Enzyme Inhibitors. IX. Hydrophobic Interactions of Some 9-Alkyladenines with Adenosine Deaminase[1,2]

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A study of a series of 9-alkyladenines as inhibitors of the enzyme, adenosine deaminase, revealed that hydrophobic bonds are important in the formation of an enzyme–inhibitor complex as far as the 9-alkyl group of the adenine derivative is concerned. The amount of inhibition of adenosine deaminase increases as the alkyl group on the 9-position of adenine is lengthened from methyl to *n*-octyl, although the nonpolar area on the enzyme appears to extend from three to seven carbon atoms (*n*-propyl to *n*-heptyl). For those adenines which are substituted at the 9-position by an *n*-propyl to a *n*-heptyl group, an average change in free energy of binding for each additional methylene group was found to be 350 cal. In addition, it has been found that if a hydroxyalkyl group is attached to the 9-position of adenine, the hydroxyl group can increase or decrease binding to the enzyme. If the hydroxyl group is located at a position two or three carbon atoms from the 9-position of adenine, an increase in binding is observed. If the hydroxyl group is located at a position five carbon atoms from the 9-position of adenine, a decrease in binding is observed.

The formation of a complex between a small molecule and a protein (*i.e.*, a receptor or an enzyme) appears to be one of the essential steps for the action of most, if not all, drugs. Consequently, a study of the factors which are important for the formation of a strong protein–inhibitor complex could lead to a better understanding of drug action and to the design of compounds which are selective in their biological action. Because a number of antibiotics which possess anticancer activity are purine nucleosides, we have been interested in determining which atoms and functional groups in purine nucleosides are important in the formation of a complex between an enzyme and its corresponding substrate. The first enzyme which was selected for such a study was adenosine deaminase.

Previous studies have been concerned with the contribution made to binding by the group at the 6-position of the purine nucleus and by the hydroxyl groups on the ribofuranosyl moiety of adenosine.[2-4] The present paper describes the syntheses of some 9-alkyl- and some 9-hydroxyalkyl-6-substituted purines and discusses the mode of binding of these inhibitors to adenosine deaminase. The specific compounds which were studied are those 6-aminopurines which are substituted at the 9-position by alkyl groups from methyl through octyl and by hydroxyalkyl groups from hydroxyethyl through hydroxypentyl.

Chemistry.—In general, the compounds were prepared by modification of known procedures. Thus, 5-amino-4,6-dichloropyrimidine was allowed to react with the appropriate amine or amino alcohol. The resulting substituted pyrimidine on treatmen twith triethyl orthoformate and hydrochloric acid gave the 9-substituted 6-chloropurine which was allowed to react with methanolic ammonia to generate the corresponding 9-substituted adenine. The specific compounds which were prepared and their physical constants are listed in the Experimental section.

Experimental[5]

The syntheses of the compounds which had previously been reported were accomplished by the procedures reported in the literature. Consequently, for those compounds, only the melting point and the literature reference are given (Table I).

9-(5-Hydroxypentyl)adenine Hydrochloride (XI).—To a mixture of 346 mg. (1.50 mmoles) of 4-(5-hydroxypentylamino)-5-amino-6-chloropyrimidine[6] in 6 ml. of triethyl orthoformate was added 0.162 ml. (1.95 mmoles) of concentrated HCl, and the mixture was stirred for 22 hr. at room temperature. After the mixture was evaporated *in vacuo* to dryness, the crude product was dissolved in 15 ml. of 20% methanolic NH_3 and heated in a stainless steel bomb at 80° for 18 hr. The volatile materials were removed *in vacuo*, and the semisolid residue was extracted with six 20-ml. portions of hot benzene. The benzene extract was evaporated *in vacuo* and gave 200 mg. of a product which was difficult to recrystallize. Therefore, the crude material was dissolved in a mixture of methanol and ether, and dry HCl was passed through the solution until precipitation was complete. The crude product was collected by filtration (163 mg., 40.8%), m.p. 150–154°, and after recrystallization from a mixture of methanol and ether gave 130 mg. (32.5%) of the pure product: m.p.[7] 164–168°; λ_{max} in mμ ($\epsilon \times 10^{-4}$), pH 1, 263 (1.25), pH 7, 264 (1.26), pH 13, 263 (1.34); ν in cm.$^{-1}$ (KBr), 3380 and 3100 (NH and OH), 2800–2300 (NH^+), 1700 (C=N^+H), 1600 and 1515 (C=N and C=C).

