Tautomerism of Purines. I. N(7)H $\rightleftharpoons$ N(9)H Equilibrium in Adenine[1]

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Abstract: Neutral adenine in water exists in two tautomeric forms, N(7)H and N(9)H adenine. The equilibrium constant, $K = C_{N(7)H}/C_{N(9)H}$, is estimated to be 0.28 at 20°, and the enthalpy of tautomerization is 0.2 kcal/mol. The interconversion between the two forms is catalyzed by H^+ ($k_{H^+} = 1.3 \times 10^{10}$ M^{-1} sec^{-1} at 10°), by OH^- ($k_{OH^-} = 0.85 \times 10^{10}$ M^{-1} sec^{-1}), and by the anion of adenine ($k_{A^-} = 3.5 \times 10^8$ M^{-1} sec^{-1}). Due to the low proportion of the N(7)HN(9)H$^+$ cation in protonated adenine, no acidic autocatalysis is observed.

Numerous works both theoretical[2] and experimental[3] have been devoted to the tautomerism of purine bases. The major importance of this question is linked to the fact that the reactivity of the biological purines will depend on the structure and relative concentration of the various species present in aqueous solutions. Formal structures of the purine molecule as well as theoretical considerations show the existence of several tautomeric forms arising either (a) from prototropy involving an extranuclear atom (e.g., keto–enol and amine–imine tautomerism), or (b) prototropy involving only ring atoms (e.g., the N(1)H, N(3)H, N(7)H, and N(9)H tautomers of purine).

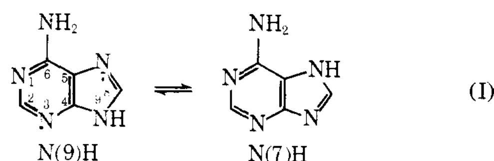
Uv[4] and ir[5] spectra of oxo- and aminopurines and their methylated derivatives *do not provide* any conclusive evidence of amine–imine or keto–enol equilibria and, consequently, suggest that these purines exist mainly in the keto and amino forms (with the exception of 1-methyladenine which consists mainly of the imino tautomer[6]).

On the other hand, tautomerism involving ring atoms only has been clearly observed. To account for the ir spectrum of hypoxanthine Brown and Mason[5] postulated an equilibrium between the N(7)H and N(9)H forms. Pfleiderer[7] proposed that the uv spectrum of guanine in water must be interpreted in terms of a contribution from N(7)H and N(9)H tautomers.

More recently, quantitative studies have led to estimations of the constant for the N(7)H $\rightleftarrows$ N(9)H equilibrium; C^{13} nmr data from concentrated aqueous solutions of purine suggest that both tautomers are present in equal proportions.[8] Fluorescence experiments conclude that adenine in 2-butanol at 170°K consists of 6% N(7)H and 94% N(9)H forms.[9]

Two striking conclusions may be drawn from the examination of the abundant literature: (a) there are few thermodynamic data for the observed tautomeric equilibria N(7)H $\rightleftarrows$ N(9)H for biological purines, (b) quantitative kinetic investigations of the interconversion are totally lacking.

These facts prompted us to approach the question of tautomerism by means of a fast kinetic method. We would now like to report an investigation of N(7)H $\rightleftarrows$ N(9)H tautomerism of adenine in water using the T-jump relaxation technique.[10]



(I)

Experimental Section

Adenine (Biochemicals) and 9-methyladenine (Cyclochemicals) were recrystallized three times from water; 7-methyladenine was prepared according to published procedures[11,12] and purified by successive crystallization from water and absolute ethanol. $NaClO_4 \cdot H_2O$ (Merck reagent grade) was used without further purification. Solutions for the kinetic runs were prepared by dissolving weighted amounts of bases in 0.2 M solutions of $NaClO_4$ in distilled water. The solutions were degassed prior to use.

T-Jump Apparatus. Temperature-jump measurements were performed using a Joule-heated double beam spectrometer (Messanlagen Studiengesellschaft). A special sample cell was designed so as to allow the solution under investigation to circulate by means of a peristaltic pump. Before entering the cell the solution stands in a thermostated glass reservoir whose volume is much larger than that of the sample cell (30 cm^3 vs. 0.4 cm^3). This system ensures rapid homogenization of both pH and temperature throughout the sample solution. The reservoir was designed to permit the immersion in the circulating liquid of a thermometer (temperature t_i) and two Beckman electrodes (G.P. glass electrode No. 39000 and calomel reference electrode No. 39402). The whole system is protected from atmospheric contact; a septum is fitted on the reservoir thus permitting the pH to be varied by injecting small amounts of NaOH or HCl. All connections are made using 2 mm i.d. PTFE or VITON (in the peristaltic pump) tubing. Filling of the system previously purged with argon was made by pumping the solution directly from the flask used for evacuation, thereby allowing minimum exposure to air.

The flow rate on the system is about 80 cm^3/min during the experiments. Blank runs were performed with a thermistance inserted into the T-jump cell and it was checked that the temperature difference between the reservoir and the sample cell is negligible using this flow rate. The replacement of the T-jumped liquid was slow enough not to interfere with the relaxation times which are observed ($\tau < 600$ μsec) and consequently the pumping of the solutions was not stopped during the temperature jump. The initial temperature (t_i) was controlled within ±0.2° by a Lauda U.K. 20 cryostat in the temperature range 0–20° and a Colora thermostat from 20° to 80°.

The temperature following the jump (t_f) was computed from t_i and the temperature jump magnitude, which was estimated as follows: the circulating system was filled with a solution showing a relaxation phenomenon of large amplitude; solutions of 3-hydroxypyridine, which exhibits tautomerism, were found convenient for this purpose. The relaxation amplitude for a given discharging voltage was recorded, and then t_i was rised with the aid of the thermostat so as to produce the same oscilloscope deflection as the relaxation experiment; the corresponding change of t_i was taken as the T-jump magnitude: it was 9 ± 0.5° in all experiments reported here. Jumps of such magnitude were found feasible in the range 0–80° (t_i) when carefully evacuated solutions were used. However, cavitation phenomena cause increasing troubles at the high temperatures.

pH Measuremnts. The pH was measured with a Beckman 1019 research pH meter; during the temperature jump the electrodes were disconnected from the pH meter to avoid damaging its high impedance preamplifier. It was found difficult to obtain reliable pH measurements in cold $NaClO_4$ solutions with a conventional reference electrode, due to partial clogging of fritted junctions by slightly soluble $KClO_4$. This was finally avoided by replacing satu-

