Acknowledgment. Partial support for this research was provided by the Department of the Army, U.S. Army Medical Research and Development Command, Contract DAMD17-78-C-8021. We are grateful to Dr. John Occolowitz of the Lilly Research Laboratories, Indianapolis, Ind., for the field desorption mass spectra, and to Dr. Lan Wong of The Ohio State University Department of Pharmacology for the low-resolution electron impact spectrum of **20b.** Assistance with some of the nomenclature was kindly provided by Professor Derek Horton.

References and Notes

(1) Some of this work has been reported. (a) Secrist III, J. A.; Cook, S. L.; Winter, Jr., W. J. "Abstracts of Papers", 174th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1977; American Chemical Society: Washington, D.C., 1977; CARB-23. (b) Cook, S. L.; Secrist III, J. A. *Carbohydr. Res.* **1976**, *52*, C3-C6.
(2) Morton, G. O.; Lancaster, J. E.; Van Lear, G. E.; Fulmor, W.; Meyer, W. E. *J. Am. Chem. Soc.* **1969**, *91*, 1535-1537.
(3) Hewitt, R. W.; Gumble, A. R.; Taylor, L. H.; Wallace, W. S. *Antibiot. Annu.* **1956-1957**, 722.
(4) Tobie, E. J. *J. Parasitol.* **1957**, *43*, 291-293.
(5) Stephen, L. E.; Gray, A. R. *J. Parasitol.* **1960**, *46*, 509-514.
(6) Jenkins, I. D.; Verheyden, J. P. H.; Moffatt, J. G. *J. Am. Chem. Soc.* **1976**, *98*, 3346-3357.
(7) Owen, G. R.; Verheyden, J. P. H.; Moffatt, J. G. *J. Org. Chem.* **1976**, *41*, 3010-3017.
(8) Verheyden, J. P. H., et al. *Ann. N.Y. Acad. Sci.* **1975**, *255*, 151-165.
(9) Verheyden, J. P. H.; Moffatt, J. G. *J. Am. Chem. Soc.* **1975**, *97*, 4386-4395.
(10) Sasaki, T.; Minamoto, K.; Kuroyanagi, S.; Hattori, K. *Tetrahedron Lett.* **1973**, 2731-2733.
(11) Sasaki, T.; Minamoto, K.; Hattori, K. *J. Am. Chem. Soc.* **1973**, *95*, 1350-1351.
(12) Sasaki, T.; Minamoto, K.; Asano, T.; Miyake., M. *J. Org. Chem.* **1975**, *40*, 106-111.
(13) Leland, D. L.; Kotick, M. P. *Carbohydr. Res.* **1974**, *38*, C9-C11.
(14) Youssefyeh, R.; Tegg, D.; Verheyden, J. P. H.; Jones, G. H.; Moffatt, J. G. *Tetrahedron Lett.* **1977**, 435-438.
(15) Rosenthal, A.; Ratcliffe, M. *Carbohydr. Res.* **1977**, *54*, 61-73.
(16) Secrist III, J. A.; Winter, Jr., W. J. *J. Am. Chem. Soc.* **1978**, *100*, 2554-2555.
(17) Chladek, S.; Smrt, J. *Collect. Czech. Chem. Commun.* **1963**, *28*, 1301-1308.
(18) Damodaran, N. P.; Jones, G. H.; Moffatt, J. G. *J. Am. Chem. Soc.* **1971**, *93*, 3812-3813.
(19) Jones, G. H.; Moffatt, J. G. *Methods Carbohydr. Chem.* **1972**, *6*, 315-322.
(20) Berkoz, B.; Chavez, E. P.; Djerrasi, C. *J. Chem. Soc.* **1962**, 1323-1329.
(21) Bedoukian, P. Z. "Organic Syntheses", Collect. Vol. III; Wiley: New York, 1955; pp 127-129.
(22) Bell, R. A.; Saunders, J. K. *Can. J. Chem.* **1970**, *48*, 1114-1122.
(23) Ducruix, A.; Pascard-Billy, C.; Eitelman, S. J.; Horton, D. *J. Org. Chem.* **1976**, *41*, 2652-2653.
(24) Benson, W. R.; McBee, E. T.; Randy, L. *Org. Synth.* **1962**, *42*, 73-75.
(25) Levy, G. C.; Nelson, G. L. "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists", Wiley-Interscience: New York, 1972.
(26) The allylic coupling constants for **2a** (0.9 Hz) and **2b** (1.2 Hz) represent another example of a reversal of the more common situation where the transoid coupling constant is smaller than the cisoid.[27]
(27) Jackman, L. M.; Sternhell, S. "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2nd ed.; Pergamon Press: Elmsford, N.Y., 1969; pp 316-328.
(28) Hassner, A.; Boerwinkle, F. P.; Levy, A. B. *J. Am. Chem. Soc.* **1970**, *92*, 4879-4883.
(29) This anhydronucleoside might be an excellent vehicle for substituent incorporation at C_4. In fact, our preliminary results, to be reported later, show that a alkylthio group can be introduced via **5e.**
(30) The peracid method was found to be very convenient for this oxidation, better than the previous literature methods. (a) O_2, Pt: Moss, G. P.; Reese, C. B.; Schofield, K.; Shapiro, R.; Todd, A. *J. Chem. Soc.* **1963**, 1149-1154. (b) $KMnO_4$: Schmidt, R. R.; Schloz, U.; Schwille, D. *Chem. Ber.* **1968**, *101*, 590-594.
(31) Perron, Y. G.; Crast, L. R.; Essery, J. M.; Grasser, R. R.; Godfrey, J. C.; Holdrege, C. T.; Minor, W. F.; Neubert, M. E.; Partyka, R. A.; Cheney, L. C. *J. Med. Chem.* **1964**, *7*, 483-487.
(32) Nowak, R. M. *J. Org. Chem.* **1963**, *28*, 1182-1187.
(33) Bartlett, P. D.; Tate, B. E. *J. Am. Chem. Soc.* **1956**, *78*, 5575-5580.
(34) Christl, M.; Reich, H. J.; Roberts, J. D. *J. Am. Chem. Soc.* **1971**, *93*, 3463-3468.
(35) (a) A systematic name for **3a** is 1-(2,3-*O*-cyclohexylidene-5-aldehydo-β-D-*ribo*-pentodialdo-1,4-furanosyl)uracil. (b) A systematic name for **3b** is 1-(2,3-*O*-cyclohexylidene-β-D-*ribo*-pentodialdo-1,4-furanosyl)uracil 5′-aldehydrol diacetate.
(36) A similar reaction utilizing pyridine as both catalyst and solvent yielded no enol acetate at room temperature after 18 h. Warming to 92 °C gave complete conversion to **2a**, however.
(37) Formation of the 2:1 $AgOAc/I_2$ complex followed by addition of 1 equiv of **2a** gave only 50% consumption of starting material, as judged by TLC.
(38) Wong, J. L.; Fuchs, D. S. *J. Org. Chem.* **1970**, *35*, 3786-3791.
(39) The two α-L-lyxo free nucleosides **20b** and **20c** began decomposing gradually almost immediately upon purification, and thus elemental analysis was not possible. It was also not possible to get a molecular ion in the mass spectra of **20c**, though this was possible on a low-resolution machine for **20b.**[40] As a result, field desorption spectra of both were taken to confirm the molecular weights.[40]
(40) Field desorption mass spectra (FDMS) were recorded on a Varian-MAT Model 731 mass spectrometer utilizing carbon dendrite emitters. The numbers in parentheses are relative peak intensities. The low-resolution electron impact spectrum of **20b** was recorded on a Hewlett-Packard Model 5985A GC-MS system.

