Proton Nuclear Magnetic Resonance Study of Hindered Internal Rotation of the Dimethylamino Group of N^6,N^6-Dimethyladenine Hydrochloride in Aqueous Solution[1]

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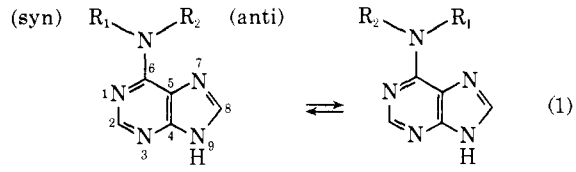
Abstract: Rates of rotational isomerism of methyl groups of N^6,N^6-dimethyladenine (diMe^{6}A) in D_2O were measured by computer simulation of coalescing methyl proton resonances (90 MHz). At DCl concentrations between 0 and 13.4 *M* three species of diMe^{6}A were detected: neutral diMe^{6}A, diMe^{6}A deuterated on N(1) [(diMe^{6}A)D$^+$)], and diMe^{6}A deuterated on N(1) and N(7) [(diMe^{6}A)D_2^{2+}]. Rates of hindered internal rotation vary in the order neutral diMe^{6}A > (diMe^{6}A)D$^+$ > (diMe^{6}A)D_2^{2+}. Isomerism of neutral diMe^{6}A is too rapid to measure by the line-shape technique under these experimental conditions. Measurable rates occur in the region of N(7) titration ($pK_a = -1.2$; 1.36–13.4 *M* DCl). In this range the rotational rate is proportional to the concentration of (diMe^{6}A)D$^+$. Activation parameters are E_a = 13.2 kcal/mol, log *A* = 11.4, $\Delta G^\ddagger$ = 15.0 kcal/mol, $\Delta H^\ddagger$ = 12.6 kcal/mol, and $\Delta S^\ddagger = -8.0$ eu (28°). It is demonstrated that rates too slow to measure by the line-shape method can be determined from the methyl proton spin-lattice relaxation time (T_1) and the extent of saturation transferred to a methyl resonance when the other methyl peak is saturated.

Hindered internal rotation resulting from partial double bond character of the C(6)–N(6) bond of N(6)-substituted adenines yields two rotational isomers shown in eq 1. Such



(1)

syn–anti [relative to N(1)] isomerism may influence the conformation and activity of biomolecules containing these modified bases. Adenine derivatives alkylated on N(6) occur as minor constituents of nucleic acids[2-4] and as components of antibiotics such as puromycin.[5] N^6-Methyladenine plays a key role in modification–restriction processes which regulate the preferrential scission of exogenous DNA in certain bacteria.[6-9] Interest in rotational isomerization of these substituted adenines is heightened by X-ray data showing that a series of seven adenine derivatives, monosubstituted on N(6), all crystallize with the substituent exclusively in the syn orientation.[10-15] Retention of such a conformation by N^6-alkylated adenines in physiological systems would block the formation of Watson–Crick base pairs. Watson–Crick base pairing has, however, been reported in the 1:1 helical complex of poly-U with N^6-monomethylated poly-A, but this complex is markedly destabilized by the presence of the methyl groups.[16,17] Further evaluation of the biological significance of such rotational isomerism of adenine derivatives requires experimental data on the dynamic and equilibrium states of these molecules in aqueous solution. Nmr evidence for hindered rotation about the exocyclic C–N bond has been presented for cytosine[18-23] and adenine[19,23,24] derivatives with methyl substituents on the amine nitrogen; however, these studies were conducted in nonaqueous solvents. Here we present total line-shape comparison measurements of rotational rates and rotational energy barrier of diMe^{6}A ($R_1 = R_2 = CH_3$) in D_2O. In addition, it is demonstrated that rotational rates too slow to measure by the line-shape technique can be determined from two parameters: the extent of transfer of saturation between methyl protons, and the methyl proton spin-lattice relaxation time. Even though rates readily measurable by either of these techniques (at 21 KG) were encountered only in very acidic solutions, the rotational energy barrier for (diMe^{6}A)D$^+$, the predominant species in the pD range 1–4, was obtained. Albert and Brown[25] determined the pK_a of (diMe^{6}A)D$^+$ in H_2O to be 3.9. We show that the pK_a of (diMe^{6}A)D_2^{2+} can be determined from displacement of the C_8H proton resonance.