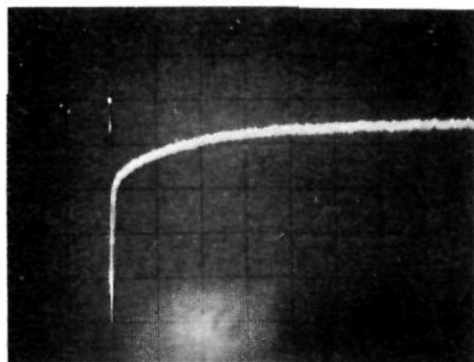


Figure 1. T-Jump relaxation spectrum of adenine in aqueous solution recorded for a wavelength of 280 nm: initial temperature (t_i) = 1°; final temperature (t_f) = 10°; pH (as measured at t_i) = 7.00; adenine concentration = 9.5×10^{-4} *M*. Vertical scale: 5×10^{-3} O.D. unit per major division. Horizontal scale: 200 μsec per major division. The signal corresponds to an increase of the solution O.D.

rated KCl solution by saturated NaCl solution in the calomel electrode. Such a modified electrode gives stable measurements, but there is evidence that its liquid junction potential is not independent of the solution in which it is immersed; thus, the emf of the cell Hg|[Hg_2Cl_2]KCl(sat)|solution X|NaCl(sat)[Hg_2Cl_2]|Hg was found to vary significantly when changing the buffer solution X. In order to estimate the systematic error thus introduced in pH measurements, the following procedure was used: after the pH meter had been standardized in the usual way with Beckman precision phosphate buffer (part No. 566008), we measured the p*K* of several indicators in 0.2 *M* $NaClO_4$ solutions; the circulating system was employed for this purpose, the T-jump detection being used as a spectrophotometer to record the variation of optical transmission when changing pH. The following values were found, at 1° (after activity correction has been made): *o*-nitrophenol 7.65 (lit.[13] 7.52); *p*-nitrophenol 7.55 (lit.[13] 7.43); 3-hydroxypyridine 5.11 (lit.[14] 5.05). This suggests that the measured pH should be corrected by about 0.1 pH unit to yield accurate values. pH measurements quoted in the text have been corrected in that way.

Although the capacitors were discharged up to a hundred time in a typical kinetic run, no alteration of the solutions is observed; this is demonstrated by the high reproducibility of the measured relaxation times.

Data Processing. The voltage output from the T-jump spectrometer is fed into a PDP-11 processor (Digital Equipment Corp.). The structure of this computer makes it specially convenient for high speed direct access to core memory. A special interface was built in our laboratory for this purpose; only a brief description of this device will be given here, as a more detailed report will appear elsewhere.[15] The signal from the T-jump is first amplified, then converted by a high speed 10 bits A/D converter (DATEL); the sampling rate, and the number of values to be transmitted in core memory as well as the delay between the triggering of the capacitors and the beginning of the acquisition process may be selected; the maximum sampling rate is 2 μsec per value. Delayed acquisition is used to eliminate very fast relaxation phenomena not to be studied. The signal to noise ratio is poor (S/N = 2 to 3) at the lowest concentrations of adenine we have used; therefore, it was found valuable to accumulate 3 to 6 relaxation curves, thereby increasing S/N by a factor of about 2. The signal thus obtained is smoothed by a procedure described by Savitzky et al.[16] and then translated so that the average of the last 20 values is put equal to zero; finally a least-square procedure is used to calculate the relaxation time by means of eq 1.

$$\ln y = -t/\tau + \ln y_0 \quad (1)$$

A more sophisticated alternative is to search for an optimum base line by incrementing all the y's in eq 1 so that a maximum for the correlation coefficient is reached; this procedure was not found to increase significantly the reproducibility of the measurements.

When the best fit is obtained for τ, the amplitude y_0, extrapolated to zero delay, is computed. From the gain of the interface, the amplitude (A) of the relaxation expressed in optical density units is then readily obtained.[17]

For each pH value at least five series of three to six curves were processed. The standard deviation is used as an estimate of the uncertainty on the relaxation time; it does not exceed 10% in most experiments reported here.

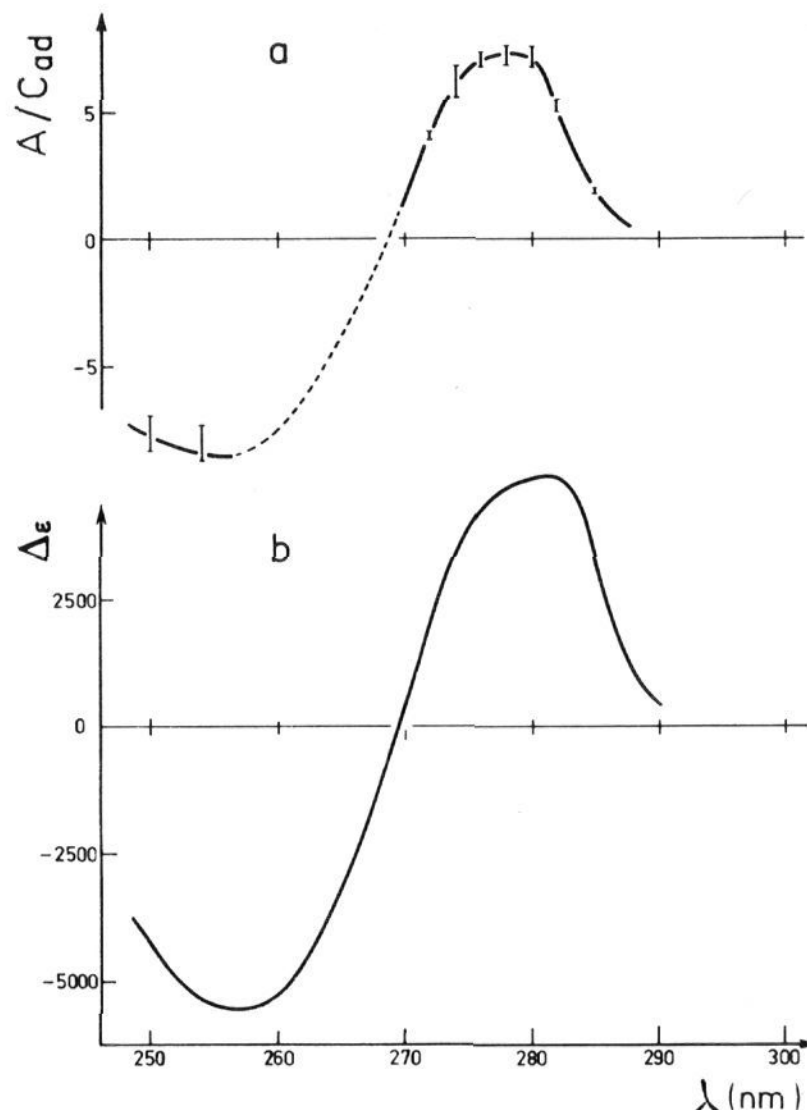


Figure 2. (a) Ratio of the relaxation amplitude to the adenine concentration versus the wavelength: t_i = 1°; t_f = 10°; uncertainties are the standard deviations. (b) Plot of the molar extinction coefficient difference ($\epsilon_{N(7)Me} - \epsilon_{N(9)Me}$) as a function of λ at 20°.

Uv Spectroscopy. Absorbance measurements were made using a Cary 16 uv spectrometer fitted with a thermostated sample cell. The spectra were recorded using a 0.025 *M* phosphate buffer as a solvent, to ensure that only neutral forms of adenines are present in solution.