lin-Benzoadenine Nucleotides. Inter- and Intramolecular Interactions in Aqueous Solutions as Observed by Proton Magnetic Resonance

Jorge R. Barrio, Fu-Tong Liu, Gene E. Keyser, Pieter VanDerLijn, and Nelson J. Leonard*

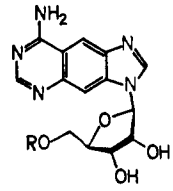
Contribution from the Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received August 13, 1978*

Abstract: The inter- and intramolecular interactions of *lin*-benzoadenine nucleotides have been examined by proton magnetic resonance. When the base is unprotonated, *lin*-benzoadenine nucleotides strongly stack in aqueous solution, with association constants of at least one order of magnitude greater than those of the corresponding adenine nucleotides. Some head-to-tail orientations of stacked *lin*-benzoadenine nucleotides were indicated by the deuterium substitution effect on relaxation times (DESERT). The relative positions of the heteroaromatic proton chemical shifts at infinite dilution (pD 8.5) and under acidic conditions (pD ~4.0) indicated the conformations of the nucleotides (anti and syn, respectively) and the site of ring protonation (the pyrimidine ring).

We have previously reported the interaction of *lin*-benzoadenine nucleotides (**1**) with enzymes and their sensitivity to the environment.[1-9] In order to understand more fully the observed properties of these adenine analogues, we have examined their inter- and intramolecular interactions by proton magnetic resonance. The accumulated data provide detailed information concerning the self-association of these compounds in aqueous solution. In addition, the relative positions of the

0002-7863/79/1501-1564$01.00/0

chemical shifts at infinite dilution (pD 8.5) and under acidic conditions (pD ~4.0) are indicative of the conformations of the nucleotides and the site of ring protonation.



1a, R = H
b, R = MP
c, R = DP
d, R = TP
e, R = cMP (involving the 3'-OH)

Experimental Section

Materials and Methods. *lin*-Benzoadenosine and its 5'-mono-, 5'-di-, 5'-tri-, and 3',5'-monophosphate, as well as P^1,P^2-di-*lin*-benzoadenosine 5'-pyrophosphate, were synthesized as previously reported.[2-4,6] A Chelex 100 column (Bio-Rad) equilibrated with 50 mM K_2HPO_4 and washed with water for 24 h was used to eliminate traces of paramagnetic impurities from the compounds. For T_1 studies a final concentration of 5 mM EDTA was found optimal[10] under the conditions used to ensure against metal ion interference. All samples were prepared in 2 mM potassium phosphate buffer (pD 8.5) in deuterium oxide (99.99%) supplied by Aldrich Chemical Co.

Proton chemical shifts, as well as spin-lattice relaxation times, were measured at 28 ± 1 °C (acetone, 2.17 ppm downfield from TSP, internal reference) using a Varian HA-220 NMR spectrometer interfaced with a NIC 80/Nicholet TT220 Fourier transform system. In the pH studies the deuterium ion concentration was varied by the addition of small amounts of DCl in 99.99% D_2O; the sample volumes were not significantly changed during these experiments. The spin-lattice relaxation times (T_1) were measured using the $(\pi\text{-}\tau\text{-}\pi/2)$ two-pulse sequence.

Determination of Association Constants. The association constants were determined using the following equation:[11]

$$\left(\frac{\Delta\delta}{C_T}\right)^{1/2} = \left(\frac{2K}{\Delta\delta_{MD}}\right)^{1/2}(\Delta\delta_{MD} - \Delta\delta) \qquad (1)$$

