave a light green solid. Recrystallization of the crude product from chloroform–hexane gave the analytical material; yield, 220 mg. (62.5%); m.p. 108–110°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 267 (1.85); pH 7, 270 (1.84); pH 13, 270 (1.84); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3350 (OH), 3200 (NH), 1600 (C=N), 1575 (C=C).

Anal. Calcd. for $C_{12}H_{19}N_5O$: C, 57.76; H, 7.68; N, 28.08. Found: C, 57.58; H, 7.71; N, 28.10.

9-(3-Hydroxypropyl)-6-benzylaminopurine (IX).—A mixture of 213 mg. (1.00 mmole) of III and 225 mg. (2.10 mmoles) of benzylamine in 10.0 ml. of 95% ethanol was heated under reflux for 3.5 hr. The reaction mixture was evaporated to dryness, and 10 ml. of chloroform was added. The benzylamine hydrochloride which precipitated was removed by filtration. The filtrate was concentrated to about 5 ml. and chilled. A second fraction of the hydrochloride salt was removed by filtration. Evaporation of the filtrate gave an oil which was crystallized from a mixture of benzene–hexane; yield, 231 mg. (81.0%); m.p. 116–118°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 268 (1.97); pH 7, 269 (2.03); pH 13, 269 (2.01); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3300 (OH), 1630 (C=N), 1580 and 1530 (aromatic).

Anal. Calcd. for $C_{15}H_{17}N_5O$: C, 63.58; H, 6.05; N, 24.72. Found: C, 63.70; H, 6.01; N, 24.89.

9-(3-Hydroxypropyl)-6-anilinopurine (X).—A mixture of 213 mg. (1.00 mmole) of III and 196 mg. (2.11 mmoles) of aniline in 10.0 ml. of 95% ethanol was heated under reflux for 3 hr. The reaction mixture was evaporated to dryness *in vacuo*, and 5.0 ml. of methanol was added. Upon chilling, a solid separated which was collected by filtration. Recrystallization of the crude material from methanol gave the pure product; yield, 170 mg. (66.7%); m.p. 185–187°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 275 (1.80); pH 7, 287 (2.12); pH 13, 287 (2.12); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3350 (OH), 3250 (NH_2), 1640 (C=N), 1580 and 1490 (aromatic).

Anal. Calcd. for $C_{14}H_{15}N_5O$: C, 62.43; H, 5.61; N, 26.00. Found: C, 62.15; H, 5.63; N, 25.78.

9-(3-Hydroxypropyl)-6-*p*-chloroanilinopurine (XI). A solution of 213 mg. (1.00 mmole) of III and 267 mg. (2.10 mmoles) of *p*-chloroaniline in 10.0 ml. of 95% ethanol was heated under reflux for 3.0 hr. The reaction mixture was evaporated *in vacuo* to constant weight and 5 ml. of methanol was added. After chilling the solution, the product precipitated and was collected by filtration; yield, 220 mg. (72.5%); m.p. 194–195°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 277 (1.85); pH 7, 291 (2.46); pH 13, 291 (2.43); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3300 (OH), 1640 (C=N), 1580 and 1490 (aromatic).

Anal. Calcd. for $C_{14}H_{14}ClN_5O$: C, 55.34; H, 4.64; N, 23.05. Found: C, 55.41; H, 4.86; N, 23.01.

9-(3-Hydroxypropyl)-6-*n*-propylaminopurine Dihydrochloride (XII). A solution of 213 mg. (1.00 mmole) of III and 1.0 ml. of *n*-propylamine in 10 ml. of 95% ethanol was heated under reflux for 3 hr. The clear solution was evaporated to a colorless oil, and 5 ml. of chloroform was added. The addition of a small amount of hexane caused the separation of propylamine hydrochloride which was removed by filtration. The filtrate was evaporated *in vacuo* and dissolved in 20 ml. of ether–chloroform (10:1). Dry HCl gas was bubbled through the chilled solution, and the white dihydrochloride salt which precipitated was removed by filtration and washed with ether; yield, 239 mg. (77.5%); m.p. 178–181° (oil bath). Recrystallization from methanol and ether gave the analytical material, m.p. 178–181°; λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 266 (1.81); pH 7, 270 (1.73); pH 13, 270 (1.67); $\tilde{\nu}$, cm.$^{-1}$ (KBr): 3400 (OH), 3000–2700 (NH_2^+), 1675 (C=N$^+$H), 1585 (C=C).