Results

Relaxation Spectrum. When the temperature of a neutral solution of adenine in the concentration range 10^{-4} to 10^{-3} *M* is rapidly raised and the optical density of the solution is recorded as a function of time, two successive phenomena are observed (Figure 1): (a) an immediate variation of the optical density; the corresponding relaxation time is shorter than the time constant of the solution heating and therefore cannot be measured with our apparatus; a similar effect is observed with solutions of 7-methyladenine and 9-methyladenine at comparable concentrations, and can probably be attributed to small variations of the molar extinction coefficient with temperature; (b) a much slower exponential variation of the optical density of the solution; this phenomenon is not observed with the methylated derivatives and its study is the major aim of the present work.

Relaxation Amplitude. Under the experimental conditions used in the kinetic runs reported here (t_i = 1°, t_f = 10°), the amplitude of the second relaxation phenomenon is found to be pH independent over the pH range 6 to 9 (however, this pH range apparently becomes narrower when the temperature is raised) and to be proportional to adenine concentration.

Using solutions of various concentrations, the amplitude-to-concentration ratio was plotted as a function of the wavelength (Figure 2a); two maxima of opposite signs are observed at 278 nm and around 260 nm.

Table I. Variation of the Relaxation Amplitude A (O.D. Units) with Temperature (t_i)[a]

t_i	$A \times 10^4$	$\sigma(A) \times 10^4$
274	34.2	1.3
284	30.4	0.7
294	23.5	1.1
304	20.3	0.9
324	12	3

[a] λ = 280 nm; pH is near neutrality; σ is the standard deviation. $C_{adenine} = 4.85 \times 10^{-4}\ M$.

Table II. Variation of the Inverse of the Relaxation Time (τ^{-1}) with pH and Adenine Concentration

$C_{adenine}$, $M \times 10^4$	pH[a]	τ^{-1} (exptl), sec^{-1} $\times 10^{-4}$	τ^{-1} (calcd), sec^{-1} [c] $\times 10^{-4}$
3.13	6.34	1.0 (0.05)[b]	1.02
3.13	6.86	0.324 (0.004)	0.348
3.13	7.83	0.22 (0.03)	0.24
3.13	8.26	0.52 (0.03)	0.49
3.13	8.46	0.78 (0.04)	0.75
7.23	5.78	3.57 (0.34)	3.53
7.23	6.33	1.01 (0.01)	1.04
7.23	6.50	0.70 (0.02)	0.72
7.23	6.69	0.453 (0.02)	0.48
7.23	6.77	0.39 (0.04)	0.42
7.23	7.40	0.202 (0.002)	0.211
7.23	7.95	0.34 (0.02)	0.344
7.23	8.30	0.72 (0.04)	0.67
7.23	8.49	1.03 (0.05)	1.01
7.23	8.57	1.18 (0.04)	1.19
7.23	8.89	2.26 (0.03)	2.44
9.45	6.06	1.95 (0.13)	1.91
9.45	6.34	1.13 (0.07)	1.02
9.45	6.44	0.91 (0.03)	0.82
9.45	6.64	0.59 (0.01)	0.54
9.45	7.78	0.298 (0.002)	0.296
9.45	7.95	0.376 (0.017)	0.381
9.45	8.17	0.59 (0.02)	0.605
9.45	8.37	0.97 (0.02)	0.86
9.45	8.55	1.40 (0.04)	1.28
11.5	6.18	1.33 (0.02)	1.46
11.5	7.00	0.283 (0.003)	0.294
11.5	8.09	0.508 (0.004)	0.533
11.5	8.44	1.09 (0.02)	1.07
11.5	8.58	1.46 (0.04)	1.48
11.5	9.11	4.72 (0.10)	4.79

[a] As measured at t_i. [b] Standard deviation. [c] Computed from eq 2 (see text).

The amplitude, measured in the pH range where it is pH independent, was found to decrease rapidly when the temperature was raised (Table I) and becomes vanishingly small at temperatures above 50°.

Relaxation Time. For t_i = 1° and t_f = 10°, the relaxation time was measured over more than 3 pH units and was found to be very sensitive to the pH (Table II). The relaxation times do not show any systematic dependence upon the adenine concentration for pH < 7.3 but at higher pH values definite evidence for a decrease of τ^{-1} was observed when adenine concentration was decreased.

The rather large enthalpy of dissociation of water causes the equilibrium concentrations of hydroxide and hydronium ions to differ considerably at t_i and at t_f when the temperature jump is 9°. For reasons to be discussed below, the concentrations $\bar{C}_{H^+}$ and $\bar{C}_{OH^-}$ corresponding to the final temperature should be considered in the interpretation of the kinetic results rather than $\bar{C}_{H^+}{}^\circ$ and $\bar{C}_{OH^-}{}^\circ$ which refer to the initial temperature. The concentrations $\bar{C}_{H^+}{}^\circ$ and $\bar{C}_{OH^-}{}^\circ$, as well as the concentrations of adenine in cationic, neutral, and anionic forms before the jump, were computed[18] from the measured pH and the dissociation constants

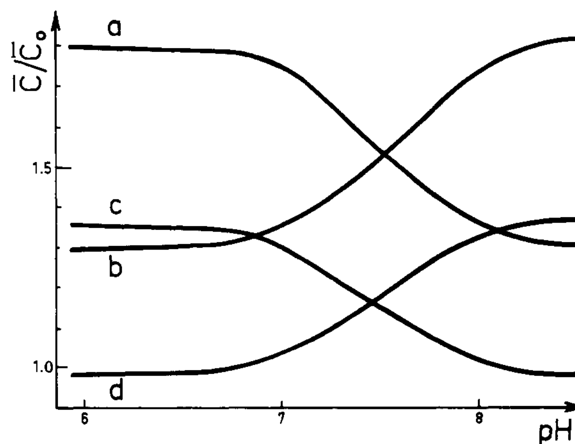


Figure 3. Ratio of the final-to-initial equilibrium concentrations of various species in adenine solution as a function of pH: t_i = 1°; t_f = 10°; concentration of adenine = $4.18 \times 10^{-4}\ M$. (a) OH^-, (b) H^+, (c) anionic adenine, (d) cationic adenine.