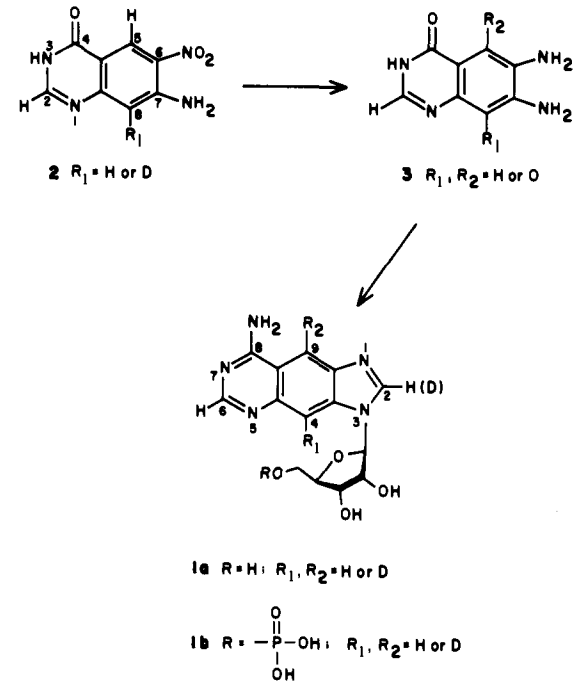
where C_T is the total concentration of *lin*-benzoadenine nucleotide, $\Delta\delta = \delta_M - \delta_{obsd}$, $\Delta\delta_{MD} = \delta_M - \delta_D$, and δ_M, δ_D, and δ_{obsd} are the chemical shifts of the examined proton in the monomer, dimer, and the experimental chemical shift determined at various concentrations of nucleotide, respectively. A plot of $(\Delta\delta/C_T)^{1/2}$ vs. $\Delta\delta$ will yield a straight line whose slope and X intercept are $(2K/\Delta\delta_{MD})^{1/2}$ and $\Delta\delta_{MD}$, respectively. This method as well as other iterative methods[12-14] assumes that there is a single minimum for the fitting of the data in the binding equation, at least in the range of values chemically possible. That this is true in our case can be demonstrated by convergence to similar values of δ_M when either an extremely high value or the highest δ_{obsd} is used for δ_{ARB} (initial δ_M). The experimental data (Figure 1) were fitted using the association constants in Table I. Although the monomer-dimer model adequately explains the data, trimers are probably formed at the highest concentration used, but calculations indicate that the association constant of the dimer (K) would not be substantially affected.

7-Amino-6-nitro-4-quinazolone-*8-d* (2, R_1 = D). A solution of 7-amino-6-nitro-4-quinazolone (**2**)[1] (200 mg, 0.97 mmol) in methanol-*d* (99+% D, 20 mL) was heated at reflux for 10 min and the solvent was removed in vacuo. The solid residue, dissolved in sulfuric acid-d_2 (99.5+% D, 98%, 4 g), was heated at 110-115 °C under a positive pressure of argon for 48 h. The solution was poured onto ice (25 g) and the resulting precipitate was filtered, washed with water (10 mL), and dried in vacuo to yield 199 mg (99%) of **2** (R_1 = D): NMR [$(CD_3)_2$SO] δ 8.66 (s, 1, ArH), 8.33 (s, 1, ArH), 7.50 (br s, 3, NH_2 and NH), 7.03 (s, 0.01, 8-ArH); MS *m/e* 207 (M^+ for $C_8H_5DN_4O_3$).

The material thus obtained was used without further purification.

6,7-Diamino-4-quinazolone-*8-d* (3, R_1 = D; R_2 = H). 7-Amino-6-nitro-4-quinazolone-*8-d* (**2**, R_1 = D) (199 mg, 0.97 mmol) was reduced according to the procedure of Leonard et al.[1] to give 160 mg (93%) of a "mixture" of **3** (R_1 = D; R_2 = H or D): NMR [$(CD_3)_2$SO] δ 7.65 (s, 1, ArH), 7.10 (s, 1, ArH), 6.65 (s, 0.4, 8-ArH), 6.0-4.0 (br s, 1, NH), 5.5 (br s, 2, NH_2), 5.0 (br s, 2, NH_2); MS *m/e* 176 and 177 (M^+).

Scheme I



6,7-Diamino-4-quinazolone-*5,8-d*$_2$ (3, R_1, R_2 = D). To a solution of 6,7-diamino-4-quinazolone (**3**, R_1 = R_2 = H) (4.40 g, 25 mmol) in dimethylformamide (50 mL)[1] was added deuterium oxide (99.5+% D, 25 mL), and the resulting mixture was reduced to dryness in vacuo. The solid residue was dissolved in sulfuric acid-d_2 (99.5+% D, 98%, 100 g) and the solution was heated at 110-115 °C under a positive pressure of argon for 20 h. The solution was poured onto ice (1 kg) and neutralized with concentrated ammonium hydroxide, and the precipitate was collected by filtration to give 4.0 g (91%) of a "mixture" of **3** (R_1 = D; R_2 = H or D): NMR [$(CD_3)_2$SO] δ 7.13 (s, 0.38, 5-ArH), 7.64 (s, 1, 2-ArH); MS *m/e* 177 and 178 (M^+), isotope ratio 3:2:0.1, d_2:d_1:d_0.

The material thus obtained was used without further purification.

***lin*-Benzoadenosine-*8-d* and -*5,8-d*$_2$ 5'-monophosphates** were prepared from **3** by the method of Leonard et al.[1-4]

Results and Discussion

Assignment of Aromatic Proton Resonances. It was necessary to assign unequivocally the proton nuclear magnetic resonances of the heteroaromatic nucleus before any conclusions could be made about intra- and intermolecular interactions or about the site of protonation of the various *lin*-benzoadenine nucleotides (**1**). It was observed that the 2-hydrogen in the series was partially exchanged for deuterium on standing at −10 °C for 3 months in buffered deuterium oxide; this transformation (and the reverse reaction) could also be accomplished by heating the nucleotides (**1**) in D_2O (H_2O) for several hours, in accord with the behavior of most benzimidazoles.[4,15] This exchange provided unequivocal assignment of the two-proton resonance. On heating **3** (R_1 = R_2 = H) (Scheme I), an early intermediate, in sulfuric acid-d_2, one hydrogen was exchanged very rapidly and a second was exchanged much more slowly on prolonged heating. To assign the two protons exchanged, compound **2** (R_1 = H), the immediate precursor to **3**, was heated in sulfuric acid-d_2 and one deuterium was incorporated. The wide separation of resonances in the ^{1}H NMR of **2** and the failure of a variety of other quinazolones to undergo deuterium exchange at position 2 under similar conditions[16] directed the assignment of the site of deuterium incorporation as position 8 in **2** (to become C-4

