Thermodynamics of Ionization of Guanosine in H_2O and D_2O*

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ABSTRACT: Spectrophotometric determination of the pK' of the guanosine cation in H_2O and D_2O shows that in both solvents the enthalpy of ionization, $\Delta H°$, is 2.15 ± 0.20 kcal/mole, whereas the entropy of ionization, $\Delta S°$, is -2.5 ± 0.6 and -4.6 ± 0.7 eu, respectively. The long-wavelength ultraviolet absorption spectrum of neutral guanosine undergoes a small blue shift in D_2O, suggesting stronger solute–solvent hydrogen bonds in this solvent. At higher concentrations of guanosine in H_2O pK' (determined by potentiometric titration) decreases with concentration, indicating intermolecular association.

Several studies of the thermally induced denaturation of deoxyribonucleic acid (DNA) and polynucleotides in D_2O have shown this solvent to have no significant effect on the transition, compared to H_2O (Crespi and Katz, 1962; Mahler *et al.*, 1963; Miles, 1960). The titration curves of DNA in D_2O are, however, steeper in this solvent for both the undenatured and denatured conformations (Bunville, 1964), and the acid-induced isothermal transition occurs at slightly lower charge densities of the helix (L. G. Bunville and S. J. Schwalbe, 1965, unpublished data). As a means of elucidating the effect of D_2O on the titration of DNA the ionic equilibria of one nucleoside, guanosine, have been studied as a function of solvent and temperature. Two reasons prompting the selection of guanosine are (a) the partial separation of the electronic transitions arising from nonbonding electrons, which are sensitive to perturbations of hydrogen-bonding solvents, from the transitions arising from bonding electrons (Clark and Tinoco, 1965), and (b) the abnormal effects of ribose on the acid pK values and heats of protonation of the purine base, compared to the very small effects in the case of adenosine or the pyrimidine nucleosides (Rawitscher and Sturtevant, 1960; Jordan, 1955).

Experimental Section

Materials. Guanosine, chromatographically pure, was obtained from the California Corp. for Biochemical Research, and was used without further purification. HCl solutions were prepared by quantitative dilution of constant-boiling HCl with either H_2O or D_2O, the latter being obtained from the Atomic Energy Commission at a nominal enrichment of 99.8%. HCl–D_2O solutions prepared in this manner are at a negligibly lower enrichment. In all cases the ionic strength was maintained at 0.100 by the addition of the appropriate quantity of NaCl.

Methods. Changes in absorbance as a function of pH were measured as difference spectra, $\Delta A = A(\text{pH}) - A(\text{pH } 6.9)$, at 290 m$\mu$ where the difference in the extinction coefficients of the protonated and unprotonated species is a maximum (Beaven *et al.*, 1955). Samples were prepared by mixing equal volumes of a guanosine stock solution and acid solution, or buffer solution at pH 6.9. The final concentration of guanosine in each case was approximately 2.7×10^{-4} M. Differences in absorbance were measured in a Beckman DU spectrophotometer, equipped with dual thermospacers, at temperatures of 15.30, 25.22, and 35.19°, the temperature of the cell compartment being measured with a thermistor mounted just below the cell carriage.

In a separate series of measurements, pK' was estimated from the difference in pH, ΔpH, between a solution of known guanosine concentration, b_0, in (0.01000 M HCl + 0.09000 M NaCl) and solvent alone. pK' is calculated from the equilibrium pH and the fraction of base protonated, $f = c_0(1 - 10^{-\Delta \text{pH}})/b_0$, where c_0 is the concentration of HCl in the solvent. Total guanosine present was estimated spectrophotometrically from aliquots of the acid solution diluted 100-fold with pH 5.6 phosphate buffer, using an extinction coefficient at 252.5 mμ of 13.65×10^3 l./mole (Beaven *et al.*, 1955). Replicate determinations on a given solution showed that pK' could be determined in this manner with a precision of ±0.02. pK' so determined is not subject to errors resulting from possible hydrolysis that might occur in the spectroscopic determination, in which the guanosine is exposed to much higher acid concentration for the determination of $\Delta A_{\max}$.

pH measurements were carried out with a Radiometer pHM4 meter, using a thermostated vessel for the sample, a glass electrode, and an external thermostated calomel electrode, electrical contact with the sample being effected by a liquid junction of saturated KCl dipping into the solution. The pH meter–electrode combination was standardized using National Bureau

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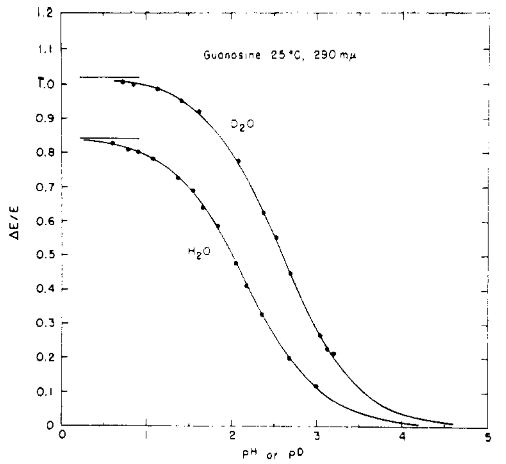

FIGURE 1: Titration curves for guanosine at 25.2°, ionic strength 0.100. Guanosine concentration, 2.7×10^{-4} M.