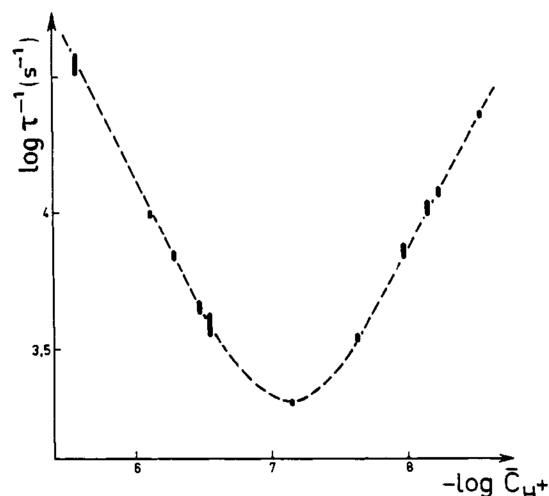


Figure 4. Log τ^{-1} as a function of $-\log \bar{C}_{H^+}$. Adenine concentration = $7.23 \times 10^{-4}\ M$; t_i = 1°; t_f = 10°; λ 280 nm.

of the cationic (K_1) and neutral (K_2) forms of adenine at the corresponding temperature evaluated from the literature data.[19] These concentrations were then used to compute the equilibrium concentrations at t_f according to the procedure described in the Appendix. A plot of $\bar{C}_{H^+}/\bar{C}_{H^+}{}^\circ$ and $\bar{C}_{OH^-}/\bar{C}_{OH^-}{}^\circ$ as a function of pH (Figure 3) for a typical adenine concentration shows that these ratios deviate considerably from unity.

The variation of τ^{-1} with the pH and the concentration of adenine was fitted by the equation

$$\tau^{-1} = k_0 + k\bar{C}_{H^+} + k'\bar{C}_{OH^-} + k''\bar{C}_{OH^-}\bar{C}_{adenine} \quad (2)$$

A least-squares fitting leads to k_0 = 210 sec^{-1}, $k = 1.3 \times 10^{10}\ M^{-1}$ sec^{-1}, $k' = 0.85 \times 10^{10}\ M^{-1}$ sec^{-1}, and $k'' = 6.5 \times 10^{12}\ M^{-1}$ sec^{-1}. Although probably real, the term k_0 contributes little to the rate law even at neutral pH where τ^{-1} is minimum, so that a plot of log τ^{-1} versus log $\bar{C}_{H^+}$ for a fixed adenine concentration shows two straight lines with unit slope, connected by a valley (Figure 4).

Discussion

The amplitude (A) of the relaxation process for a two-species equilibrium as monitored by uv spectroscopy is readily derived

$$A = C[lK/(1 + K)^2]\Delta\epsilon\Delta T\Delta H_0/RT^2 \quad (3)$$

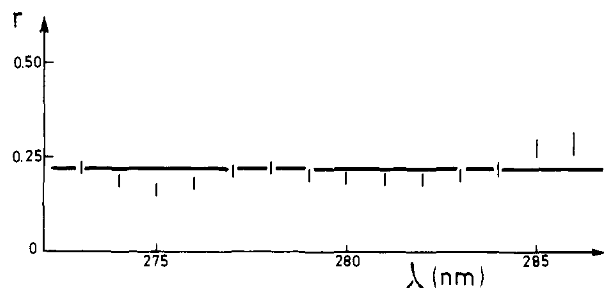


Figure 5. $r = (\epsilon_{\text{adenine}}^{\lambda} - \epsilon_{N(9)Me}^{\lambda+2.5})/(\epsilon_{N(7)Me}^{\lambda+2.5} - \epsilon_{N(9)Me}^{\lambda+2.5})$ as a function of λ (see text).

where C is the total concentration, l is the optical length, ΔT is the magnitude of the temperature jump, $\Delta\epsilon$ is the difference between the molar extinction coefficients of each species, ΔH_0 is the reaction enthalpy, and K is the equilibrium constant.

Thus, for such a process, the amplitude-to-concentration ratio for a given temperature and ΔT is proportional to the difference of the molar extinction coefficients of the two interconverting species.

The close similarity of the wavelength dependence of the amplitude-to-concentration ratio (Figure 2a) with the differential spectrum of 7-methyladenine and 9-methyladenine (Figure 2b) demonstrates that the observed relaxation is associated with the reequilibrium of the N(7)H and N(9)H tautomers of adenine in water.

The pH independence of the amplitude and its proportionality to adenine concentration are consistent with this interpretation.

Estimate of the Thermodynamic Parameters of the N(7)H ⇄ N(9)H Equilibrium. The overall molar extinction coefficient for adenine is

$$\epsilon_{\text{adenine}} = (1 - \alpha)\epsilon_{N(9)H} + \alpha\epsilon_{N(7)H} \qquad (4)$$

where α is the molar fraction of N(7)H form in the mixture; (4) may be rewritten as

$$\alpha = (\epsilon_{\text{adenine}} - \epsilon_{N(9)H})/(\epsilon_{N(7)H} - \epsilon_{N(9)H})$$

Thus the computation of α requires the knowledge of the molar extinction coefficients of the individual tautomers.

Now, it is apparent (Figure 2) that the differential spectrum for the methylated derivatives (Figure 2b) is somewhat red shifted with respect to that of the unsubstituted adenines (Figure 2a). Indeed when the two plots are scaled to the same maximum values, it is found that curve a parallels curve b in the spectral range 276–286 nm with a *constant* blue shift of about 2.5 nm. This suggests that the spectra of N(7)H and N(9)H forms of adenine can be obtained, at least in this wavelength range, simply by blue shifting by 2.5 nm the spectra of the methylated derivatives.[20] It is found moreover (Figure 5) that the ratio

$$r = \frac{\epsilon_{\text{adenine}}^{\lambda} - \epsilon_{N(9)Me}^{\lambda+2.5}}{\epsilon_{N(7)Me}^{\lambda+2.5} - \epsilon_{N(9)Me}^{\lambda+2.5}} = \frac{\epsilon_{\text{adenine}}^{\lambda} - \epsilon_{N(9)Me}^{\lambda+2.5}}{(\Delta\epsilon)_{Me}^{\lambda+2.5}}$$

is roughly constant in that spectral range.

From Figure 5, we estimate α at room temperature to be 0.22 and the equilibrium constant $K = \bar{C}_{N(7)H}/\bar{C}_{N(9)H} = \alpha/(1 - \alpha)$ to 0.28. The equilibrium enthalpy is estimated to 210 cal M^{-1} by means of eq 3. The entropy variation is -1.8 cal M^{-1} deg^{-1}. Although Figure 5 allows only a rough estimation of K, the computed ΔH_0 is rather independent of it and is, in all cases, positive and close to zero; if the uncertainty on K is assumed to be ±20%, ΔH_0 lies between 190 and 230 cal and ΔS between -1.90 and -1.77 cal M^{-1} deg^{-1}.

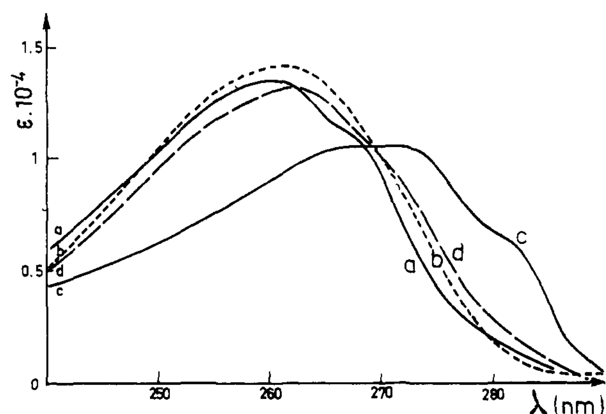


Figure 6. Spectra of (temperature 20°): (a) adenine; (b) 9-methyladenine; (c) 7-methyladenine; (d) a "weighted" spectrum of 22% 7-methyladenine and 78% 9-methyladenine.