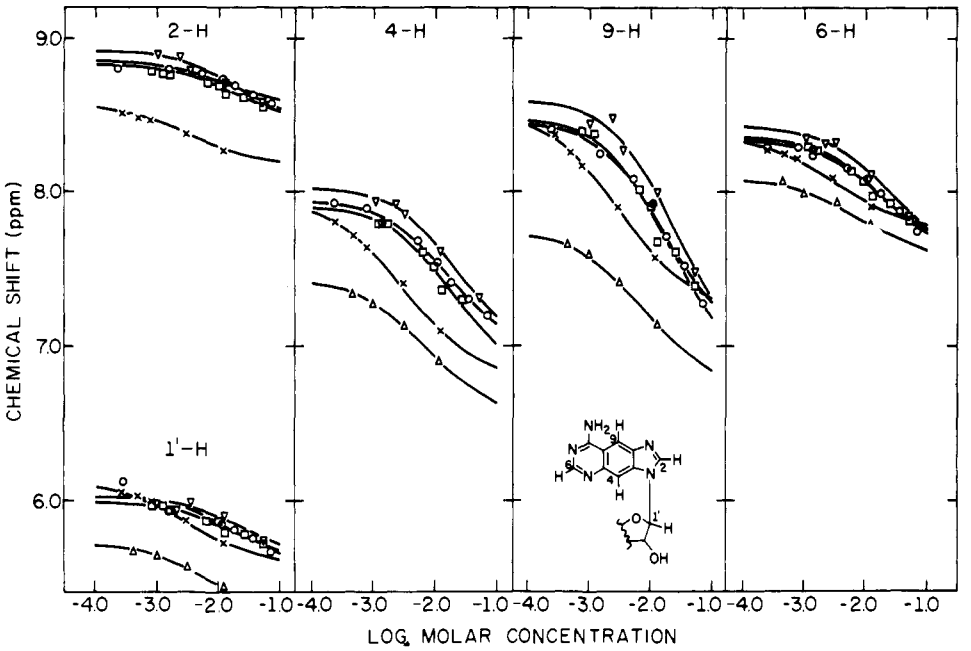
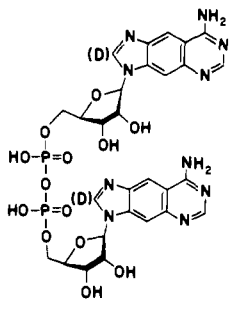


Figure 1. Chemical shifts of the base protons, 2-, 4-, 9-, and 6-H, and anomeric proton, 1′-H, as a function of the logarithm of the molar concentration of: (×) *lin*-benzo-cAMP (**1e**); (○) *lin*-benzo-AMP (**1b**); (□) *lin*-benzo-ADP (**1c**); (▽) *lin*-benzo-ATP (**1d**); (△) bis-5′,5′-(*lin*-benzo-AMP-*2-d*) (**1f**).

in **1**). By reduction of **2**-*8-d* (R_1 = D) to a "mixture" of **3** (R_1 = H or D; R_2 = H), it was shown that the position of rapid deuterium incorporation in **3** ($R_1 = R_2$ = H) was in fact C-8, as was predicted from the electronic character of substituents about the benzenoid portion of the molecule. This observation, in combination with the failure of similar quinazolones to undergo deuteration at C-2[16] under the conditions employed, defined the site of partial exchange as position 5 (to become C-9 in **1**). Deuterium was incorporated fully into position 8 and to an extent of 60% at position 5 by heating **3** ($R_1 = R_2$ = H) in sulfuric acid-d_2 at 110–115 °C for 20 h. The half-life of deuterium incorporation at C-8 under these conditions was approximately 10 min.

The deuterated "mixture" of **3** was carried through synthetic sequences described earlier[1-4] to a "mixture" of *lin*-benzoadenosine-*4-d* and -*4,9-d*$_2$ (**1a**) and thence to a "mixture" of 5′-monophosphates (**1b**), as shown by ^{1}H NMR. No exchange of deuterium was observed throughout the sequence, within the error of NMR detection.

By the incorporation of ca. 30% deuterium into position 2 of the "mixture" of **1b** (R_1 = D; R_2 = H or D) (by controlled heating in D_2O) it was possible to monitor the effects of dilution and changing pH on *lin*-benzo-AMP (**1b**) or related derivatives (**1c–f**) by NMR with a single sample, greatly reducing the number of experiments and variables involved in those determinations.



1f

Self-Association of *lin*-Benzoadenine Nucleotides. Vertical intermolecular stacking interactions in bases, nucleosides, and mono- and polynucleotides have been studied using vapor phase osmometry, ^{1}H NMR, ^{13}C NMR, sedimentation equilibrium experiments, and ultraviolet hypochromism measurements.[17,18] Instructive results have come from the ^{1}H NMR studies which provided elaborate information concerning the extent and mode of association.[19-29] *lin*-Benzoadenine nucleotides are expected to self-associate extensively in aqueous solution in their nonprotonated forms. With the deuterium-labeling results available, we determined the nature and extent of the molecular aggregations by means of the ^{1}H NMR spectra. Plots of chemical shifts vs. concentration showed that the shifts of the aromatic protons and the anomeric proton, 1′-H, changed appreciably with concentration while those of the pentose protons, especially the 5′-H's, which are farthest away from the base moiety, were not appreciably concentration dependent. The effect is similar to that observed for the chemical shifts of purine nucleosides and nucleotides,[17] where, however, a limit of either one or two base protons may be observed and where solutions have not been studied to the limiting low concentrations that we have examined here. The chemical shifts of the four aromatic protons and the 1′-H of the *lin*-benzoadenine nucleotides are plotted vs. the log of the molar concentration, over the range 2×10^{-4} to 5×10^{-2} M, in Figure 1.

