Studies of Tautomers and Protonation of Adenine and Its Derivatives by Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy[1a]

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Abstract: High-resolution nitrogen-15 NMR spectra of adenine have been obtained both at the *S*-adenosylmethionine level and for a uniformly enriched sample prepared from *S*-adenosylmethionine isolated from yeast grown with $^{15}NH_4Cl$ as the principal nitrogen source. Specific ^{15}N labeling at N1, N3, N6′, N7, and N9 provided unequivocal assignments of the chemical shifts as well as elucidation of the position of the tautomeric equilibrium. For comparison, the nitrogen resonances of several adenine derivatives were determined at the natural-abundance level. The protonation sites of many of these substances were determined from the effect of pH on the nitrogen chemical shifts. Both adenine and its conjugate acid clearly exist as the N9-H tautomers in aqueous solution. Conversion of adenine to its conjugate base ($pK_a \sim 10$) results in a 56 ppm downfield shift of the N9 resonance.

High-resolution nitrogen-15 NMR spectroscopy can provide substantial information about the character of the nitrogens of nucleosides and nucleotides.[2,3] Observations on adenine and its derivatives are of special interest because of the ubiquitous nature of adenine in biological systems.[4]

There is considerable difficulty in detecting nitrogen-15 NMR signals at the natural-abundance level because of the low gyromagnetic ratio of this isotope (−0.101 relative to protons) and its low natural abundance (0.37%). The difficulties are compounded by the low solubility of adenine in common solvents. Because of these factors and the importance of making unequivocal spectral assignments, we have taken ^{15}N NMR spectra of adenine both generally and specifically enriched with nitrogen-15. The spectra of a number of adenine derivatives were taken at the natural-abundance level.

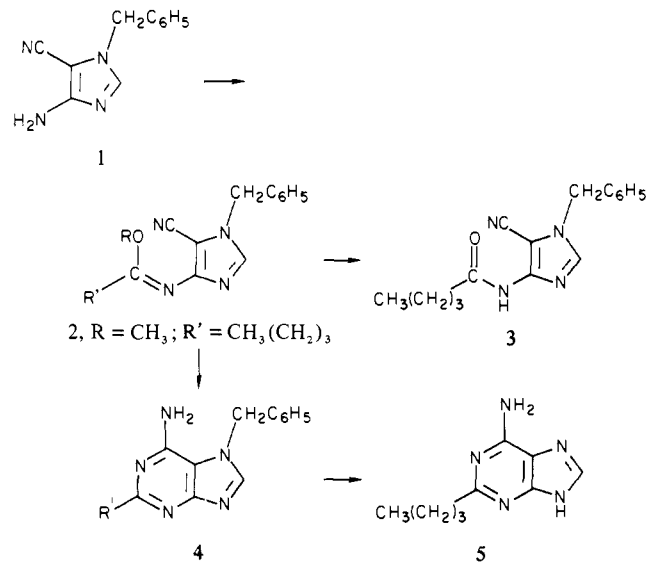
Experimental Section

Most of the nitrogen spectra were obtained at a frequency of 18.25 MHz with a Bruker WH-180 pulse spectrometer that has been described elsewhere.[5] Several of the spectra in which the changes in the nitrogen resonances of adenine were measured as a function of pH were taken at 50.69 MHz with a Bruker WM-500 spectrometer. A 0.1 M $D^{15}NO_3$ solution in D_2O provided both the reference standard and the external field-frequency lock for the natural-abundance spectra. For the labeled adenines, a capillary containing a 1 M $D^{15}NO_3$ solution in D_2O provided the reference standard, whereas D_2O or deuteriodimethyl sulfoxide was used as an internal field-frequency lock. The reported chemical shifts are in parts per million upfield from the resonance of external $D^{15}NO_3$. The natural-abundance spectra were taken in dimethyl sulfoxide solvent at concentrations of 0.43–4.2 M. Spectra of specifically labeled ^{15}N adenines were taken in dimethyl sulfoxide and aqueous solutions at concentrations of 0.042–0.14 M.

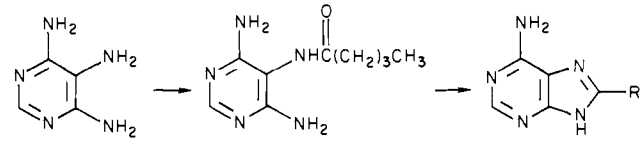
The normal operating conditions for the natural-abundance spectra employed pulse widths of 30–45 μs (30°–50° flip angle) and pulse delays of 10–20 s. The samples were run at about 30 °C with either gated proton decoupling or no decoupling. As reported earlier,[2] proton decoupling does not usually enhance the signal intensity of ^{15}N resonances of the azine nitrogens of this type of sample through operation of the nuclear Overhauser effect. For some of the natural-abundance spectra of adenine near neutral pH values, the samples were heated to about 50 °C to enhance the solubility. Changes in the nitrogen shifts resulting from protonation in dimethyl sulfoxide solutions were determined from titration curves of chemical shifts vs. added trifluoroacetic acid.

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H magnetic resonance spectra were recorded on an EM-390 spectrometer with tetramethylsilane as an internal standard. Low-resolution mass spectra were obtained with a Varian MAT CH-5 spectrophotometer. High-resolution mass spectra were obtained with a Varian MAT 731 spectrophotometer coupled with a 620i computer and STATOS recorder. The ultraviolet spectra were obtained with a Beckman Acta Model M VI spectrophotometer; microanalyses were performed by Josef Nemeth and his associates at the

Scheme I



Scheme II



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University of Illinois, who also weighed samples for quantitative ultraviolet absorption spectra. Thin-layer chromatography was carried out on EM silica gel f-254 plates (thickness, 0.25 mm). The solvent systems used were chloroform–ethanol (1:1 v/v), and ethyl acetate–methanol (9:1 to 3:2 v/v). Brinkman 0.05–0.2-mm silica gel was used for column chromatography.