Several authors have estimated ΔH_0 for reaction I from theoretical computations.[25,26] Most of them agree that the N(9)H tautomer should be intrinsically more stable than the N(7)H one. Our estimates for the enthalpy of tautomerization and for the entropy are thus consistent with a larger heat of hydration for the N(7)H tautomer, which indeed has been shown both theoretically[25,26] and experimentally[27] to have a larger dipole moment.

It might have seemed more straightforward to estimate the thermodynamic parameters of reaction I from the variation of the relaxation amplitude with the temperature at constant wavelength and for a given temperature jump magnitude. Differentiating AT^2 obtained from eq 3 with respect to $1/T$ leads to

$$\Delta H_0 = -\frac{R}{1 - 2K}\frac{\partial \ln AT^2}{\partial(1/T)} \qquad (5)$$

where K is defined as the ratio of *minor to major* tautomer concentrations.

In deriving (5) it has been assumed that ΔH_0 and $\Delta\epsilon$ are temperature independent and that ln $(1 + K)$ may be approximated by K. From the values quoted in Table I, AT^2 is found to decrease when the temperature increases so that a negative value is obtained for ΔH_0; from eq 5, $\Delta H_0 = -2.5$ kcal M^{-1}, if K is assumed to be very small, and is even less otherwise. This implies either: (a) the minor tautomer is indeed exothermic with respect to the major one; as the sign of the amplitude clearly shows (Figure 2) that the equilibrium is shifted toward N(7)H when the temperature is raised, N(7)H *is* the endothermic species and thus hypothesis (a) would mean that N(7)H is the predominant form of adenine in water; this conclusion is quite unlikely in view of the very different uv spectra of adenine and 7-methyladenine (Figure 6); or (b) the hypothesis that $\Delta\epsilon$ and/or ΔH_0 are constant when the temperature is varied is not valid.

It is unlikely that a change of $\Delta\epsilon$ with temperature causes the observed variation of $(AT)^2$ with $(1/T)$, since this would necessitate a temperature dependence for $\Delta\epsilon$ far larger than that observed for $(\Delta\epsilon)_{Me}$. Thus it must be assumed that ΔH_0 is temperature dependent; differentiating AT^2 from eq 3 then leads to the expression

$$\Delta H_0 = -\frac{1}{1 - 2K}\left[R\frac{\partial \ln AT^2}{\partial(1/T)} + \frac{RT^2}{\Delta H_0}\frac{\partial\Delta H_0}{\partial T}\right] \qquad (6)$$

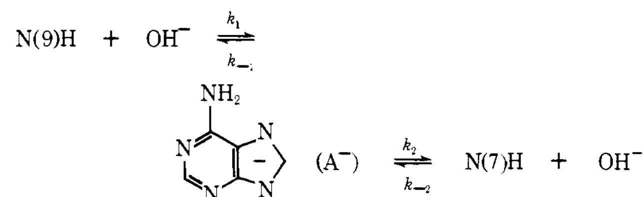
Inserting in eq 6 the estimates of K and ΔH_0 previously obtained leads to $(1/\Delta H_0)(\partial\Delta H_0/\partial T) = -1.6\%$, this quite large value being in turn consistent with a ΔH_0 close to zero. Thus the variation of the amplitude with temperature brings additional evidence for an almost athermic reaction;

$\partial\Delta H_0/\partial T$ is found to be negative so that the molar specific heat of the more polar N(7)H tautomer is smaller than that of N(9)H; a similar effect is observed in the tautomerism of β hydroxypyridines.[28]

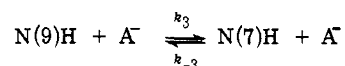
Mechanism of N(7)H ⇌ N(9)H Interconversion. The variation of the relaxation amplitude with the concentration and the pH is consistent with the first-order reequilibration N(9)H ⇄ N(7)H. Thus, if N(7)H and N(9)H are interconverted through several reaction pathways, the inverse of the relaxation time can be expressed as $\tau^{-1} = \Sigma_i(\vec{k}_i + \overleftarrow{k}_i)$ where $\vec{k}_i$ and $\overleftarrow{k}_i$ are the first-order rate constants for the reaction along route i.

Now, to account for the dependence of the relaxation time on the pH and the concentration the mechanism in Schemes I–III is proposed.

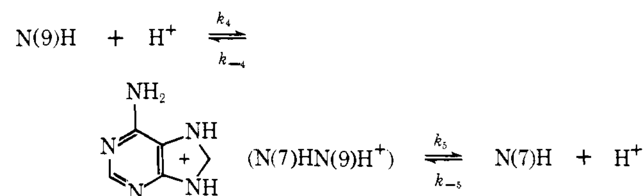
Scheme I. Base Catalysis by OH^-



Scheme II. Base Catalysis by the Adeninate Anion (A^-)



Scheme III. Acid Catalysis by H^+



The intermediates A^- and $N(7)HN(9)H^+$ are present at minute concentrations. When the steady state approximation is applied to A^- and $N(7)HN(9)H^+$, the following contributions of the different reaction schemes to the relaxation time are obtained.

$$(\vec{k}_{\mathrm{I}} + \overleftarrow{k}_{\mathrm{I}}) = \left[\frac{k_1k_2}{k_{-1} + k_2} + \frac{k_{-1}k_{-2}}{k_{-1} + k_2}\right]\bar{C}_{\mathrm{OH^-}}$$

$$(\vec{k}_{\mathrm{II}} + \overleftarrow{k}_{\mathrm{II}}) = (k_3 + k_{-3})\bar{C}_{\mathrm{A^-}}$$

$$(\vec{k}_{\mathrm{III}} + \overleftarrow{k}_{\mathrm{III}}) = \left[\frac{k_4k_5}{k_{-4} + k_5} + \frac{k_{-4}k_{-5}}{k_{-4} + k_5}\right]\bar{C}_{\mathrm{H^+}}$$

Since $\bar{C}_{\mathrm{A^-}} = (K_2/K_{\mathrm{W}})\bar{C}_{\mathrm{adenine}}\bar{C}_{\mathrm{OH^-}}$, it is seen that this reaction mechanism accounts for the observed dependence of τ^{-1} upon concentration and pH (eq 2).