The solid curves represent the computer fit to the data based on a monomer–dimer model. All of these protons show upfield shifts with increasing concentration, with the 4-H and 9-H showing the greatest changes. This observation indicates the association of these nucleotides by means of vertical stacking.[17] It was previously demonstrated for purine nucleosides and nucleotides that hydrogen bonding does not play an important role in the mechanism of association.[21] Iteration of the data provided the chemical shifts at infinite dilution, as shown in Table I.[8]

Association Constants. A comparison of the association constants with the corresponding values for adenine nucleotides[12,30,31] shows that the stacking interaction is approximately one order of magnitude stronger for the corresponding *lin*-benzoadenine nucleotides (Table I).[32] This is due in large part to the strengthening of the stacking or π interactions by the additional (central) ring in the *lin*-benzoadenine nucleo-

Table I. *lin*-Benzoadenine Nucleotides: Chemical Shifts at Infinite Dilution and Association Constants

compound	2-H	4-H	6-H	9-H	1′-H	K, M^{-1}
lin-benzo-cAMP	8.56	7.93	8.37	8.50	6.12	250
lin-benzo-AMP	8.83	7.94	8.36	8.46	6.05	42
lin-benzo-ADP	8.82	7.91	8.37	8.48	5.99	36
lin-benzo-ATP	8.91	8.03	8.44	8.61	6.03	28
bis-5′,5′-[*lin*-benzo-AMP-*2-d*]		7.42	8.10	7.74	5.71	100

Table II. Typical Differential Relaxation Rates (DRR) of 4-H, 6-H, 9-H, and 1′-H between *lin*-Benzo-5′-AMP and 2-Deuterium-Substituted *lin*-Benzo-5′-AMP

compound	2-H	4-H	6-H	9-H	1′-H
lin-benzo-AMP ($1/T_1$, s^{-1})	0.92	1.06	0.28	0.38	1.38
lin-benzo-AMP-*2-d* ($1/T_1$, s^{-1})		1.06	0.23	0.38	0.86
DRR, s^{-1}		0.00	0.05	0.00	0.52

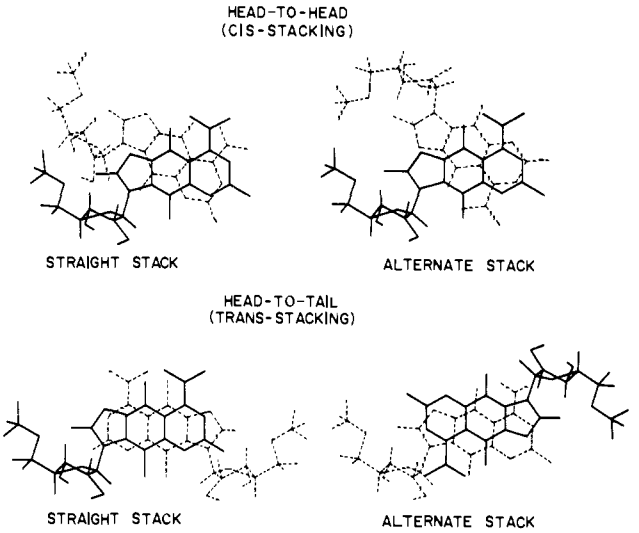


Figure 2. Possible intermolecular stacking orientations for *lin*-benzoadenine nucleotides. Extent of overlap is not implied.

tides. As with the adenine nucleotides,[10] the charge repulsions of the phosphate chains inhibit dimer formation. Thus, the strongest association constant is found with *lin*-benzo-cAMP (**1e**), the singly charged phosphate of which is geometrically restricted from intramolecular interaction with the base.[8]

The strong stacking interaction of the *lin*-benzoadenine moiety is also indicated by the chemical shift at infinite dilution (δ_M) of a "dimer" of *lin*-benzo-AMP, namely P^1,P^2-di-*lin*-benzoadenosine-*2-d* 5′-pyrophosphate (**1f**) (Table I). The anomeric and base protons are at higher field than those of the related monomer **1b**. The strong intramolecular stacking is also reflected in the spectroscopic properties of this dimer. We have previously reported[3,4] that the hypochromism of the dimer is 23% as compared with 9% for the corresponding dimer of AMP. Similarly, the fluorescence of the *lin*-benzoadenine moiety in the dimer **1f** is quenched dramatically ($\Phi_F = 0.005$). That the breaking of intramolecular stacking leads to a large increase in fluorescence intensity is shown by its quantum yield in ethanol ($\Phi_F = 0.25$), a denaturing solvent which is known to break stacking interactions. The quantum yields of *lin*-benzo-AMP (**1b**) are identical in water and ethanol ($\Phi_F = 0.44$).[33]

Stacking Orientations. It may be assumed that the greater changes in chemical shift with concentration for 4-H and 9-H over 2-H and 6-H (Table I) reflect a greater influence of the magnetic anisotropy of an associated heteroaromatic ring on the central protons. This, in turn, is considered indicative of the preferred average orientation of the nucleotide bases in the dimeric stacks (Figure 2). The change in chemical shift with concentration for the anomeric 1′-H appears to be slightly greater than for 2-H. Similar results were observed in the adenine nucleotides for 1′-H and 8-H.[21] In addition, the change for the 6-H in the pyrimidine ring is greater than for 2-H, the proton on the imidazole ring. A similar situation occurs for cAMP, AMP, and ATP,[31] indicating that the pyrimidine rings are more involved in stacking than the imidazole rings in the adenine nucleotide series.

Further information concerning the preferred stacking orientation of the nucleotides in dimeric association can be obtained using the deuterium substitution effect on relaxation times (DESERT).[34] In this method the differential relaxation rate (DRR) of a nucleus i is observed before and after specific deuterium substitution of a proton k, and DRR is related to the distance between the two nuclei i and k. At a 1.8×10^{-2} M concentration of *lin*-benzo-AMP (**1b**) in solution, in which significant proportions of monomer and dimer exist, measurement of the T_1 relaxation times of the 4-H, 6-H, and 9-H before and after deuteration of the 2-H shows that the DRRs of 4-H and 9-H are zero, while that of 6-H is 0.05 (Table II).[35] That is, the fraction of the differential relaxation rate to the total relaxation rate of the 6-H is about 18%. (For 8-deuterated 5′-AMP, the corresponding fraction for the 2-H in that molecule is ">10%".[34]) Because in the monomer 6-H is the farthest of all the aromatic protons from 2-H, such an effect can only result from at least partial head-to-tail stacking (straight or alternate stack) (Figure 2), in which 2-H and 8-H are in close proximity.