Anal. Calcd. for $C_{11}H_{19}Cl_2N_5O$: C, 42.86; H, 6.21; Cl, 23.01. Found: C, 42.72; H, 6.06; Cl, 22.73.

7-(3-Hydroxypropyl)-6-aminopurine Hydrochloride (XIII). A mixture of 160 mg. (0.760 mmole) of IV in 15 ml. of 2% methanolic ammonia was heated in a steel bomb at 75° for 48 hr. The volatile materials were removed *in vacuo*, and the residue

(5) M. Ikehara, E. Ohtsuka, S. Kitagawa, K. Yagi, and Y. Tonomura, *J. Am. Chem. Soc.*, **83**, 2679 (1961).

(6) J. A. Montgomery and C. Temple, Jr., *ibid.*, **83**, 630 (1961).

(7) The infrared spectra were determined on a Perkin-Elmer Model 137 spectrophotometer; the ultraviolet spectra and enzyme rates were determined on a Perkin-Elmer Model 4000A spectrophotometer. The melting points, unless noted otherwise, were determined on a Kofler Heizband and are corrected.

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

was triturated with acetone. The insoluble ammonium chloride was removed by filtration and evaporation of the filtrate gave the oily product; yield, 112 mg. The crude product was dissolved in ether and a small amount of methanol. Dry HCl was bubbled through the chilled solution, and the white salt was collected by filtration; yield, 65 mg. (32.4%); m.p. 180–183° (oil bath); λ_{max}, mμ ($\epsilon \times 10^{-3}$): pH 1, 254 (9.35); pH 7, 257 (9.35); pH 13, 263 (10.0); $\bar{\nu}$, cm.$^{-1}$ (KBr): 3400 (OH), 3000–2700 (NH_2^+), 1690 (C=N$^+$H), 1570 (C=C).

Anal. Calcd. for $C_8H_{12}ClN_5O$: C, 41.83; H, 5.26; Cl, 15.44. Found: C, 41.65; H, 5.05; Cl, 15.23.

Reagents and Assay Procedure.—Adenosine and adenosine deaminase were purchased from the Sigma Chemical Company. The general method of assay has been described by Kaplan[9] and involves measuring the rate of disappearance of the absorption band of adenosine at 265 mμ. All enzymatic reactions were performed in 0.05 *M* phosphate buffer at pH 7.6 and 25°. The substrate and the stock solutions of all reagents were prepared in 0.05 *M* phosphate buffer at pH 7.6. For the assay, the cell contained a total volume of 3.1 ml. which was 0.066 m*M* with respect to adenosine. To study inhibition, appropriate amounts of buffer were excluded from the cells and were replaced by an equal volume of a solution of the inhibitor in phosphate buffer.