The assumption that the observed relaxation phenomenon can be associated with an N(9)H ⇄ N(7)H first-order interconversion process, through the proposed mechanism, can be further substantiated by a theoretical calculation of the relaxation spectrum, following the procedure described in the Appendix.[29]

Base Catalysis by OH^-. The rate constants k_2 and k_{-1} may be expressed as

$$k_2 = k_{-2}(\bar{C}_{\mathrm{N(7)H}}\bar{C}_{\mathrm{OH^-}}/\bar{C}_{\mathrm{A^-}})$$

and

$$k_{-1} = k_1(\bar{C}_{\mathrm{N(9)H}}\bar{C}_{\mathrm{OH^-}}/\bar{C}_{\mathrm{A^-}})$$

using these expressions, $(\vec{k}_{\mathrm{I}} + \overleftarrow{k}_{\mathrm{I}})$ may be rewritten as

$$(\vec{k}_{\mathrm{I}} + \overleftarrow{k}_{\mathrm{I}}) = \frac{k_1k_{-2}\bar{C}_{\mathrm{OH^-}}}{(k_{-2}\bar{C}_{\mathrm{N(7)H}} + k_1\bar{C}_{\mathrm{N(9)H}})/(\bar{C}_{\mathrm{N(7)H}} + \bar{C}_{\mathrm{N(9)H}})} \quad (7)$$

it is apparent that the denominator in eq 7 is the average rate constant, $k_{\mathrm{OH^-}}{}^{\mathrm{av}}$, for the proton abstraction from adenine by OH^-. Thus, the coefficient of $\bar{C}_{\mathrm{OH^-}}$ in the expression of τ^{-1} (eq 2) is $k_1k_{-2}/k_{\mathrm{OH^-}}{}^{\mathrm{av}}$.

As amply demonstrated by Eigen,[30] the exothermic reactions of "normal" acids and bases with OH^- an H^+, respectively, are diffusion controlled; thus, differences in reactivity toward OH^- of two acids of the same charge type are likely to be due mainly to steric factors. Because of the close similarity of the structure of N(7)H and N(9)H adenines, it may be assumed, as a first approximation, that $k_1 = k_{-2} = k_{\mathrm{OH^-}}{}^{\mathrm{av}}$. From eq 2, their common value is $0.85 \times 10^{10}\ M^{-1}\ \mathrm{sec}^{-1}$ at 10°, in good agreement with the value of $1.0 \times 10^{10}\ M^{-1}\ \mathrm{sec}^{-1}$ previously found for $k_{\mathrm{OH^-}}{}^{\mathrm{av}}$ at 25°.[31]

Base Autocatalysis. The concentration of the adeninate anion, $\bar{C}_{A^-}$, can be computed from the dissociation constant of adenine

$$\bar{C}_{\mathrm{A^-}} = (K_2/K_{\mathrm{W}})\,\bar{C}_{\mathrm{adenine}}\bar{C}_{\mathrm{OH^-}}$$

then, from eq 2, $(k_3 + k_{-3})$ is found equal to $3.5 \times 10^8\ M^{-1}\ \mathrm{sec}^{-1}$; since $k_3/k_{-3} = K = 0.28$, it is found that $k_3 = 0.75 \times 10^8\ M^{-1}\ \mathrm{sec}^{-1}$ and $k_{-3} = 2.7 \times 10^8\ M^{-1}\ \mathrm{sec}^{-1}$. These values are well below the diffusion limit, and compare favorably with those reported for the purine–purinate system from NMR measurements.[32]

Acid Catalysis. The contribution from Scheme III to the inverse of the relaxation time can be written analogously to that of Scheme I

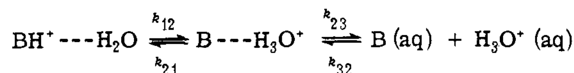
$$(\vec{k}_{\mathrm{III}} + \overleftarrow{k}_{\mathrm{III}}) = \frac{k_4k_{-5}}{k_{\mathrm{H^+}}{}^{\mathrm{av}}}\bar{C}_{\mathrm{H^+}}$$

where $k_{\mathrm{H^+}}{}^{\mathrm{av}} = (k_4\bar{C}_{\mathrm{N(9)H}} + k_{-5}\bar{C}_{\mathrm{N(7)H}})/(\bar{C}_{\mathrm{N(9)H}} + \bar{C}_{\mathrm{N(7)H}})$ is the average rate constant for the formation of the $N(7)HN(9)H^+$ cation from H^+ and adenine. Again, it may be assumed, as a first approximation, that $k_4 = k_{-5} = k_{\mathrm{H^+}}{}^{\mathrm{av}}$. From eq 2 our experimental value for this constant is $1.3 \times 10^{10}\ M^{-1}\ \mathrm{sec}^{-1}$.

Before commenting on this result, we shall briefly discuss protonation of adenine in aqueous solutions. Adenine has four basic sites, namely N1, N3, N7, or N9, and the exocyclic N10, so that the adeninium cation exists as a mixture of several tautomeric species.[33] There is little sound experimental evidence concerning the relative abundance of these species, although it is now thought that protonation of the amino group does not occur to a large extent.[19,34] Our experiments apparently rule out the possibility of $N(7)HN(9)H^+$ cation being a predominant form of protonated adenine, since in that case the acidic autocatalysis would be observed; this conclusion is supported by theoretical calculations[35] on the adeninium cation.

Let us now turn back to the discussion of acid catalysis. The reaction of hydronium ion with "normal" bases is likely to be ultrafast once the encounter complex has been formed;[36] thus, it is reasonable to accept that when several basic sites are present each of them has a nearly equal chance to capture the incoming proton if no steric hindrance exists; their relative basicities are reflected only by the different lifetimes of the cations thus formed. It is then improbable that the value observed for k_4 or k_{-5} is indeed the rate constant for proton fixation on the *sole* imidazole ring, since in that case the rate constant for overall reaction of adenine and H^+ would be improbably large.[37] Thus, the $N(7)HN(9)H^+$ cation must be formed from adenine and H^+ by some other mechanism than direct protonation on the imidazole ring.

A possible explanation may lay in the popular two-step mechanism[30,38] for acid dissociation of an acid BH^+ in water



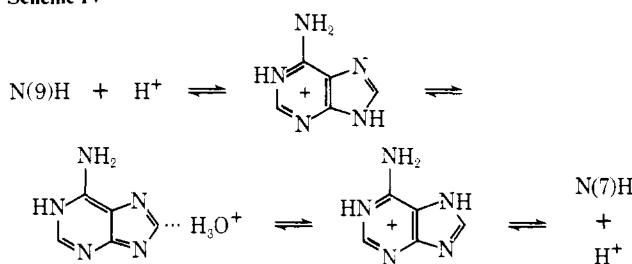
$$BH^+ \cdots H_2O \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \cdots H_3O^+ \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} B\,(aq) + H_3O^+\,(aq)$$

where the intermediate $B \cdots H_3O^+$ is present at low concentration. If the reaction between B and H_3O^+ is diffusion controlled, then $k_{21} \gg k_{23}$; this means that the acid BH^+ experiences many ionizations followed by back capture of the proton, before finally dissociation takes place. Now, when **B** has several basic sites, it is suggested that the proton ionizing from one basic center can occasionally be trapped back on another, and thereby that interconversion of the tautomeric species occurs efficiently without acid dissociation; the adeninium cation may then exist under all its possible tautomeric forms during its lifetime.