Anal.[8] Calcd. for $C_{10}H_{16}ClN_5O \cdot 0.5H_2O$: C, 45.03; H, 6.42; Cl, 13.29. Found: C, 44.75; H, 6.46; Cl, 13.08.

After the hemihydrate was dried at 100° *in vacuo*, it melted at 167–170° and was analyzed for the anhydrous material.

Anal. Calcd. for $C_{10}H_{16}ClN_5O$: C, 46.60; H, 6.26; Cl, 13.76; N, 27.18. Found: C, 46.57; H, 6.28; Cl, 13.90; N, 27.06.

Reagents and Assay Procedure.—Adenosine and adenosine deaminase were purchased from the Sigma Chemical Co. The assay procedure has previously been described and is a modification of the general procedure described by Kaplan.[9] All enzymatic reactions were performed at 25° in 0.05 *M* phosphate buffer

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(2) For the previous paper of this series, see H. J. Schaeffer, D. Vogel, and R. Vince, *J. Med. Chem.*, **8**, 502 (1965).

(3) H. J. Schaeffer, S. Marathe, and V. Alks, *J. Pharm. Sci.*, **53**, 1368 (1964).

(4) H. J. Schaeffer and P. S. Bhargava, *Biochemistry*, **4**, 71 (1965).

(5) 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 Heizbank and are corrected.

(6) This compound was prepared according to the procedure of B. R. Baker and P. Tanna, whose method will be published.

(7) This melting point was determined in an oil bath.

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

(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.

TABLE I

DERIVATIVE OF ADENINE

Substituent	M.p., °C.	Lit. m.p., °C.	Ref.
9-Methyl (I)	306–308	310	*a*
9-Ethyl (II)	194	194–195	*b*
9-*n*-Propyl (III)	174–175	173	*c*
9-*n*-Butyl (IV)	136–138	138–139	*d*
9-*n*-Pentyl (V)	132–135	133–135	*e*
9-*n*-Hexyl (VI)	145–146	146	*e*
9-*n*-Heptyl (VII)	126	127	*e*
9-*n*-Octyl (VIII)	131	131	*e*
9-(2-Hydroxyethyl) (IX)			*e*, *f*
9-(3-Hydroxypropyl) (X)			*e*, *g*
9-(4-Hydroxybutyl) (XI)			*e*, *g*

[a] R. K. Robins and H. H. Lin, *J. Am. Chem. Soc.*, **79**, 490 (1957). [b] J. A. Montgomery and C. Temple, Jr., *ibid.*, **79**, 5238 (1957). [c] C. Temple, Jr., C. L. Kussner, and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 866 (1962). [d] J. A. Montgomery and C. Temple, Jr., *J. Am. Chem. Soc.*, **80**, 409 (1958). [e] Ref. 4. [f] M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull* (Tokyo), **9**, 27 (1961). [g] M. Ikehara, E. Ohtsuka, S. Kitagawa, K. Yagi, and Y. Tonomura, *J. Am. Chem. Soc.*, **83**, 2679 (1961).

at pH 7.6. The stock solution of the enzyme, substrate, and all inhibitors were prepared in 0.05 *M* phosphate buffer at pH 7.6. For each assay, the cell contained a total volume of 3.1 ml. which was 0.066 m*M* with respect to the substrate. Sufficient amounts of adenosine deaminase were employed so that the initial rate of reaction gave a change of approximately 0.8 optical density units/min. at 265 mμ. The ratio of the m*M* concentration of inhibitor to the m*M* concentration of substrate for 50% inhibition $[I/S]_{0.5}$, *i.e.*, the inhibition index, was used to compare the inhibitory properties of the various compounds. In order to determine the concentration of inhibitor required for 50% inhibition, a plot of V_0/V *vs.* I was made where V_0 = initial velocity of the uninhibited enzymatic reaction, V = initial velocity of inhibited reaction at various inhibitor concentrations, and I = the various concentrations of inhibitor.[10] Each plot was made at five different inhibitor concentrations; each point for each plot is an average of at least three assays, and each reported $[I/S]_{0.5}$ is an average of at least two different determinations of the concentration of inhibitor required for 50% inhibition of adenosine deaminase.