Results and Discussion

Previously it was found[4] that V and VI were competitive inhibitors of adenosine deaminase with K_i values of 3.0×10^{-5} *M* and 9.8×10^{-5} *M*, respectively. Enzymatic evaluation of compounds VII–XI revealed that they were all essentially noninhibitory at concentrations 2–3 times that of the substrate. These results establish that adenosine deaminase has little bulk tolerance for groups on the 6-amino group of the purine nucleus. For example, the replacement of the 6-amino group by a 6-methylamino group increased the K_i by a factor greater than 3. Thus, it would appear that it will not be feasible to prepare active-site-directed irreversible inhibitors at the 6-position of the purine nucleus. It might be suggested that even though the enzyme has little bulk tolerance for branch chain groups at the 6-position of the purine nucleus, it might tolerate straight-chain groups. Therefore, the 6-*n*-propylamino analog (XII) was synthesized, and it, too, was essentially noninhibitory at concentrations 2–3 times that of the substrate. Consequently, adenosine deaminase has little bulk tolerance for either branched or unbranched groups on the 6-amino group of the purine nucleus. Finally, it was found that 7-(3-hydroxypropyl)-6-aminopurine (XIII) was noninhibitory against adenosine deaminase. This fact may be rationalized if it is assumed that the enzyme has little bulk tolerance for a group at the 7-position of the purine nucleus or that an essential binding group at the 9-position is absent. At the present time, however, it is not possible to answer this question unambiguously.

(9) N. O. Kaplan in "Methods in Enzymology," Vol. II, S. P. Colowick and N. O. Kaplan, Ed., Academic Press Inc., New York, N. Y., 1955, p. 473.

Analogs of Tetrahydrofolic Acid. XVII.[1,2] On the Mode of Binding of the *p*-Aminobenzoyl Moiety of N-(2-Amino-4-hydroxy-6-methyl-5-pyrimidylpropyl)-*p*-aminobenzoyl-L-glutamic Acid to Dihydrofolic Reductase

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The binding of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (IV) to dihydrofolic reductase is tightened by introduction of carboxyl (V) or carboxyglycyl groups (VI) in the *para* position. Since the benzene ring of IV probably binds to the enzyme in a charge–transfer complex with the benzene ring being an electron acceptor, the electron-withdrawing *p*-carbonyl group of V and VI could tighten binding by making the benzene ring a better electron acceptor. Strong evidence to support this hypothesis has now been obtained by comparison of IV–VI and the *p*-(4-chloro-3-oxo-1-butenyl) (X) and *p*-(4-chloro-3-oxobutyl) (VIIIb) derivatives of IV as inhibitors of dihydrofolic reductase.

The pyrimidyl analog of tetrahydrofolic acid (III)[3] binds to folic reductase ($K_i = 2.0 \times 10^{-6}$) better than the substrate, folic acid ($K_m = 10 \times 10^{-6}$). Removal of the carboxy-1-glutamate residue as in IV[4] increases K_i to 63×10^{-6}; it can be calculated readily from $-\Delta F = RT \ln K$ that the loss in free energy of binding by removal of the carboxy-L-glutamate is only 22% of III. About one-half of the binding of the carboxy-L-glutamate residue is due to the *p*-carboxyl as shown by $K_i = 13 \times 10^{-6}$ for V.[4] The α-carboxyl of III would appear not to contribute to binding since the K_i of VI is also 13×10^{-6}; thus the other half of the binding of the carboxy-L-glutamate residue of III is due to the γ-carboxyl.[4] Evidence was presented that both the *p*- and γ-carbonyls were probably complexed to the enzyme by hydrogen bonding.[4]

It was pointed out[4] that it should be possible to obtain an active-site-directed irreversible inhibitor[5,6] of folic reductase if the proper type of functional group for formation of a covalent bond could be placed on III positioned where the *p*- or γ-carbonyl normally occur. Several chloromethyl ketones (VIII and X) were synthesized to evaluate this possibility, since halomethyl ketones have been previously used for

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(2) For the previous paper of this series, see B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **53**, 1137 (1964).

(3) Papers VI and VII of this series: B. R. Baker and C. E. Morreal, *ibid.*, **51**, 596 (1962); *ibid.*, **52**, 840 (1963).

(4) Paper X of this series: B. R. Baker, D. V. Santi, P. I. Almaula, and W. C. Werkheiser, *J. Med. Chem.*, **7**, 24 (1964).

(5) B. R. Baker, *Cancer Chemotherapy Rept.*, **No. 4**, 1 (1959).

(6) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964), a review.