If this "proton scrambling" mechanism is operative in the adeninium cation, k_4 and k_{-5} then must represent the rate constant for *overall* protonation of N(9)H and N(7)H adenine, rather than for protonation on the sole imidazole ring, and, indeed, the value found for $(k_4k_{-5})/k_{H^+}{}^{av}$ compares favorably with the rate constant for protonation of imidazole[38,39] and possibly for overall protonation of purine.[32]

In addition to Scheme III, several reactions schemes for the acid-catalyzed N(7)H ⇄ N(9)H interconversion may formally be written if proton scrambling occurs; for example, see Scheme IV.

Scheme IV



Such types of mechanism have been proposed to explain the kinetics of proton exchange in acidic solutions of purine.[32] However, tautomers of adenine lacking hydrogen on the imidazole ring are likely to be very unstable compared to the N(7)H or N(9)H tautomer,[26] so that Scheme IV is probably a rather high energy process. It seems more probable that the imidazole ring always retains a proton during reversible ionization of the adeninium cation, thereby making the $N(7)HN(9)H^+$ species a necessary intermediate in the N(9)H ⇄ N(7)H acid-catalyzed interconversion.

Conclusion

The temperature-jump technique has proved successful in investigating tautomerism in aqueous adenine both in neutral and protonated forms; our preliminary observations on guanine solutions show that it can be usefully applied to other purines as well. The rate of interconversion of the two tautomers of neutral adenine was found to be catalyzed by acids and bases; in the neutral pH range where the rate is the slowest, the equilibrium process is virtually completed within 1 msec at 10°. The proposed mechanism for this interconversion involving the exchange of a proton between two nitrogen atoms in water is quite similar to the well-known mechanism of the keto-enol interconversion of carbonyl compounds[40] although the rate constants involved here are considerably larger. The pH-independent term in the rate law is very small; this shows that the intramolecular, water-catalyzed mechanism, which probably holds for the interconversion of the various adeninium cations, contributes little to the N(7)H ⇄ N(9)H exchange in adenine. However, the respective spatial positions of the proton donor and proton acceptor groups in adenine, pointing in opposite directions, clearly do not favor such a mechanism; it may nevertheless hold for a more favorable geometry, and thus our failure to detect the amine-imine equilibrium in adenine or methylated analogs cannot be considered as definite evidence against the existence of such tautomerism (although it seems unlikely both on a theoretical and an experimental basis).[2,3] In that case, N(1) and N(10) would be linked by a chain of H bonds involving only one or two water molecules and thus a proton exchange between these two sites is likely to proceed via a very fast concerted mechanism rather than a acid- and base-catalyzed one.

Acknowledgment. We are much indebted to J. C. Fontaine, J. J. Meyer, and P. Levoir for technical help with the electronics and the programming of the data processing system.

Appendix

Computation of the Relaxation Times and Amplitudes for a Given Mechanism. A general formalism allowing the computation of the relaxation spectrum associated with a reaction mechanism of any complexity has been derived by Castellan.[41] This treatment may also be used to evaluate the respective amplitudes, expressed as concentration changes, associated with the successive relaxation times. The notations are from ref 41.

Consider a reaction mechanism involving N chemical species and consisting of R elementary chemical reactions, among which R' are linearly independent. The stoichiometric coefficient, $\nu_{i\alpha}$ of the chemical species i in the reaction α will be taken as negative for the reactants and positive for the products. When the system is slightly displaced from equilibrium, this mechanism is characterized by a relaxation spectrum. The inverses of the relaxation times may be defined as the solutions of the eigenvalue problem

$$\mathbf{BU} = \mathbf{U}\boldsymbol{\lambda} \quad (1)$$

where **B** is a symmetrical matrix (several other forms of this eigenvalue problem are given in ref 41; however, diagonalization of a symmetrical matrix has distincts advantages for machine computation) expressed as

$$\mathbf{B} = \mathbf{r}^{1/2}\mathbf{G}\mathbf{r}^{1/2} \quad (2)$$

r is a $R \times R$ diagonal square matrix, the nonzero elements of which are the "exchange currents" associated with the corresponding chemical reaction; **G** is a $R \times R$ symmetrical matrix defined by

$$(G)_{\alpha\beta} = (G)_{\beta\alpha} = \sum_{i=1}^{N} \frac{\nu_{i\alpha}\nu_{i\beta}}{C_i}$$

where C_i is the molar concentration of the chemical species i.

The difference between the "reaction coordinate" of the reaction α at a given time and its final equilibrium value may be computed from the eigenvalues and eigenvectors of **B**

$$\Delta\xi_\alpha = r_\alpha{}^{1/2} \sum_\epsilon U_{\alpha\epsilon} A_\epsilon e^{-\lambda_\epsilon t} \quad (3)$$

where $y_\epsilon = A_\epsilon e^{-\lambda_\epsilon t}$ is the "normal coordinate of relaxation" corresponding to the relaxation time $\tau_\epsilon = 1/\lambda_\epsilon$. (Equation 3 is equivalent to eq 70 of ref 41.)

If some elementary reactions can be expressed as linear combinations of others, viz., if $R' < R$ then **B** has only R' nonzero eigenvalues. To the remaining $(R - R')$ zero roots correspond zero amplitude normal coordinates (cf. Castel-

lan's eq 83) so that only the R' nonzero modes need to be considered in the summation over ϵ in eq 3.

The difference between the concentration C_i of the species i at a given time and its final equilibrium value may be computed by

$$C_i - \bar{C}_i = \sum_{\alpha=1}^{R} \nu_{i\alpha}\Delta\xi_\alpha = \sum_{\epsilon=1}^{R'} A_\epsilon e^{-\lambda_\epsilon t} \sum_{\alpha}^{R} U_{\alpha\epsilon}\,\nu_{i\alpha}\,r_\alpha^{1/2} \quad (4)$$

Thus, the overall concentration change of i during the relaxation mode ϵ is proportional to the coefficient $b_{i\epsilon} = \sum_\alpha U_{\alpha\epsilon}\nu_{i\alpha}r_\alpha^{1/2}$. The comparison of the different $b_{i\epsilon}$ for a given relaxation mode ϵ gives information on the "composition" of this mode in terms of concentration changes.

Now, the determination of the *absolute* concentration changes accompanying the successive relaxation modes necessitates the determination of their respective amplitudes, A_ϵ. If the concentrations corresponding to the *initial* equilibrium $\bar{C}_i°$ together with the final ones are known, the A_ϵ may be obtained by solving the linear system

$$\bar{C}_i° - \bar{C}_i = \sum_\epsilon A_\epsilon b_{i\epsilon} \quad (5)$$

(5) is a set of N equations, R' of them being independent, of the R' unknowns A_ϵ.

A general program was written in Fortran to build the symmetric matrix **B** from the final concentrations $\bar{C}_i$, computed as below, and approximate values of the rate constants. Rate constants of 10^{10} M^{-1} sec^{-1} were used for all the elementary exothermic reactions,[30] except those involving two different forms of the adenine molecule; a value of 10^9 $M-1$ sec^{-1} was chosen in that case to account for the smaller diffusion coefficients of large molecules compared to those of H^+ and OH^-.