From a study of the intermolecular interactions of AMP, Evans and Sarma[36] concluded that the preferred self-association of two nucleotide molecules occurs with the bases aligned head-to-head and face-to-back in straight vertical stacks involving almost 100% base overlap. In these stacks, the ribose groups are close but the phosphate groups are well separated, presumably in order to eliminate steric hindrance and reduce electrostatic repulsion. The conclusion that the preferred self-association involves the formation of head-to-head, face-to-face (alternate stack) dimers was reached by Ts'o et al.[37] and by Berger and Eichhorn.[38]

Sugar–Base Torsion Angle, χ_{CN}. The comparison of δ_M's of *lin*-benzo-AMP (**1b**), *lin*-benzo-ADP (**1c**), *lin*-benzo-ATP (**1d**), and *lin*-benzo-cAMP (**1e**) in water (pH 8.5, 28 °C) indicates that 2-H is deshielded by about 0.26–0.35 ppm in **1b–d** relative to **1e**. The deshielding results directly from the effect of the ionization of the secondary phosphate on the 2-H[22] and is indicative of the anti conformation for the *lin*-benzoadenine 5′-nucleotides.[22,24] The specific interaction of the 5′-phosphate group with the 2-H, which occurs when the nucleotide is in an anti conformation, is abolished in the 3′,5′-monophosphate (**1e**). This conclusion is further substantiated by the observation that 4-H, 6-H, and 9-H in **1b–e** have similar δ_M's, which can be rationalized on the basis that these protons reside in an environment far away from the ribofuranose phosphate(s), as is true in anti conformations. The similarities in the 1′-H chemical shifts for the series also agree with this finding. Specific influences of the phosphate(s) on the 8-H in the ^1H NMR spectra of purine nucleotides have been recognized,[39] and the mechanism of the deshielding effect has been thoroughly investigated in that series.[22] Smaller deshielding effects (~0.1 ppm) were noted for the adenine nucleotides. The quantitative differences between the *lin*-benzoadenine and the adenine nucleotides are in part a reflection of the different acidities of the imidazole protons.[15,40] Work on purine 5′-nucleotides does provide some reliable evidence that this de-

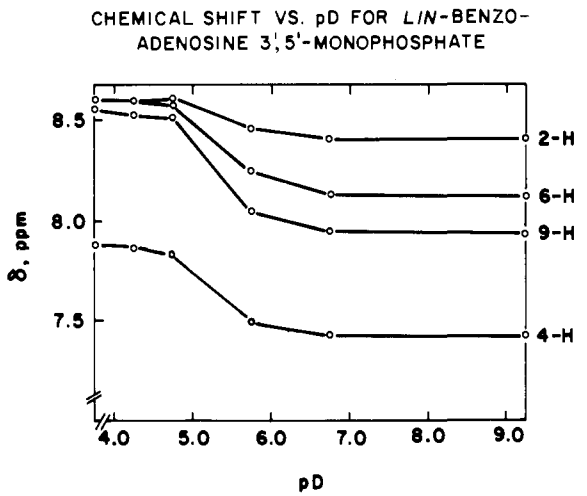


Figure 3. Chemical shifts of the base protons of *lin*-benzoadenosine 3′,5′-monophosphate (**1e**) as a function of pD in 3×10^{-3} M aqueous solution (D_2O).

shielding effect of the phosphate is reduced when the acidity of the sensitive protons is decreased.[22]

Base Protonation. The effects of pH (pD) on the chemical shifts of *lin*-benzoadenosine 3′,5′-monophosphate (**1e**) in 3×10^{-3} M aqueous solution (D_2O) are shown in Figure 3. Analogous effects were observed for **1b–d.** N-Protonation of the *lin*-benzoadenine moiety caused the downfield shift of the base C-H's by the intrinsic deshielding due to the positive charge and by the decrease in the intermolecular association of the bases.[22,41] From the differential chemical shifts ($\delta_{M_{pD\ 8.5}} - \delta_{pD\ 4.0}$) for the *lin*-benzoadenine nucleotides, as shown in Table III, several correlations can be adduced, mainly due to the discriminative effects of the phosphates on the base protons. 6-H has similar chemical shifts for all four nucleotides (**1b–e**) at pH 8.5 (Table I). Similar downfield shifts were observed in all four cases upon protonation of the base. The absence of downfield shift for 2-H upon protonation provides additional evidence that at pD 4.0 the base is protonated on the pyrimidine ring. The different pK_a values observed for the series **1a–e** (**1a** = 5.6; **1e** = 5.6; **1b** = 7.6; **1c** = 7.3; and **1d** = 7.1)[3,4] provide evidence for the unique response to the conformation around the N_3–C_1' bond and proximity of the phosphate side chain. If the 8-NH_2 group were to bind the proton, this would destroy the CT interaction between the amino group and the ring, and a blue shift in the fluorescence emission spectra would be expected upon protonation.[42] However, there is actually an increase in CT character upon acidification, and involvement of the free amino group in the resonance-stabilized cation seems important, consistent with protonation at N-5 or N-7 rather than at N-1 or 8-NH_2.

At pD 8.5, the influence of the secondary phosphate ionization on the chemical shifts of 2-H was observed for **1b–d** compared with **1e** (Table I). At pD 4.0, this influence was removed, and the chemical shifts of 2-H were similar for the whole series **1b–e**, centered at 8.60 ppm (cf. Tables I and III). Acid did not change the chemical shifts of 4-H in the 3′,5′-monophosphate **1e** appreciably, whereas it produced a substantial deshielding of 4-H in **1b–d.** It is interesting that in *lin*-benzo-AMP (**1b**) (pK_a = 7.6), the 4-H first experiences a dramatic downfield shift, ~0.85 ppm at 3×10^{-3} M, upon changing the pD from 8.5 to the range 6.5–6.0. The two negative charges on the α-phosphate at pD 6.5–6.0 produce a deshielding effect on 4-H that is not observed in **1c** and **1d** at similar pD. Upon lowering the pD to 4.0, protonation of the secondary phosphate occurs and an upfield shift (0.25 ppm) is observed. The *net* downfield shift for the 4-H of *lin*-benzo-AMP in going from pD 8.5 to 4.0 is 0.60 ppm at 3×10^{-3} M.