Results and Discussion

In a recent paper which was concerned with the mode of binding by a hydroxyl group attached to a 9-alkyl group of a 6-substituted purine, the suggestion was made that the area of the enzyme where the 9-alkyl substituent is bound is relatively nonpolar, consequently, hydrophobic bonds probably play an important role in the formation of an enzyme-inhibitor complex, at least as far as the 9-substituent is concerned.[2] That the postulate is correct can be seen by an examination of Table II since it has been found that the amount of inhibition of adenosine deaminase increases as the alkyl group at the 9-position of adenine is lengthened from methyl to *n*-octyl (I through VIII). In fact, if one calculates the differences in the free energy of binding for each added methylene group, one finds an average value of 350 cal./methylene group in the compounds from 9-*n*-propyladenine (III) through 9-*n*-heptyladenine (VII). We believe that these data define this nonpolar area of adenosine deaminase from about three–seven carbon atoms. The actual size or shape of this nonpolar region on the enzyme cannot be stated accurately because the compounds which were employed in this study are

TABLE II

INDEX OF INHIBITION AND FREE ENERGY OF BINDING TO ADENOSINE DEAMINASE OF CERTAIN 9-ALKYLADENINES

Compd.[a]	No.	$[I/S]_{0.5}$[b]	ΔF/additional carbon atom, cal.
Ad-Me	I	7.3 ± 0.3	...
Ad-Et	II	6.2 ± 0.2	98
Ad-Pr	III	3.3 ± 0.1	362
Ad-Bu	IV	2.3 ± 0.04	215
Ad-Pent	V	1.4 ± 0.0	292
Ad-Hex	VI	0.70 ± 0.03	408
Ad-Hept	VII	0.32 ± 0.0	459
Ad-Oct	VIII	0.24 ± 0.01	168

[a] In each case, the abbreviation Ad means an adenine group with the indicated group substituted at the 9-position. None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*.

ones which possess a large number of conformational possibilities. Recently, Belleau and Lacasse[11] have discussed the importance of hydrophobic bonds in the formation of complexes with acetylcholinesterase and have indicated, based on their work and others, that the affinity to the enzyme can be increased by hydrophobic forces by a maximum of 730 cal./methylene group.

This nonpolar region of adenosine deaminase does, however, appear to have a maximum length of seven carbons since the ΔF/methylene group in 9-*n*-octyladenine is only 168 cal. compared to the average of about 350 cal. The reason that the ΔF/carbon atom in comparing 9-ethyladenine (II) to 9-methyladenine (I) is also below the average of 350 cal. is probably a reflection of the fact that the area of the enzyme which is removed about two carbon atoms from the 9-position of adenine is the site to which a hydroxyl group binds in, for example, 9-(2-hydroxypropyl)adenine ($[I/S]_{0.5}$ = 0.25).[2] Since this region of the enzyme which binds the hydroxyl group must be relatively polar compared to the area where hydrophobic bonding occurs, it follows that the 9-ethyl group will not exhibit as strong a hydrophobic bond as will the analogs with longer alkyl chains.

Furthermore, it has been found that a hydroxyl group attached to an alkyl chain at the 9-position of adenine can cause repulsion to binding. Thus, it can be seen in Table III that the binding of the inhibitor increases in going from a 9-(2-hydroxyethyl)- to a 9-(3-hydroxypropyl)adenine and then decreases as the 9-substituent is changed to a 4-hydroxybutyl

TABLE III

INDEX OF INHIBITION OF SOME 9-(HYDROXYALKYL)ADENINES WITH ADENOSINE DEAMINASE

Compd.[a]	No.	$[I/S]_{0.5}$[b]
Ad-CH_2CH_2OH	IX	1.1 ± 0.05
Ad-$(CH_2)_3OH$	X	0.70 ± 0.08
Ad-$(CH_2)_4OH$	XI	1.94 ± 0.14
Ad-$(CH_2)_5OH$	XII	3.0 ± 0.12

[a] In each case, the abbreviation Ad means an adenine group with the indicated group substituted at the 9-position. None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*.

(10) B. R. Baker and H. S. Sachdev, *J. Pharm. Sci.*, **52**, 933 (1963).