Adenine and adenosine were commerically available and were used without further purification. Uniformly labeled adenine was obtained from *S*-adenosylmethionine isolated from yeast grown on 99% ^{15}N-enriched ammonium chloride by the elegant procedure described by Schlenk and co-workers.[6] 2-Butyladenine (**5**) and 8-butyladenine (**8**) were synthesized by methods outlined below (Schemes I and II). The remaining adenine derivatives were prepared by published procedures.[7-9]

1-Benzyl-5-cyano-4-((1-methoxypentylene)amino)imidazole (2). A mixture of 4-amino-1-benzyl-5-cyanoimidazole (**1**)[10] (1.75 g, 8.8 mmol), trimethyl orthopentanoate[11] (4.30 g, 26.7 mmol), and formic acid (100 μL) was stirred and heated under nitrogen in a water bath (~70 °C) until one liquid phase was obtained (~10 min). The solution was allowed to cool, and the stirring was continued overnight. After addition of 1,4-dioxane (20 mL), the solution was neutralized with sodium bicarbonate. The resulting mixture was concentrated under reduced pressure and, when the residue was subjected to column chromatography on silica gel (100 g) with hexane–ethyl acetate (4:1) as eluant, it gave analytically pure **2**, 2.38 g (91%) as a colorless oil that crystallized upon standing: mp 55 °C; ^1H NMR ($CDCl_3$) δ 0.90 (t, 3, J = 7 Hz, CH_3), 1.13–1.80 (m, 4, $(CH_2)_2$), 2.18 (t, 2, J = 8 Hz, CH_2), 3.90 (s, 3, CH_3O), 5.20 (s, 2, Ar CH_2), 7.27–7.50 (m, 6, Ar H); MS, *m/e* (rel abundance; 10 eV) 296 (M^+, 26), 281 ($M^+ - CH_3$, 3), 267 ($M^+ - C_2H_5$, 3), 264 ($M^+ - CH_3OH$, 3), 254 ($M^+ - C_3H_6$, 100), 239 ($M^+ - C_4H_9$, 38), 197 ($C_{11}H_9N_4^+$, 30), 91 ($C_7H_7^+$, 89). Anal. Calcd for $C_{17}H_{20}N_4O$: C, 68.90; H, 6.80; N, 18.90. Found: C, 69.03; H, 6.89; N, 18.76.

Anhydrous conditions must be maintained during formation of the imidate **2** prior to neutralization to prevent hydrolysis to **1-benzyl-5-cyano-4-(pentanoylamino)imidazole (3)**, which has a melting point of 128 °C (recrystallized from benzene); ^1H NMR (CD_3OD) δ 0.93 (t, 3, J = 7 Hz, CH_3), 1.13–1.83 (m, 4, $(CH_2)_2$), 2.37 (t, 2, J = 8 Hz, CH_2), 5.27 (s, 2, CH_2), 7.33 (s, 5, Ar H), 7.80 (s, 1, 2-H); MS, *m/e* (rel abundance; 10 eV) 282 (M^+, 9), 240 ($M^+ - C_3H_6$, 1), 198 ($M^+ - C_5H_8O$, 100), 91, ($C_7H_7^+$, 69). Anal. Calcd for $C_{16}H_{18}N_4O$: C, 68.06; H, 6.43; N, 19.84. Found: C, 67.95; H, 6.42; N, 19.85.

7-Benzyl-2-butyladenine (4). A mixture of **2** (1.00 g, 3.4 mmol) and liquid ammonia (30 mL) was heated at 70 °C for 24 h in a 300-mL general-purpose Parr bomb. The bomb was cooled in a dry ice–isopropyl alcohol bath for 1 h and then opened, and the excess ammonia was allowed to evaporate as the bomb and its contents were gradually warmed to room temperature. The residue was dissolved in ethanol (50 mL), filtered, and concentrated under reduced pressure to give **4** as a chromatographically homogeneous, white solid (0.88 g, 93%): mp 185 °C (recrystallized from 1,4-dioxane); ^1H NMR (CD_3OD) δ 0.93 (t, 3, J = 7 Hz, CH_3), 1.17–1.90 (m, 4, $(CH_2)_2$), 2.73 (t, 2, J = 8 Hz, CH_2), 4.80 (s, 2, NH_2), 7.10–7.33 (m, 5, Ar H), 8.20 (s, 1, 8-H); MS, *m/e* (rel abundance; 10 eV) 281 (M^+, 3), 280 (1), 266 ($M^+ - CH_3$, 6), 252 ($M^+ - C_2H_5$, 14), 239 ($M^+ - C_3H_6$, 100), 91 ($C_7H_7^+$, 16); high-resolution MS, *m/e* 281.1621 (calcd for $C_{16}H_{19}N_5$). Anal. Calcd for $C_{16}H_{19}N_5 \cdot 0.25H_2O$: C, 67.23; H, 6.87; N, 24.50. Found: C, 67.41; H, 6.85; N, 24.40.