Experimental Section

Commercial samples of 6-dimethylaminopurine (diMe^{6}A, Sigma Chemical Co., St. Louis, Mo.), deuterium chloride (Thompson Packard, Inc., Little Falls, N.J.), and deuterium oxide (Stohler Isotopes, Rutherford, N.J.) were used in these studies. Proton nmr spectra were measured on a Bruker HX-90-18 spectrometer employing an internal standard, tetramethylammonium chloride (TMAC, Eastman Kodak Co., Rochester, N.Y.). The pD (pH meter reading + 0.40) was adjusted with DCl and NaOD. Solutions at pD > 0 contained 1 *M* KCl in order to maintain constant ionic strength. The Hammet H_0 function for DCl (D_0)[26] was obtained from data for HCl[27] assuming $D_0 = H_0$. D_0 has not yet been measured for high concentrations of DCl, but data indicate that $D_0 = H_0$ for DCl and HCl concentrations up to 1 *M*.[28] Also, $D_0 = H_0$ for concentrated D_2SO_4 and H_2SO_4 (0.6–12 *M*).[28]

Results

The theoretical basis for measurement of chemical exchange rates from the shape of nmr spectra[29,30] and the application of this technique to the measurement of rates of hindered internal rotation of a broad range of molecules[30-34] have been extensively reviewed. Theory predicts that the magnetically distinct methyl groups yield two well-resolved and sharp resonances when the rate of rotation about the C(6)–N(6) bond of diMe^{6}A is slow on the nmr time scale. As the rate of internal rotation becomes increasingly rapid, these resonances first broaden and coalesce and then sharpen again. In the intermediate range of broadened nmr spectra, the mean lifetime (τ) of a methyl group in the syn or anti orientation can be determined by fitting experimental spectra to theoretical curves generated by equations derived by Gutowsky and Holm.[35]

Proton nmr spectra of diMe^{6}A in neutral D_2O and in dimethyl sulfoxide solution (both at 28°) exhibited a single sharp methyl resonance of six-proton intensity, indicating rapid internal rotation of the dimethylamino group. Esti-

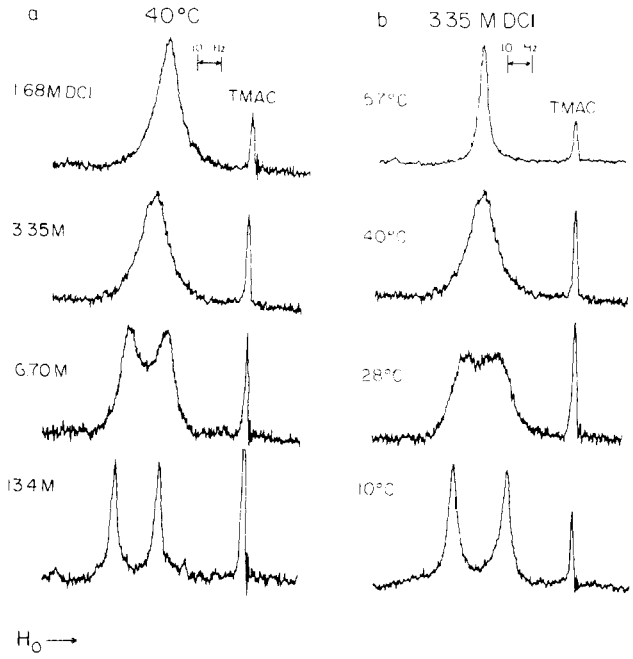


Figure 1. Methyl proton resonances of 0.15 *M* diMe^{6}A in D_2O (a) at 40° in various concentrations of DCl and (b) in 3.35 *M* DCl at various temperatures.

mates of τ could not be obtained from these spectra by the line-shape technique. A gradual transition to spectra characteristic of intermediate rotational rates occurs at progressively higher concentrations of DCl (Figure 1a). τ varies between 7 and 140 msec as the DCl concentration increases from 1.67 to 13.4 *M*.