(11) B. Belleau and G. Lacasse, *J. Med. Chem.*, **7**, 768 (1964).

and finally to a 5-hydroxypentyl group. Both IX and X are bound to the enzyme more strongly than the corresponding nonhydroxylated compounds (II and III), which is a demonstration of the importance of the OH group to the formation of an enzyme–inhibitor complex. That X is bound more tightly to the enzyme than IX is a consequence of the increase in hydrophobic bonds in X. Comparison of XI and IV reveals that these compounds are bound equally, whereas the index of inhibition of XII is higher than V. The lesser inhibition of adenosine deaminase by XII relative to V can be rationalized if it is assumed that the OH group on the terminal position of the pentyl group would be located near the nonpolar region of the enzyme, and thus, would interfere with the formation of hydrophobic bonds. Whereas it is true that the hydroxypentyl group of XII could undergo folding so that the hydroxyl group could reach the hydroxyl binding site utilized by IX and X, this folding would be energetically unfavorable; therefore, the energy that could be gained by OH binding at the site two carbons removed from the 9-position of the adenine nucleus is more than offset by the energy required for folding the pentyl chain and by the energy lost by repulsion to binding in the hydrophobic region of the enzyme. In the case of XI and IV, these two opposing forces are very nearly equal, *i.e.*, the energy lost by repulsive forces of the hydroxyl group in the nonpolar region of the enzyme and the energy required to fold the butyl chain of XI nearly equals the energy gained by OH binding to the site on the enzyme utilized by the hydroxyl group of IX and X.

Finally, on the basis of this work, it appears that the 9-alkyl group of the various adenine derivatives is binding to the enzyme outside of the active site. It may be that the adenine moiety of these inhibitors is binding to the same site on the enzyme which is normally utilized by the adenine portion of the substrate, adenosine. Because of the length and flexibility of the 9-alkyl group, it can bridge to a nonpolar region of the enzyme to which the normal 9-substituent (β-D-ribofuranosyl) of the substrate is neither attracted nor able to reach. We believe that it should be possible to synthesize inhibitors which take advantage of the polar and nonpolar regions of adenosine deaminase.

Glutamic Acid Analogs. The Synthesis of 3-Alkylglutamic Acids and 4-Alkylpyroglutamic Acids[1a,b]

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3-Alkylglutamic acids, designed as metabolic antagonists of glutamic acid, were prepared by the acid hydrolysis of their corresponding 4-alkyl-5,5-dicarbethoxy-2-pyrrolidinones. The pyrrolidinones were formed from the Michael condensation of ethyl β-alkyl-α,β-unsaturated esters with diethyl acetamidomalonate. The Michael condensation of diethyl alkylidenemalonates and diethyl acylaminomalonates in refluxing ethanol formed 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones. Acid hydrolysis of these pyrrolidinones and subsequent pyrolysis of the reaction residue gave 4-alkylpyroglutamic acids.

For some time we have been interested in the synthesis of analogs of glutamic acid with the idea that they might possess antimetabolic activity. Of particular interest are the glutamic acids which have aliphatic substituents on positions 2, 3, and 4. A review[2] of the literature on this type of glutamic acid analog reveals that only a limited number has been reported.

The mechanism of glutamic acid activation and glutamine synthesis as described by Meister, *et al.*,[3] depicts an enzyme site which makes use of at least two points of attack; the amino group and the α-carboxyl group. The possibility of a steric restriction by alkyl substituents on the carbon skeleton to inhibit enzyme action exists, since mapping of the enzyme site remains incomplete. The proximity of a β-substituent on glutamic acid to the functional groups which are actively involved in the metabolic reactions of glutamic acid may produce the nonpolar steric impedance desired.

The synthesis of glutamic acids with alkyl substituents on the carbon skeleton has been accomplished by several routes, many of which are not applicable to universal substitution of the skeletal carbons of glutamic acid. The preparation of 2-methylglutamic acid by the hydrolysis of γ-cyano-γ-valerolactam[4] requires a Strecker-type synthesis with ethyl levulinate to prepare the valerolactam. Another synthesis of 2-methylglutamic acid has been reported by Pfister, *et al.*,[5] by the hydrolysis of the hydantoin prepared from the corresponding methyl ketone. 2-, 3-, and 4-Methylglutamic acids were prepared by the Schmidt reaction.[6] Another synthesis of 4-methylglutamic acid[7] and of 3-

(1) (a) Presented at the 144th National Meeting of the American Chemical Society, Los Angeles, Calif., April 1963, Abstracts, p. 18L. (b) This work was supported by a grant (GM-08644) from the National Institutes of Health, Public Health Service. (c) To whom inquiries should be addressed.

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