2-Butyladenine (5). To a suspension of **4** (2.80 g, 10 mmol) in liquid ammonia (250 mL) was added sodium (460 mg, 20 mg-atom) with vigorous stirring. Debenzylation was complete after the solution stayed blue for 1 min, and a pinch of ammonium chloride was added to react with the excess sodium. The ammonia was allowed to evaporate, and the resulting residue was dissolved in 50 mL of 2 N hydrochloric acid. The acidic solution was warmed on a steam bath, treated with decolorized carbon, and filtered. The filtrate was brought to pH 8 with concentrated aqueous ammonia, whereupon **5** precipitated as a white solid. The solid was crystallized from methanol for microanalysis (1.04 g). Partial evaporation of the mother liquor afforded an additional 200 mg of **5** (total 1.26 g, 66%): mp >200 °C dec; pK_a[12] (66% $HCON(CH_3)_2$ 4.3, 11.3; $((CH_3)_2SO)$ 15.0; UV max (0.1 N HCl) 266 nm (ε 12950); UV max (0.05 N Na_2PO_4 buffer) 262 nm (ε 13050); UV max (0.1 N NaOH) 271 nm (ε 12250); ^1H NMR ($(CD_3)_2SO$) δ 0.93 (t, 3, J = 7 Hz, CH_3), 1.17–1.93 (m, 4, $(CH_2)_2$), 2.67 (t, 2, J = 7 Hz, CH_2), 7.00 (s, 2, NH_2), 8.03 (s, 1, 8-H); MS, *m/e* (rel abundance; 10 eV) 191 (M^+, 13), 176 ($M^+ - CH_3$, 13), 162 ($M^+ - C_2H_5$, 26), 149 ($M^+ - C_3H_6$, 100). Anal. Calcd for $C_9H_{13}N_5$: C, 56.53; H, 6.85; N, 36.62. Found: C, 56.48; H, 6.83; N, 36.65.

4,6-Diamino-5-(pentanoylamino)pyrimidine (7). A mixture of 4,5,6-triaminopyrimidine (**6**) sulfate[13] (8.0 g, 33.2 mmol) and sodium hydroxide (2.65 g, 66.4 mmol) in 150 mL of water (through which nitrogen has been bubbled) was stirred for 1 h, and then the water was removed under reduced pressure. The resulting solid was treated with pentanoic anhydride[14] (18.3 g, 98.1 mmol), and the mixture was heated under nitrogen at 120 °C until formation of products was complete (8 h), as judged by thin-layer chromatographic analysis. The solid was cooled, dissolved in water, and made acidic with 1.5 N hydrochloric acid. The aqueous layer was washed with dichloromethane and neutralized with 2 N sodium hydroxide solution, and the water was removed under reduced pressure. Methanol (500 mL) was added to the residue, the mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting solid was recrystallized from water to give **7** as a white solid, 3.78 g (55%): mp 267 °C dec; ^1H NMR ($(CD_3)_2SO$) δ 0.88 (t, 3, J = 6 Hz, CH_3), 1.07–1.80 (m, 4, $(CH_2)_2$), 2.30 (t, 2, J = 7 Hz, CH_2), 5.73 (br, 4, NH_2), 7.70 (s, 1, 2-H), 8.47 (br, 1, NH); MS, *m/e* (rel abundance; 10 eV) 209 (M^+, 100), 167 ($M^+ - C_3H_6$, 14), 125 (38). Anal. Calcd for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.47. Found: C, 51.60; H, 7.28; N, 33.70.

8-Butyladenine (8). A suspension of **7** (3.87 g, 18.5 mmol) and *p*-toluenesulfonic acid monohydrate (50 mg, 0.3 mmol) in diphenyl ether (100 mL) was heated under nitrogen at 220 °C until formation of the products was complete (5 h), as judged by thin-layer chromatographic analysis. The mixture was cooled, mixed with diethyl ether (100 mL), and filtered. The filtrate was evaporated under reduced pressure to give **8** (3.31 g, 95%) as a white solid after sublimation (210 °C, 0.05 mm): mp 255 °C (recrystallized from water); pK_a[12] (66% $HCON(CH_3)_2$) 3.8, 11.7; $((CH_3)_2SO)$ 15.3; UV max (0.1 N HCl) 266 nm (ε 15400); UV max (0.05 N Na_2HPO_4 buffer) 263 nm (ε 15500); UV max (0.1 N NaOH) 271 nm (ε 14900); ^1H NMR ($(CD_3)_2SO$) δ 0.90 (t, 3, J = 7 Hz, CH_3), 1.07–1.90 (m, 4 $(CH_2)_2$), 2.73 (t, 2, J = 7 Hz, CH_2), 7.20 (br, 2, NH_2), 8.03 (s, 1, 8-H), 12.50 (br, 1 NH); MS, *m/e* (rel abundance;

Table I. Nitrogen-15 Chemical Shifts of Adenine and Derivatives in Dimethyl Sulfoxide (ppm Upfield from External $D^{15}NO_3$)

compound	N1	N3	N7	N9	N6'
adenine (natural-abundance)	140	145	149	205	296
adenine (^{15}N labeled)		144.9[a]	148.1[b]	206.3[c]	
2-butyladenine	143.5	149.8	148.8	208.2	297.8
8-butyladenine	142.6	146.9	152.7	207.3	296.1
adenosine	138.6	151.8	133.8	205.0	292.5
N^6-benzyladenosine	144.6	153.2	135.9	205.6	286.3
9-ethyladenine	140.8	150.2	136.0	210.0	294.9
7-ethyladenine	138.6	132.3	216.2	131.4	297.0
N^6,N^6-dimethyladenine	141.5	150.1	132.4	217.3	300.3
N^6,N^6-diethyladenine	142.6	150.6	130.3	218.8	271.5

[a] From the spectrum of [3-^{15}N]adenine. [b] From the spectrum of [7-^{15}N]adenine. [c] From the spectrum of [9-^{15}N]adenine.

Table II. Nitrogen-15 Chemical Shifts of Specifically Labeled Adenines in Neutral Water (ppm Upfield from External $D^{15}NO_3$)

	N1	N3	N7	N9	N6'
adenine	151.1[a] (150.3)[f]	152.0[b] (153.3)[f]	160.6[c] (160.3)[f]	203.7[d] (204.2)[f]	297.8[e]
$^2J_{^{15}N-H}$, Hz	14.6	13.2	11.7	10.2	

[a] [1-^{15}N]Adenine. [b] [3-^{15}N]Adenine. [c] [7-^{15}N]Adenine. [d] [9-^{15}N]Adenine. [e] [6'-^{15}N]Adenine. [f] Parenthetical values are those calculated from the fits to the experimental points in Figure 6.