B was then diagonalized using the Givens procedure to obtain the relaxation spectrum; the "composition" of each successive relaxation was investigated by computing the coefficients $b_{i\epsilon}$ from the eigenvectors of **B**. The absolute amplitude of each normal mode was then obtained by solving the system 5.

Computation of the Concentrations Corresponding to the Final Equilibrium. It will be assumed that a set of R' independent chemical reactions has been chosen; the equilibrium constant of the independent reaction λ will be denoted $\bar{K}_\lambda°$ and $\bar{K}_\lambda$ at the initial and final temperatures, respectively. The final concentration, $\bar{C}_i$, of the species i can be written as

$$\bar{C}_i = \bar{C}_i° + \sum_{\lambda=1}^{R'} \nu_{i\lambda}\bar{x}_\lambda \quad (6)$$

where the $\bar{x}_\lambda$ must satisfy the following system

$$\bar{K}_\mu = \prod_{i=1}^{N} (\bar{C}_i)^{\nu_{i\mu}} = \prod_{i=1}^{N}\left[\bar{C}_i° + \sum_{\lambda=1}^{R'} \nu_{i\lambda}\bar{x}_\lambda\right]^{\nu_{i\mu}} \quad (\mu = 1\ldots R') \quad (7)$$

(7) is a system of R' nonlinear equations of the R' unknowns $\bar{x}_\lambda$. It may be solved for the $\bar{x}_\lambda$ by minimizing the following expression

$$F = \sum_{\mu=1}^{R'}\left[\bar{K}_\mu - \prod_{i=1}^{N}\left[\bar{C}_i° + \sum_{\lambda=1}^{R'} \nu_{i\lambda}x_\lambda\right]^{\nu_{i\mu}}\right]^2 \quad (8)$$

with respect to the x_λ; F should be zero for $x_\lambda = \bar{x}_\lambda$. A general routine of minimization of a several-variable function was used for this purpose, starting from $x_\lambda = 0$ as trial values for the $\bar{x}_\lambda$. The values of the $\bar{C}_i$ were finally obtained from the computed $\bar{x}_\lambda$ by eq 6.

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Interaction of Metal Ions with 8-Azapurines. Synthesis and Structure of Tetrachlorobis-2-[(5-amino-4-carboxamidinium)[1,2,3]triazole]copper(II) Monohydrate

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Abstract: The reaction of copper(II) chloride with 8-azaadenine in 0.36 *M* hydrochloric acid solution brings about ring opening at C(2) of the azapurine to form tetrachlorobis-2-[(5-amino-4-carboxamidinium)[1,2,3]triazole]copper(II) monohydrate, $[CuCl_4(N_6C_3H_7)_2]\cdot H_2O$, hereinafter referred to as $[CuCl_4(HACT)_2]\cdot H_2O$. The composition and geometry of this complex have been verified by single-crystal X-ray crystallography using data collected by counter methods on an automatic diffractometer. The material crystallizes in the monoclinic space group $C2/c$ with four molecules in a cell of dimensions a = 15.153 (14) Å, b = 6.962 (4) Å, c = 18.274 (16) Å, and β = 121.47 (6)°. The observed and calculated densities are 1.90 (3) and 1.93 g cm^{-3}, respectively. Least-squares refinement of the structure has led to a final value of the conventional R factor (on F) of 0.042 using 1131 independent intensities. The complex consists of monomeric $CuCl_4$ $(HACT)_2$ units which are hydrogen bonded to the water molecules. The geometry at the copper(II) centers is tetragonally distorted octahedral, the base plane being formed by two trans chlorine atoms and two trans N(8) atoms of the $HACT^+$ ligands, while the axial ligands are the remaining chlorides; the copper atoms lie on a crystallographic inversion center. The in-plane Cu–N and Cu–Cl distances of 2.049 (3) and 2.258 (2) Å, respectively, are normal, as is the axial Cu–Cl separation of 2.967 (2) Å. The triazole portion of the ligand is planar, but the copper atom and the N and C atoms of the amidine moiety are out of this plane. The ligand bond lengths suggest four principal resonance contributors to the bonding in $HACT^+$. The hydrogen bonding in the crystals is complex, with all available donor and acceptor atoms apparently participating. Since neither adenine nor 8-azaguanine undergoes hydrolysis in weakly acidic solution, it appears probable that copper coordination at N(8) may assist ring opening at C(2) in 8-azapurines.

The interactions of metal ions with nucleic acid constituents have been the subjects of numerous recent chemical, spectroscopic, and crystallographic studies;[2-16] this demonstrated interest in this field is due primarily to the biological significance of metal-nucleic acid and peptide-metal-nucleic acid interactions in living systems.[15,17-19] Crystallographic studies have demonstrated that individual transition metal ions can bind in a variety of different ways to a given purine or pyrimidine base, which may in part explain the observation[19] that some metal ions (e.g., Cu^{2+}, Cd^{2+}, Hg^{2+}) lower the thermal denaturation temperature of DNA while others (e.g., Zn^{2+}, Mn^{2+}, Mg^{2+}) have the reverse effect. Thus, for example, Zn^{2+} binds to N(7) in adenine[10] while Cu^{2+} binds[5] to N(9); moreover, Zn^{2+} binds to N(9) in guanine.[11] Thus, these two metal ions would be expected to produce different effects on the hydrogen bonding scheme in a polynucleotide and, hence, a different degree of stabilization (or destabilization).

The syntheses and structures of aza analogs of the nucleic acid constituents have also been widely studied during the past 2 or 3 years,[20-31] mainly as a result of the demonstrated but varying potency of some of these species (notably 8-azaguanine) as antineoplastic agents.[32,33] On the basis of structural models, it has been suggested that the biological activity of the ortho aza nucleosides (i.e., 6-azapyrimidine or 8-azapurine nucleosides) may result from changes in their hydrogen bonding ability brought about by conformation changes.[34] This postulate has recently received partial confirmation from theoretical[35] and crystallographic studies, which have shown that the conformations of ortho aza nucleosides about the glycosyl bond are in a different angular range than are those of the naturally occurring nucleosides.[26,27,29,30]

Since azapurines and azapyrimidines, therefore, are in common use in medical therapy, it is apparent that the interactions of naturally occurring metal ions with these bases are also of great importance. To our knowledge, however, no such studies have been reported to date, although we have noted in the literature[31] that $HgCl_2$ interacts sufficiently with 6-azauracil to allow the dissolution of this insoluble base in aqueous medium. Similarly, Zn^{2+} and Cu^{2+} form complexes with 6-azauracil and 6-azathymine, respectively, in aqueous solution,[36] but in all of these cases it is the uncomplexed base which crystallizes from the solution.

In order to overcome this lack of information, we have initiated a program of synthetic and structural chemistry to