For each acid concentration rates of internal rotation ($1/\tau$) were determined. At various temperatures Figure 1b shows representative spectra of diMe^{6}A in 3.35 *M* DCl. Table I indicates that at each acid concentration rates of internal rotation are more rapid at higher temperatures.

Titration curves showing the dependence of the C_2H and C_8H chemical shifts on acidity appear in Figure 2. D_0 replaces pD at DCl concentrations greater than 1 *M*.[26] Above pD 3 the C_2H and C_8H chemical shifts depend not only on titration of N(1) but also on stacking interactions of the heterocyclic ring system. However, stacking interactions are expected to be negligible between charged species such as (diMe^{6}A)D$^+$ and (diMe^{6}A)D_2^{2+}.[36] Therefore, the second dissociation constant, pK_a', can be obtained in highly acidic media. Equation 2[26] describes the relationship between D_0

$$D_0 = pK_a' - \log ([(diMe^6A)D_2^{2+}]/[(diMe^6A)D^+]) \quad (2)$$

and concentrations of (diMe^{6}A)D$^+$ and (diMe^{6}A)D_2^{2+}. The pK_a' (−1.2) was determined from D_0 at half displacement of the C_8H resonance (Figure 2), since eq 1 indicates that $D_0 = pK_a'$ when [(diMe^{6}A)D_2^{2+} = (diMe^{6}A)D$^+$].

Titration curves were obtained at each temperature. Values of *f*, the mole fraction of (diMe^{6}A)D$^+$, are included in Table I. No significant temperature dependence of the pK_a' was observed.

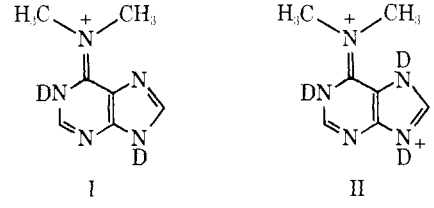
When rotation of the dimethylamino group is too slow to perturb the shape of the methyl resonance, τ can be determined by a transfer of saturation experiment, provided that τ is comparable to T_1, the spin-lattice relaxation time.[37-40] Under these conditions saturation of one of the diMe^{6}A methyl groups will result in partial saturation of the other. In Figure 3, α and β denote the lower and upper field methyl peak, respectively. Equation 3 governs this effect[41]

$$(M_0^\alpha - M_z^\alpha)/M_0^\alpha = T_1/(T_1 + \tau) \quad (3)$$

where M_z^α and M_0^α are, respectively, the intensities of the α peak with and without application of saturating radiofrequency power to the β peak. The left-hand side of eq 3, the fractional decrease in the intensity of the observed α peak as a result of saturation transfer, was 0.54 in 13.4 *M* DCl at 28°. M_0^α was measured by applying a strong radiofrequency off-resonance, 21 Hz to low field of the α peak (Figure 3a). Saturation of the β peak (21 Hz to high field of the α resonance) yielded M_z^α (Figure 3b). T_1, determined by the inversion recovery technique,[42,43] is 0.73 sec. The calculated value of τ is 0.62 sec.

Discussion

Rates of internal rotation of diMe^{6}A in neutral D_2O and in dimethyl sulfoxide solution were too rapid to measure by techniques employed in this study. The decrease in isomerization rate in more acidic media may originate in part from contributions of resonance forms such as I and II



which would augment the double bond character of the C(6)–N(6) bond of (diMe^{6}A)D$^+$ and (diMe^{6}A)D_2^{2+}, respectively. In addition, examination of space-filling models suggests that steric hindrance between the anti-methyl and the deuteron on N(7) may further restrict rotation of methyl groups.