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(12) Cf. pK_a of adenine (66% $HCON(CH_3)_2$) 3.7, 10.9; $((CH_3)_2SO)$ 14.2. The pK_a values in 66% $HCON(CH_3)_2$–34% H_2O were obtained through the courtesy of Dr. R. F. Eizember, Lilly Reserach Laboratories, Eli Lilly and Co., Indianapolis, IN and those in $(CH_3)_2SO$, courtesy of Prof. F. G. Bordwell, Department of Chemistry, Northwestern University, Evanston, IL.
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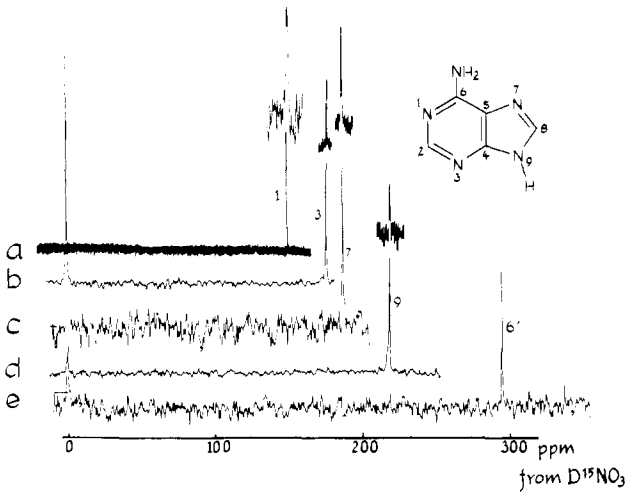


Figure 1. Comparison of resonances in ^{15}N NMR proton-decoupled and coupled (above) spectra of specifically labeled adenines in water. The strong peak on the far left is of $D^{15}NO_3$ in an external capillary: (a) [1-^{15}N]adenine, (b) [3-^{15}N]adenine, (c) [7-^{15}N]adenine, (d) [9-^{15}N]-adenine, (e) [6′-^{15}N]adenine.

10 eV) 191 (M^+, 35), 163 (10), 162 (9), 150 (16), 149 ($M^+ - C_3H_6$, 53). Anal. Calcd for $C_9H_{13}N_5$: C, 56.53; H, 6.85; N, 36.62. Found: C, 56.45; H, 6.65; N, 36.39.

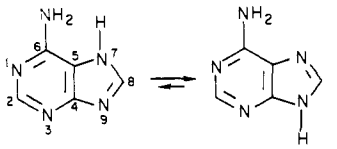
Results and Discussion

The nitrogen-15 chemical shifts obtained for adenine and eight of its derivatives are listed in Table I. The values for adenine were obtained at the natural-abundance level in dimethyl sulfoxide over an 18-h period while maintaining the sample at about 50 °C. The assignments of the chemical shifts for adenine could be established unequivocally from adenine labeled specifically at N1, N3, N7, N9, and N6′ (the NH_2) with 95% ^{15}N.[9] The nitrogen shifts for adenine in water solution were also measured for the labeled materials, as shown in Table II (see Figure 1).

The nitrogens of the imidazole ring of adenine are either "pyrrole-like" or "azine- or pyridine-like". It is known[2,15,16] that "pyrrole-like" nitrogens in aromatic rings come into resonance at much higher magnetic fields than do "pyridine-like" nitrogens. Because N9 is 56 ppm upfield from N7 in dimethyl sulfoxide and 43 ppm upfield in water, the tautomeric equilibrium for adenine, in both solvents, favors the proton residing on N9 (**9b**). The smaller chemical shift difference between N7 and N9 for adenine in water compared with that between N7 and N9 in dimethyl sulfoxide might indicate that the equilibrium in water is substantially more favorable for the N7-H tautomer than it is in dimethyl sulfoxide. This conclusion requires qualification because, as has been shown by comparison of adenosine and adenosine phosphates[2] where there is no complication from tautomeric equilibria, the solvent change from dimethyl sulfoxide to water causes the azine nitrogen resonance to move 10 ppm upfield and the N–R resonance to move 2.0 ppm upfield. A simple way to estimate K for the N7-H $\rightleftarrows$ N9-H equilibrium for adenine in dimethyl sulfoxide is to compare the N7 and N9 shifts with those of 7- and 9-ethyladenine in the same solvent (Table I). The problem with this is that the N7 and N9 resonance give disparate answers because account must be taken of the effect of alkyl substitution on the N–H resonances. One way to do this is to assume that analysis of the observed N7 and N9 shifts of adenine should give the same percentages of N7-H and N9-H tautomers. Furthermore, it is assumed that the effect on an NH(7) resonance by converting it to an N7-R resonance should be comparable to the corresponding change for N9. Call the shift change effect x ppm. Then, for Me_2SO_4 solutions, the percent of N7-H = [obsd N7 shift − (shift N7-Et + x)]/[(shift N7-Et + x) − shift N7] × 100, where shift N7 is the resonance position of N7 for 9-ethyladenine. The corresponding equation for the percent of N7-H calculated for the N9 shifts is [(shift N9-Et + x) − obsd N9 shift]/[(shift N9-Et + x) − shift N9] × 100, where shift N9 is the resonance position of N9 for 7-ethyladenine. Combination of these equations gives x = 8 ppm and 13.5% of the N7-H tautomer of adenine in dimethyl sulfoxide solution. The pattern of alkyl substitution effects on ^{15}N resonances is erratic[15,16] but is generally downfield. Certainly, 8 ppm is a very reasonable value to correct for alkyl substitution. The percentage of N7-H tautomer determined from the ^{15}N chemical shifts agrees well with the values of 15% from ^{13}C chemical shifts[17] and 19% from $^{13}C,H$ spin–spin couplings.[18]