Table III. Differential Chemical Shifts ($\delta_{M_{pD\ 8.5}} - \delta_{pD\ 4.0}$) in *lin*-Benzoadenine Nucleotides[a]

compound	2-H	4-H	6-H	9-H[b]	1′-H
lin-benzo-cAMP (**1e**)	0.00	+0.05	−0.25	−0.05	−0.15
lin-benzo-AMP (**1b**)	+0.20	−0.60	−0.15	+0.10	0.00
lin-benzo-ADP (**1c**)	+0.25	−0.60	−0.15	+0.15	0.00
lin-benzo-ATP (**1d**)	+0.30	−0.45	−0.15	+0.20	0.00

[a] Values are within a precision of ±0.03 ppm. [b] See ref 41.

These effects can be explained if in the N-protonated form the syn conformation is preferred in comparison with the anti conformation in the neutral form. In the N-protonated syn conformation, a better stabilization of the positive charge on the base results from the intramolecular coulombic interaction between first ionized phosphate and protonated base at low pH. In the *lin*-benzoadenine nucleotide series, this conformational assignment can be made because 4-H is present and is sensitive to the proximity of the phosphate. In the adenine nucleotide series, by contrast, the spatial relationship of the α-phosphate relative to 2-H is such that phosphate monoanion/N-protonated pyrimidine ring interaction leading to the presence of any syn conformation cannot be detected by the same method.

lin-Benzoadenosine 3′,5′-monophosphate (**1e**) probably retains the anti conformation in acid, as indicated by the constant chemical shift of 2-H and the downfield shift of 1′-H (Table III) in going from pH 8.5 to 4.0. The protonated base would have an effect on the chemical shift of 1′-H only in an anti conformation. This conformation might still be preferred in acid because intramolecular phosphate stabilization of the positive charge of the base is sterically impossible; this is also reflected in the pK_a value of **1e**, which is the same as that of *lin*-benzoadenosine.[8]

Conclusions

When the base is unprotonated, the *lin*-benzoadenine nucleotides strongly stack in aqueous solution, with association constants of at least one order of magnitude higher than those of the corresponding adenine nucleotides. The presence of phosphates in the 5′ side chain slightly decreases this stacking interaction. The anti conformation is preferred at pD 8.5, but at least for **1b–d**, the syn conformation induced by intramolecular charge stabilization is predominant in acidic pH, i.e., below the pK_a of the base. The 6-H is a "monitor" of charge (protonation takes place in the pyrimidine ring), 4-H is sensitive to phosphate ionization and thus is indicative particularly of the syn conformation, and 2-H is responsive to the anti conformation.

Acknowledgment. This work was supported by Research Grant No. GM-05829 from the National Institutes of Health, U.S. Public Health Service. One of us (G.E.K.) held an Eli Lilly and Co. Fellowship during 1977-1978.

References and Notes

(1) N. J. Leonard, A. G. Morrice, and M. A. Sprecker, *J. Org. Chem.*, **40**, 356 (1975).
(2) N. J. Leonard, M. A. Sprecker, and A. G. Morrice, *J. Am. Chem. Soc.*, **98**, 3987 (1976).
(3) D. I. C. Scopes, J. R. Barrio, and N. J. Leonard, *Science*, **195**, 296 (1977).
(4) N. J. Leonard, D. I. C. Scopes, P. VanDerLijn, and J. R. Barrio, *Biochemistry*, **17**, 3677 (1978).
(5) R. F. Kauffman, H. A. Lardy, J. R. Barrio, M. del C. G. Barrio, and N. J. Leonard, *Biochemistry*, **17**, 3686 (1978).
(6) M. J. Schmidt, L. L. Truex, N. J. Leonard, D. I. C. Scopes, and J. R. Barrio, *J. Cyclic Nucleotide Res.*, **4**, 201 (1978).
(7) J. R. Barrio, M. del C. G. Barrio, N. J. Leonard, T. E. England, and O. C. Uhlenbeck, *Biochemistry*, **17**, 2077 (1978).
(8) P. VanDerLijn, J. R. Barrio, and N. J. Leonard, *Proc. Natl. Acad. Sci. US.A.*, **75**, 4204 (1978).
(9) P. VanDerLijn, J. R. Barrio, and N. J. Leonard, *J. Biol. Chem.*, **253**, 8694 (1978).
(10) Y.-F. Lam and G. Kotowicz, *Can. J. Chem.*, **55**, 3620 (1977).