In the acidity range in which readily measurable rates of isomerization of diMe^{6}A occur, the kinetics of rotation can be described by the following scheme

$$(diMe^6A)D_2^{2+} \underset{k_{12}}{\overset{k_{21}}{\rightleftharpoons}} (diMe^6A)D^+ + D^+ \quad (4)$$

k_2 ↘ rotation ↙ k_1

Table I. Summary of Rate and Titration Data

	DCl concentration[a]							
	1.68 *M*		3.35 *M*		6.71 *M*		13.4 *M*	
T, °C	$1/\tau$, sec^{-1}	*f*[b]	$1/\tau$, sec^{-1}	*f*	$1/\tau$, sec^{-1}	*f*	$1/\tau$, sec^{-1}	*f*
10	16.7	0.804	11.1	0.429	4.00	0.125		0.018
28	69.0	0.811	40	0.432	13.3	0.126	1.61[c]	0.018
40	143	0.822	91	0.430	28.6	0.112	7.14	0.019
57	471	0.811	333	0.443	125	0.104	23.8	0.019
73							83.3	

[a] Uncorrected for titration of diMe^{6}A. [b] Mole fraction of (diMe^{6}A)D$^+$. [c] Measured by transfer of saturation.

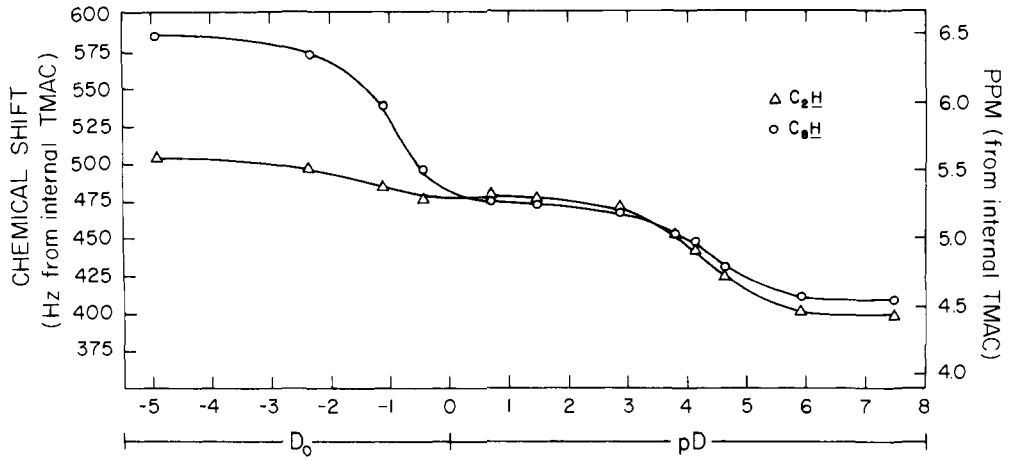


Figure 2. Chemical shifts of C_2H and C_8H resonances of 0.15 *M* diMe^{6}A in D_2O at 28° in media of varying acidity. D_0 was determined from DCl concentration corrected for titration of diMe^{6}A.

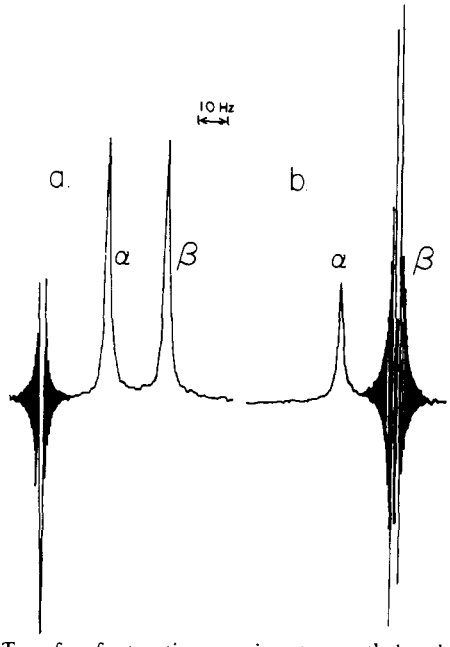


Figure 3. Transfer of saturation experiment on methyl peaks of 0.15 *M* diMe^{6}A in 13.4 *M* DCl at 28°: (a) a strong radiofrequency was applied 21 Hz to low field of the α peak: (b) the radiofrequency was then shifted to coincide with the β peak. 21 Hz to high field of the α peak. The same radiofrequency power was employed in both experiments.