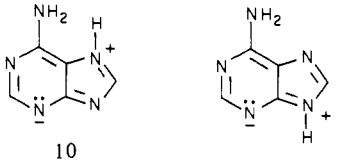
For water solutions, the situation is more complex because the 7- and 9-ethyladenines are too insoluble to have their ^{15}N spectra taken in water. However, a rough calculation is possible by virtue of the fact that the shifts of N7 and N9 of either AMP or ATP in neutral water are 9 ppm downfield and 7 ppm upfield, respectively, of the corresponding nitrogens of 9-ethyladenine in dimethyl sulfoxide solution. If the same pattern is assumed for the shifts of the isomer of AMP or ATP with the ribosyl group attached to N7, then, for this isomer, we expect shift N7-R $\simeq$ 209 ppm and shift N9 $\simeq$ 140 ppm. Calculation analogous to that for adenine in dimethyl sulfoxide solution yields x = 13 ppm and N7-H = 20%. That x = 13 ppm compared to 8 ppm for the ethyladenines is very reasonable is reflected by the 5-ppm difference between the N9 shifts of adenosine and 9-ethyladenine in dimethyl sulfoxide solution (Table I). Considering the approximate nature of the calculations, we do not regard the small apparent solvent effect on the tautomeric equilibrium to be worth detailed speculation, although there is good agreement here with the 22% of N7-H tautomer determined by T-jump relaxation for adenine in water solution.[19] In any case, the free energy difference between the forms is on the order of 1 kcal.

The question as to the position of equilibrium between **9a** and **9b** has received much theoretical and experimental attention, and the result obtained here by way of ^{15}N NMR is in harmony with the generally accepted predominance of the N9-H tautomer (**9b**).[20]



9a **9b**

As with other purines,[21] it seems likely that an important effect in stabilizing the N9-H over the N7-H tautomer is N3 vs. N9 electron-pair repulsions in the latter form. The N9-H tautomer might also be stabilized relative to the N7-H tautomer by virtue of the fact that the electron-attracting nitrogen N3 has a less electron-attracting nitrogen two σ bonds away in the N9-H form than is the case for the N7-H form. A related consideration is that the resonance form **10** of **9a** has positive and negative charges



10 **11**

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Table III. Dependence of ^{15}N Shifts of Adenosine on Trifluoroacetic Acid Concentration in Dimethyl Sulfoxide (ppm Upfield from External $D^{15}NO_3$)

molar equiv acid	N1	N3	N7	N9	N6'
0	138.6	151.8	133.8	205.0	292.5
0.25	162.7	152.7	133.7	203.3	290.6
0.50	181.9	151.6	132.9	201.2	288.5
0.75	198.4	151.6	132.2	199.4	286.5
0.90	206.9	150.8	131.5	198.1	285.3
1.0	209.9	150.8	129.6	198.0	284.7

Table IV. Dependence of ^{15}N Shifts of 9-Ethyladenine on Trifluoroacetic Acid Concentration in Dimethyl Sulfoxide (ppm Upfield from External $D^{15}NO_3$)

molar equiv acid	N1	N3	N7	N9	N6'
0	140.8	150.2	136.0	210.0	294.9
0.33	180	150.2	135	206	291
0.66	214	150.3	135.5	203	286
1.33	218	150.2	133.5	202.4	285.5

farther apart than does resonance form **11** of **9b**. Specific solvent interactions are probably not important in view of the reported dominance of **9b** in the gas phase.[20]

The chemical shifts of 2-butyladenine (**5**) and 8-butyladenine (**8**) were assigned to specific nitrogens by comparison with the ^{15}N shifts of adenine or through the nitrogen–hydrogen coupling constants. The N7 resonance in 2-butyladenine could be distinguished from N1 and N3 on the basis of the longer relaxation times observed for nitrogen atoms in the imidazole ring of adenine. The N1 and N3 resonances in 8-butyladenine were assigned on the basis of their nitrogen–hydrogen coupling constants of 18 and 15 Hz, respectively. This assignment is in agreement with the ^1H spectra of ^{15}N-labeled adenine derivatives in dimethyl sulfoxide, which have shown that two-bond nitrogen–hydrogen coupling constants are about 16 Hz when the nitrogen atoms are in the pyrimidine ring and about 10 Hz when in the imidazole ring.[7] However, the difference between these coupling constants is less in D_2O, pH ~8.0,[9] and therefore not always clearly diagnostic as to which ring is involved.

The ^{15}N NMR spectra of N^6,N^6-diethyladenine and N^6,N^6-dimethyladenine gave chemical shifts for N7 downfield from those of N1 and N3; in fact, quite like those for adenines with alkyl or sugar subtituents on the N9 nitrogen. These shifts suggest that with *N*,*N*-dialkyl substituents, the tautomeric equilibrium is shifted far toward the N9-H tautomer. One possible explanation for the change in the position of the equilibrium is steric hindrance between an alkyl group on the N6′ nitrogen and an N7-H proton.