(11) J.-L. Dimicoli and C. Hélène, *J. Am. Chem. Soc.*, **95**, 1036 (1973).
(12) J. Granot and D. Fiat, *J. Am. Chem. Soc.*, **99**, 4963 (1977).
(13) A. Cornish-Bowden and D. E. Koshland, Jr., *Biochemistry*, **9**, 3325 (1970).
(14) C. W. Wharton, A. Cornish-Bowden, K. Brocklehurst, and E. M. Crook, *Biochem. J.*, **141**, 365 (1974).
(15) J. A. Elvidge, J. R. Jones, C. O'Brien, and E. A. Evans, *Chem. Commun.*, 394 (1971).
(16) 7-Chloro-, 6-nitro-, and 6-amino-4-quinazolone all failed to undergo deuteration at the 2 position after 24 h at 110–115 °C.
(17) P. O. P. Ts'o in "Basic Principles in Nucleic Acid Chemistry", Vol. 1, P. O. P. Ts'o, Ed., Academic Press, New York, 1974.
(18) T. Schleich, B. P. Cross, B. J. Blackburn, and I. C. P. Smith, in "Structure and Conformation of Nucleic Acids and Protein-Nucleic Acid Interactions", M. Sundaralingam and S. T. Rao, Eds., University Park Press, Baltimore, Md., 1975.
(19) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *J. Am. Chem. Soc.*, **86**, 4182 (1964).
(20) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **87**, 5241 (1965).
(21) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **89**, 3612 (1967).
(22) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Am. Chem. Soc.*, **90**, 1042 (1968).
(23) S. I. Chan and J. H. Nelson, *J. Am. Chem. Soc.*, **91**, 168 (1969).
(24) F. E. Evans and R. H. Sarma, *FEBS Lett.*, **41**, 253 (1974).
(25) F. E. Evans, C.-H. Lee, and R. H. Sarma, *Biochem. Biophys. Res. Commun.*, **63**, 106 (1975).
(26) C. Giessner-Petre and B. Pullman, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **261**, 2521 (1965).
(27) C. Giessner-Petre and B. Pullman, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **268**, 1115 (1969).
(28) C. Giessner-Petre and B. Pullman, *J. Theor. Biol.*, **27**, 87 (1970).
(29) C. Giessner-Petre and B. Pullman, *J. Theor. Biol.*, **27**, 341 (1970).
(30) W. Egan, *J. Am. Chem. Soc.*, **98**, 4091 (1976).
(31) S. Fau, A. C. Storer, and G. G. Hammes, *J. Am. Chem. Soc.*, **99**, 8293 (1977).
(32) The major difficulty in this study is the low water solubility at room temperature of *lin*-benzoadenosine, an appropriate reference for this work. This could be overcome because *lin*-benzoadenosine 3′,5′-monophosphate (**1e**), which shows increased water solubility, is a close model for the riboside. Both have similar spectroscopic properties[3,4] and pK_a[3-8] values because intramolecular interaction cannot occur between cyclic phosphate and base.
(33) P. VanDerLijn, J. R. Barrio, and N. J. Leonard, manuscript in preparation.
(34) T. Imoto, *Biochim. Biophys. Acta*, **475**, 409 (1977).
(35) The relaxation times of 2-H and 9-H were shortened in the presence of minute amounts of paramagnetic ions, while no large differences were observed for 4-H. Such a differentiation between protons could be an indication of the proximity of the metal ion to 2-H and 9-H, probably because of its binding to N-1[8] (for analogy to the adenine nucleotides, see A. T. Tu and M. J. Heller, in "Metal Ions in Biological Systems", Vol. 1, H. Sigel, Ed., Marcel Dekker, New York, 1974, pp 1–51).
(36) F. E. Evans and R. H. Sarma, *Biopolymers*, **13**, 2117 (1974).
(37) P. O. P. Ts'o, M. P. Schweizer, and D. P. Hollis, *Ann. N.Y. Acad. Sci.*, **158**, 256 (1969).
(38) N. A. Berger and G. L. Eichhorn, *Biochemistry*, **10**, 1847 (1971).
(39) C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.*, **82**, 222 (1960).
(40) F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, **29**, 1988 (1964).
(41) There are very small concentration effects on the chemical shifts of 2-H, 4-H, and 6-H in *lin*-benzo-AMP (**1b**) at pD 4.5 in the concentration range 2.5×10^{-2} to 7.5×10^{-4} M. Dilution in the same range results in 9-H being shifted downfield by 0.4 ppm. At 3×10^{-3} M the differences observed for the 9-H among **1b–e** (Table III) may be due to incomplete dissociation of stacking.
(42) S. G. Schulman, P. J. Kovi, G. Torosian, H. McVeigh, and D. Carter, *J. Pharm. Sci.*, **62**, 1823 (1973).

Conformational Study of the Dipeptide Arginylglutamic Acid and of Its Complex with Nucleic Bases

Gérard Lancelot,* Roger Mayer, and Claude Hélène

Contribution from the Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orleans Cedex, France. *Received April 3, 1978*

Abstract: Proton and ^{13}C magnetic resonance spectra are reported for Ac-Arg-Glu-NHEt, Boc-Arg-Glu-NHEt, and protected Arg and Glu peptides. An intramolecular complex with two hydrogen bonds is found between side chains of arginine and glutamic acid. In all protected peptides containing a carboxylate group, a hydrogen bond is found between COO^- and the peptidic NH (Glu). Replacing the carboxylate group (COO^-) by the acid function (COOH) leads to the vanishing of the intramolecular hydrogen bond. NMR investigation of the interaction of the dipeptide with nucleic acid bases has shown that only guanine forms a strong intermolecular complex. Guanine forms a complex with two hydrogen bonds with carboxylate groups and disrupts the intramolecular Arg-Glu complex. No strong interaction was seen between bases and arginine.

The specific recognition between nucleic acids and proteins is one of the fundamental molecular processes involved at every step of genetic expression. The most striking examples include the recognition of operators by repressors, of promoters by RNA polymerase, and of DNA base sequences by restriction endonucleases. It may be asked whether there are general rules which govern this recognition process.

Several authors have attempted to solve the problem of protein–nucleic acid recognition in terms of models based on structural complementarity between double-stranded DNA or RNA and the antiparallel β structure in proteins.[1-4]

The present work is based on the idea that besides an overall structural complementarity the interacting regions of the two macromolecules involved in a protein–nucleic acid complex must establish point interactions between the available chemical groups of both components. There are several ways by which peptides can interact specifically with other molecules. These include electrostatic interactions, which are very common but would not lead to highly specific binding, stacking interactions involving aromatic amino acids and bases,[5-9] and hydrogen-bonding interactions.[10-14]

It has been suggested by Seeman et al.[10] that a single hydrogen bond is unable to discriminate with any great precision a particular base pair in a nucleic acid double helix. However, two hydrogen bonds in the same functional group provide a mechanism for fixing the position of the two bonds relative to each other with a much higher degree of precision. An interesting analogy has been made by Davies,[15] who found many highly specific polynucleotide interactions, all of which utilize hydrogen bond pairs as the basis of specificity in the interaction. These analogies enabled Seeman et al.[10] and Hélène[11] to propose several types of complementary pairs involving nucleic acid bases or base pairs and some amino acid side chains. Among these side chains, two are expected to play a particularly important role. These are the carboxylate anions of glutamic and aspartic acids and the guanidinium cation of arginine. The former could form a highly specific hydrogen-bonded complex with guanine and experimental evidence is

0002-7863/79/1501-1569$01.00/0