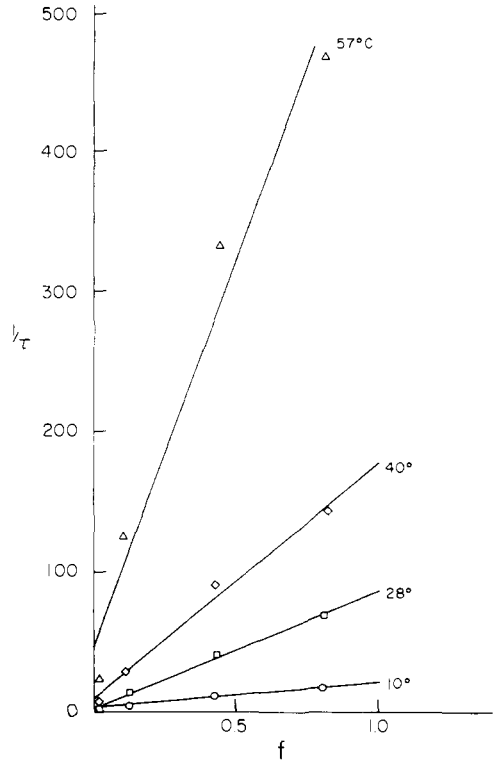


Figure 4. $1/\tau$ as a function of f at various temperatures. Lines are least-squares best fits to eq 5.

where (diMe^{6}A)D_2^{2+} and (diMe^{6}A)D^+ have the same orientation of methyl groups. Isomerization of the mono- and dideuterated species may be treated as unidirectional reactions since the nmr technique is sensitive to the lifetime of a given configuration of methyls. The first-order rate constants for isomerization of (diMe^{6}A)D^+ and (diMe^{6}A)D_2^{2+} are k_1 and k_2, respectively. The decrease in rotational rates at higher acid concentrations (Table I) indicates that $k_1 \gg k_2$.

Single sharp time-averaged C_2H and C_8H peaks indicate that k_{12} and k_{21} are large relative to k_1 and k_2. It can readily be demonstrated that when rotational isomerization is rate limiting

$$1/\tau = k_2 + (k_1 - k_2)f \qquad (5)$$

Figure 4 demonstrates, in agreement with eq 5, that $1/\tau$ varies linearly with f at each temperature. Least-squares values of k_1 and k_2 are summarized in Table II. k_2 is approximately zero within the limits of experimental error. Consequently (diMe^{6}A)D^+ appears to be the only significant species undergoing internal rotation in this acidity range.

The possibility that in strongly acidic solution neutral diMe^{6}A serves as a transition state for internal rotation was also considered but was excluded on the basis of the following observation. Between D_0 −0.54 (1.67 *M* DCl) and pD 1.50 (28°) the broad and coalesced methyl resonances remain essentially unaltered and indicate no appreciable

Table II. Summary of Rate Constants for Internal Rotation of (diMe^{6}A)D^+ (k_1) and (diMe^{6}A)D_2^{2+} (k_2) (±S.D.)