The nitrogen resonances for N^6-benzyladenosine and 9-ethyladenine were assigned by comparison with the assignments for adenosine.[2] The ^{15}N NMR spectrum of 7-ethyladenine shows three closely grouped signals at the lower field end. The signal at 131.4 ppm is assigned to N9 because of its nitrogen–hydrogen coupling-constant value ($^2J_{NH}$ = 11.3 Hz).[6] The signals of 138.6 and 132.3 ppm, which are assigned to N1 and N3, respectively, agree with what is expected for the previously mentioned population averaging between adenine tautomers **9a** and **9b** and also with the chemical shifts of 7-ethyl- and 9-ethyl-6-chloropurine.[22]

The protonation sites of the nitrogens in nucleic acid bases, nucleosides, and other analogous compounds are important in connection with their biological activities.[3] Previous ^{15}N studies have shown that protonation occurs only on N1 in adenosine and adenosine phosphates,[2,3] N1 of vitamin B_1 (which, it should be noted, corresponds to N3 of adenosine),[23] and on N7 of the imidazole ring in guanosine and guanosine phosphates.[2,3] Such determinations of protonation sites are easily made by ^{15}N NMR

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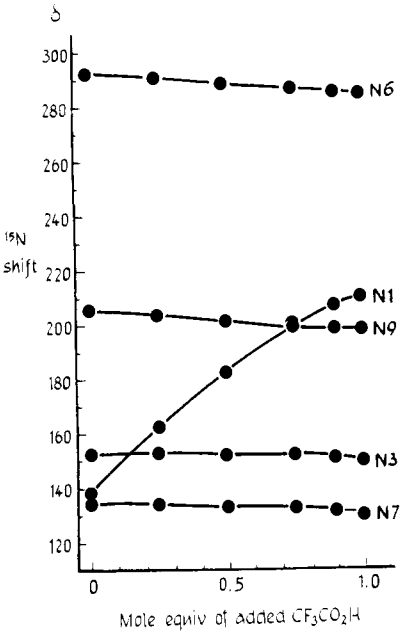

Figure 2. Dependence of the ^{15}N shifts of adenosine on trifluoroacetic acid concentration in $(CH_3)_2SO$ solution.

Table V. Influence of 3 Molar Equiv Trifluoroacetic Acid on ^{15}N Chemical Shifts of Adenine Derivatives (ppm Upfield from External $D^{15}NO_3$) in Dimethyl Sulfoxide

compound	molar equiv acid	N1	N3	N7	N9	N6'
N^6-benzyladenosine	0	144.6	153.2	135.9	205.6	286.3
	3	221.8	157.4	139.2	199.2	275.5
N^6,N^6-diethyl-adenine	0	142.6	150.6	130.3	218.8	271.5
	3	176.5	190.7	148.6	198.7	252.5
N^6,N^6-dimethyl-adenine	0	141.5	150.1	132.4	217.3	300.3
	3	176.4	189.6	157.2	191.1	280.1
adenine	0	140	145	149	205	296
	1.0	187.8	164.3	147.0	193.3	276
	1.0		161.0[a]			

[a] From the spectrum of [3-^{15}N]adenine.

Table VI. Dependence of ^{15}N Shifts of 7-Ethyladenine on Trifluoroacetic Acid Concentration in Dimethyl Sulfoxide (ppm Upfield from External $D^{15}NO_3$)

molar equiv acid	N11	N3	N7	N9	N6'
0	138.6	132.3	216.2	131.4	297.0
0.14	147.9	147.2	214.1	132.6	294.1
0.27	156.6	149.5	213.3	135.7	290.0
0.70	180.0	161.8	208.4	140.2	278.1
1.52	192.4	162.2	207.9	141.3	276.2
2.7	193.5	161.5	207.9	142.4	276.6
3.1	196.2	161.2	207.6	142.6	276.9

because it is well-known[2,14] that protonated nitrogens in pyridine and purine rings come into resonance at fields 50–100 ppm higher than for the corresponding unprotonated nitrogens. The shift changes measured on protonation of adenosine and 9-ethyladenine are given in Tables III and IV, respectively. The results in Table III agree with those reported previously[2] which indicate that adenosine protonates mainly at N1 in dimethyl sulfoxide because as the amount of added trifluoroacetic acid reaches 1 molar equiv, the N1 resonance has shifted upfield by 71.3 ppm, while the resonances of N7, N9, and N6′ move slightly downfield and that of N3 remains essentially unchanged (see Figure 2).

Like adenosine, 9-ethyladenine protonates predominantly at N1, with an upfield shift of 77.2 ppm. Once again the resonances

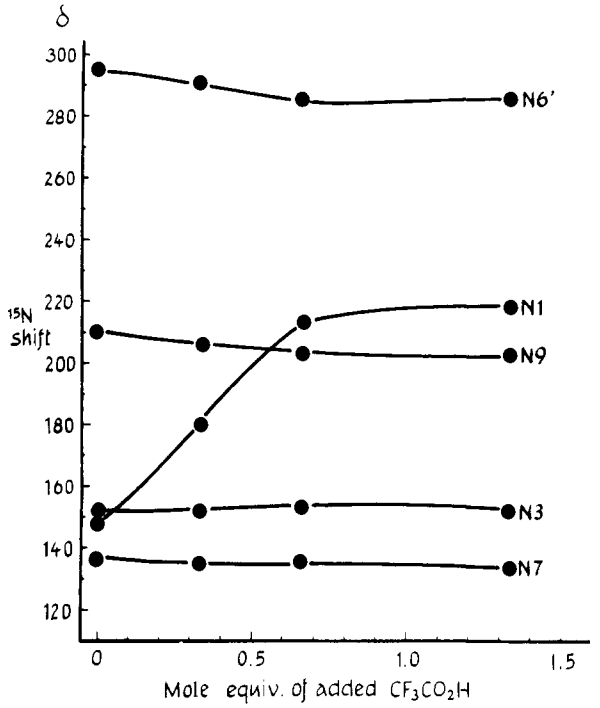

Figure 3. Dependence of the ^{15}N shifts of 9-ethyladenine on trifluoroacetic acid concentration in $(CH_3)_2SO$ solution.

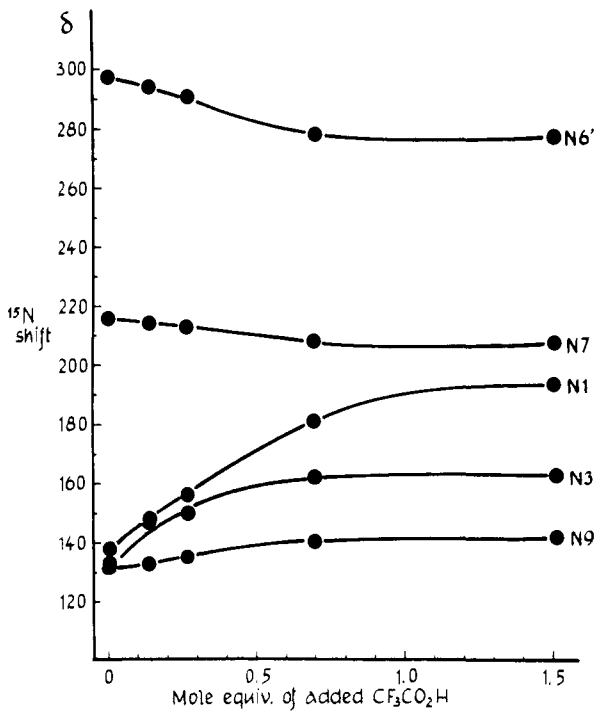

Figure 4. Dependence of the ^{15}N shifts of 7-ethyladenine on trifluoroacetic acid concentration in $(CH_3)_2SO$ solution.

of N7, N9, and N6′ move downfield while that of N3 remains substantially unchanged (see Figure 3).

Substitution on the N6′ nitrogen atom of adenosine has, at most, a small effect on the protonation site. Thus N^6-benzyladenosine protonates predominantly at N1, and the nitrogen resonance responds with an upfield shift of 77.2 ppm. In contrast to adenosine, the N3 and N7 nitrogen resonances for *N*-6′-benzyladenosine move slightly upfield (~4 ppm) while those for N9 and N6′ shift downfield (Table V). This suggests that N3 and N7 protonate sufficiently to give a small upfield shift effect that more than cancels the expected downfield effect resulting from protonation on N1.

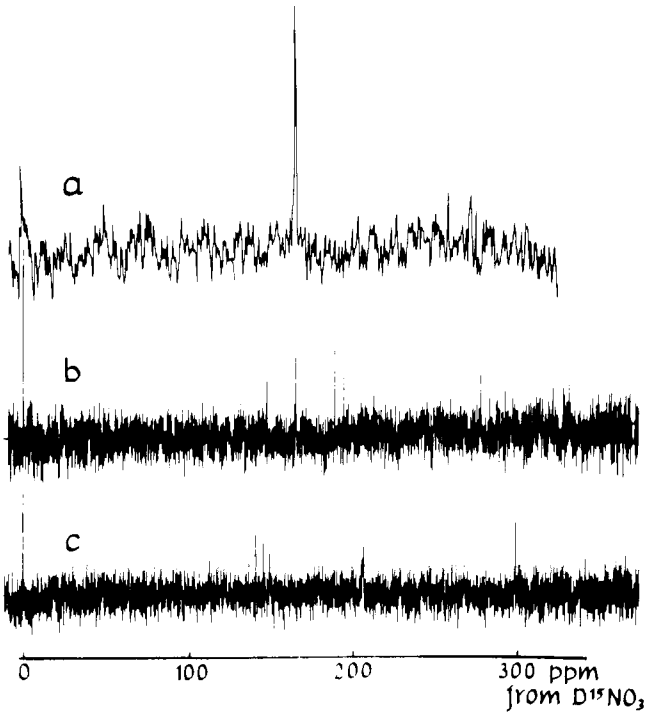

Figure 5. ^{15}N NMR spectra of adenine in dimethyl sulfoxide with gated proton decoupling (no NOE): (a) [3-^{15}N]adenine with 1 molar equiv CF_3CO_2H, (b) natural-abundance spectrum with 1 molar equiv CF_3CO_2H, (c) natural-abundance spectrum with no CF_3CO_2H.

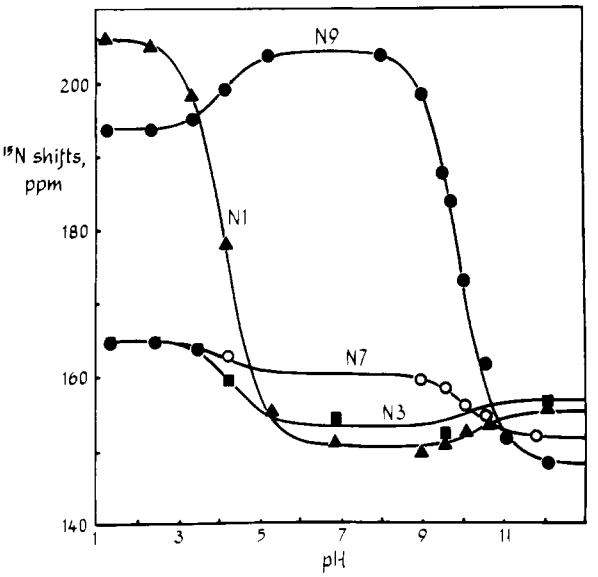

Figure 6. Changes in the ^{15}N resonance positions of adenine in aqueous solution with pH. The lines connecting the points were calculated by an iterative procedure involving finding the best nonlinear least-squares fit to the experimental points as a function of the pK_a values of adenine and the shifts of the nitrogens at the isoelectric point. The pK_a values derived from the curves were 4.2 ± 0.1 and 10.1 ± 0.2. The shifts derived for the different nitrogens at the isoelectric pH are given in Table II.