T, °C	k_1, sec^{-1}	k_2, sec^{-1}
10	20.8 ± 3.7	2.2 ± 1.3
28	86.0 ± 4.8	1.8 ± 1.5
40	177 ± 19	9.0 ± 6.1
57	598 ± 102	48.7 ± 32.5

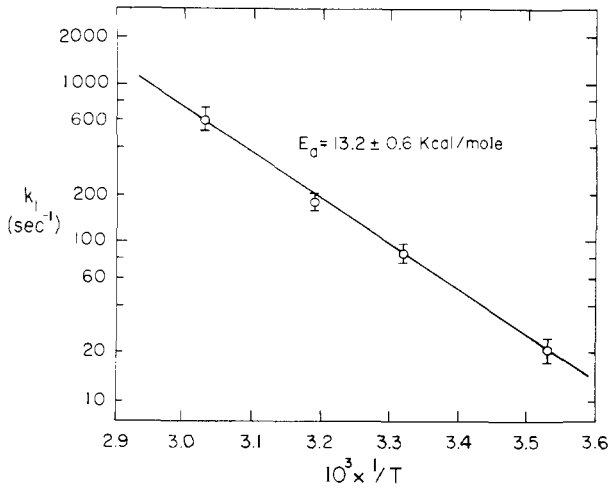


Figure 5. Arrhenius plot of k_1, determined from Figure 4, *vs.* $1/T$. The least-squares best line, E_a, and its standard deviation are shown.

change in the isomerization rate. This is consistent with rotation occurring *via* (diMe^{6}A)D$^+$, whose mole fraction remains about unity over this range of acid concentration. If neutral diMe^{6}A were contributing significantly, a large increase in rotational rate would be anticipated, since the mole fraction of this species increases from *ca.* 2×10^{-5} to *ca.* 2×10^{-3}, between D_0 −0.54 and pD 1.50, respectively [assuming an approximate dissociation constant of 4.2 for (diMe^{6}A)D$^+$; see Figure 2].

The rotational energy barrier of only (diMe^{6}A)D$^+$ could be obtained under these experimental conditions. An Arrhenius plot ($k_1 = Ae^{-E_a/RT}$) is shown in Figure 5, from which values of E_a = 13.2 kcal/mol and log A = 11.4 were obtained by linear least-squares analysis. This activation energy is comparable to barriers obtained for methylated cytosines, 15-18 kcal/mol,[22] and methylated adenines, 11.8[19] and 12.2[24] kcal/mol. Molecular orbital calculations of Berthod and Pullman[44] indicate a barrier of 16 kcal/mol for N(6)-methyladenine. For (diMe^{6}A)D$^+$ in D_2O, the Eyring equation (eq 6) yields values of ΔG^*, ΔH^*, and

$$k_1 = (kT/h)e^{-\Delta G^*/RT} \quad (6)$$

ΔS^*, which are summarized in Table III. ΔG^* is essentially independent of temperature. Hence, the energy barrier is primarily enthalpic; ΔH^* constitutes about 84% of the activation free energy. Comparable negative values of ΔS^* have been reported for other rotational isomerizations.[30]

These studies demonstrate that the rate of syn-anti isomerism increases in the order (diMe^{6}A)D$_2^{2+}$ < (diMe^{6}A)D$^+$ < neutral diMe^{6}A. Definitive conclusions about the physiological role of N(6)-substituted adenines require extension of these measurements to the neutral species and to various other adenine derivatives. Higher magnetic fields should permit measurements of more rapid rates of isomerization. However, to the extent that diMe^{6}A is representative of other N(6)-substituted adenines, these experiments suggest that rapid reorientation of these derivatives about the C(6)–N(6) bond probably occurs under physiological conditions, unless rotation is restricted by factors such as formation of hydrogen bonds.

Table III. Activation Free Energies, Enthalpies, and Entropies of (diMe^{6}A)D$^+$ (±S.D.)

T, °C	ΔG^*, kcal/mol	ΔH^*, kcal/mol	ΔS^*, eu
10	14.8 ± 0.1	12.6 ± 0.6	−7.8 ± 2.5
28	15.0 ± 0.0	12.6 ± 0.6	−8.0 ± 2.2
40	15.1 ± 0.1	12.6 ± 0.6	−8.0 ± 2.2
57	15.2 ± 0.1	12.5 ± 0.6	−8.2 ± 2.1

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References and Notes

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