With 7-ethyladenine, there is compelling evidence for more than one protonation site (Table VI). When 1.5 molar equiv of trifluoroacetic acid was added, the nitrogen resonances of N1, N3, and N9 all moved upfield by 53.8, 29.9, and 9.9 ppm, respectively, while the N7 and N6′ resonances moved downfield by 8.3 and 20.8 ppm, respectively (see Figure 4). These results are quite decisive in showing that the N7-R forms have a rather enhanced basicity of the N3 nitrogen compared with N9-R forms.

Similar behavior is also observed for adenine itself, which was found to protonate in dimethyl sulfoxide at both N1 and N3 to give upfield shifts of 47.8 and 19.3, respectively. Here, the res-

onances of N7, N9, and N6′ shifted downfield by 2, 11.7, and 10 ppm, respectively. The N3 and N1 resonances were identified by protonation of adenine specifically labeled at N3 (see Figure 5). On protonation of adenine labeled at N3 with ^{15}N, the N3 shift changed to 161.0 ppm. This allows the 164.3 ppm resonance to be assigned to N3 in the natural-abundance spectrum of the protonated form. The discrepancy of chemical shifts for N3 in these samples of 3.3 ppm can be reasonably attributed to the large differences in the concentrations.

Protonation of N^6,N^6-diethyladenine and N^6,N^6-dimethyladenine was found to occur principally on N1, N3, and N7, all resonances of which moved upfield, while those of N9 and N6′ shifted downfield. The degree of protonation decreased in the order N3, N1, and N7. Apparently, substitution on the N6′ nitrogen by *N*,*N*-alkyl groups has considerable influence on the preferred site of protonation. The predominance of protonation at N3 over N1 may be the result of the steric interactions between *N*,*N*-dialkyl group and the proton attached to N1.

The behavior of the ^{15}N resonances of adenine in aqueous solution (Figure 6) is worthy of additional comment. On the acid side, the pattern of protonation shifts is generally similar to that observed for dimethyl sulfoxide with upfield shifts of N1 and N3 of 51 and 11 ppm, respectively, and a downfield shift of N9 of 10.5 ppm, the resonance of N7 moving slightly upfield rather than slightly downfield.

Comparison of these results with those for adenosine phosphates[2] indicates some degree of protonation of N3 and N7 as well as of N1. Regardless of the partial protonation of N7, which causes the N9 resonance to move somewhat downfield, there can be no question that in acid solution, as in neutral solution, the N9-H tautomer predominates.

In basic solution, the N9 resonance of adenine undergoes a dramatic downfield shift of 56 ppm on formation of the corresponding anion. Curiously, at about pH 11, all of the ring-nitrogen resonances fall within a 5 ppm range. Large downfield shifts are customary on removal of a σ-bonded proton attached to an aromatic system through enhancement of the second-order paramagnetic effect associated with the presence of σ unshared pairs,[15] and it is interesting that the downfield shift increment on removal of the N9-H proton is comparable to that which occurs on the removal of the proton from N1 of adenine's conjugate acid. A downfield ^{15}N shift has also been reported for the ionization of imidazole in basic solution.[24]

Registry No. 1, 4059-12-5; **2**, 84133-05-1; **3**, 84133-06-2; **4**, 84133-07-3; **5**, 84133-08-4; **6**, 118-70-7; **7**, 84133-09-5; **8**, 84133-10-8; trimethyl orthopentanoate, 13820-09-2; pentanoic anhydride, 2082-59-9; adenine, 73-24-5; adenosine, 58-61-7; N^6-benzyladenosine, 4294-16-0; 9-ethyladenine, 2715-68-6; 7-ethyladenine, 24309-36-2; N^6,N^6-dimethyladenine, 938-55-6; N^6,N^6-diethyladenine, 6284-24-8; [1-^{15}N]adenine, 79364-53-7; [3-^{15}N]adenine, 79364-54-8; [7-^{15}N]adenine, 79364-55-9; [9-^{15}N]-adenine, 56777-22-1; [6′-^{15}N]adenine, 19713-11-2; ^{15}N, 14390-96-6.

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Estimation of the Energy Barrier to Syn–Anti Interconversion in a Pyrimidine Nucleotide: Ultrasonic Investigation of Syn–Anti Glycosyl Isomerization in Cytidine Cyclic 2′,3′-Monophosphate with Bound Ethidium Bromide[1]

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Abstract: Ultrasonic relaxation studies were performed on aqueous solutions of cytidine cyclic 2′,3′-monophosphate in the absence and presence of ethidium bromide in the 15–95 MHz frequency range. The relaxation observed in the presence of ethidium bromide (but not in its absence or in aqueous solutions of cytosine–ethidium bromide) could be assigned to a unimolecular conformational isomerization. Consistent with earlier results, this relaxation could be assigned to the syn–anti glycosyl isomerization process. The findings strongly suggest that the barrier to syn–anti interconversion is diminished in cytidine cyclic 2′,3′-monophosphate upon addition of ethidium bromide and that even in the absence of this dye the barrier cannot be very large, although it is larger than in purine nucleotides. The heterostacking constant between the nucleotide and the dye was determined to be 100 M^{-1} according to both ultrasonic and ^1H NMR methods.

Ultrasonic relaxation measurements have been demonstrated to be capable of monitoring the glycosyl syn–anti conformational equilibrium in nucleosides[4,5] and in a mononucleotide: adenosine cyclic 3′,5′-monophosphate (cyclic AMP).[6,7] It was demonstrated that stacking and the glycosyl conformational equilibrium are interrelated[7,8] and self-stacking can lead to an increase of the barrier to glycosyl interconversion.[7] On the other hand, heterostacking of 2′-deoxyadenosine to 3-indoleacetic acid or to ethidium bromide surprisingly led to a decrease in the conformational barrier.[9] To date most of the research was performed on purine

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