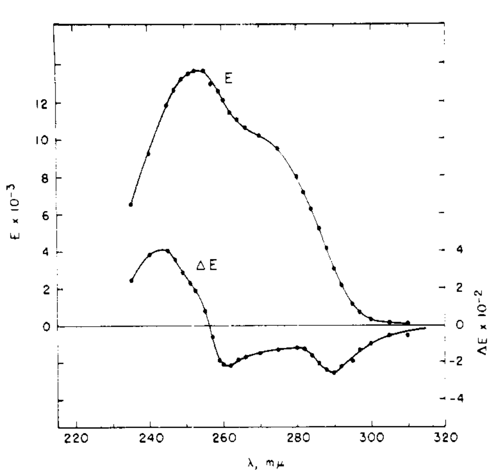

FIGURE 2: Spectrum of guanosine in H_2O, upper curve, and difference spectrum of guanosine in D_2O, atom fraction D of 0.91.

TABLE I: pK' of Guanosine in H_2O and D_2O.[a]

Temp (°C)	H_2O pK'	H_2O $(\Delta E/E)^b$	D_2O pK'	D_2O $(\Delta E/E)^b$
15.3	2.231 (0.017)[c]	0.860	2.655 (0.020)	1.04
25.2	2.174 (0.018)	0.843	2.593 (0.012)	1.02
35.2	2.122 (0.011)	0.826	2.554 (0.014)	0.993

[a] Spectrophotometric titration, ionic strength 0.100. [b] 290 mμ. [c] Average error.

of Standards buffers, pH values as redefined by Bates (1962); a 0.05 M potassium acid phthalate buffer of pH 4.008 was used for routine standardization. For solutions with D_2O as solvent, the same procedure was used, except that pH was obtained by adding 0.400 to the pH indicated by the meter (Salomaa *et al.*, 1964).

Difference spectra of neutral guanosine were obtained in buffer-free, salt-free solutions of varying proportions (by weight) of D_2O and H_2O. A solution (1 ml) of guanosine in H_2O ($A_{252.5\ m\mu}$ approximately 20) was added to 10 ml of a mixture of H_2O and D_2O of known composition. Volume additivity was assumed. Difference spectra were taken at room temperature at a constant slit width of 0.22 mm. The difference spectra in pure D_2O were obtained by extrapolation. Errors in extinction coefficient differences were estimated from absorbance differences in replicate samples prepared in H_2O alone, for which errors in dilution factors (gravimetrically determined) were negligible; the resulting maximum error at 252.5 mμ is ±30 l./mole.

Results

The results for the spectrophotometric titrations (Table I) were calculated as the apparent pK, $pK_{1a}' = pH - \log[(\Delta A_{max} - \Delta A)/\Delta A]$ (Hermans *et al.*, 1960), for the dissociation of a proton from the guanosine cation: $BH^+ = B + H^+$. Since differences in pK' and its temperature coefficient in the two solvents are the primary consideration, no corrections were made for the effect of ionic strength (0.100 M) on the activity coefficients of the various ionic species present. The variation of ionic activity coefficients with temperature will be smaller than the uncertainty in determining pK', and the difference between activity coefficients in D_2O and H_2O will likewise be negligible, owing to the very small difference in dielectric constant (Kirshenbaum, 1951). The thermodynamic quantities for the ionization of the guanosine cation are summarized in Table II, calculated as

$$\Delta H^\circ = 4.575 \frac{dpK}{d(1/T)}$$

$$\Delta F^\circ = 4.575TpK$$

$$\Delta S^\circ = \frac{\Delta H^\circ}{T} - 4.575pK$$

where ΔF° and ΔS° are correct to within an additive constant.

Several features of the protonation difference spectra of guanine are noteworthy. First, in both solvents ΔA_{max} decreases with increasing temperature. The decrease in absorbance at 290 mμ is similar to what would be expected for the hydrolysis of guanosine to guanine, the extinction coefficient of protonated guanine being similar to that of neutral guanosine (Beaven *et al.*, 1955). That this decrease arises from hydrolysis is unlikely. From the rate constant for hydrolysis in 0.1 M HCl (Venner, 1965) the time estimated for the hydrolysis of 1% of the guanosine would be of the order of 200 min (at 37°), much longer than the time required for measurement of absorbances. Furthermore, the decrease in absorbance is partially reversible, so that the presence of equilibria other than that of protonation would seem reasonable. The titration curves at 25° in the two solvents are shown in Figure 1, and the calculated pK' values and the values of $(\Delta\epsilon_{max}/\epsilon)$ at 290 mμ given in Table I.

In addition to the variation of ΔA_{max} with temperature, which is observed in both solvents, the relative increase in extinction coefficient, $(\epsilon_{BH} - \epsilon_B)/\epsilon_B$, is substantially larger in D_2O (1.02) than in H_2O, (0.843) at 25°. To further examine the origin of this difference, the difference spectra of the neutral base in D_2O was determined as $\Delta\epsilon = \epsilon_B(D_2O) - \epsilon_B(H_2O)$, the results as shown in Figure 2. Thus, solvent perturbation of the spectra of neutral guanosine is seen to contribute to the observed protonation difference spectra.

The pK' of guanosine in H_2O has been variously reported as 2.2 in dilute solution (spectrophotometric titration) (Cohn, 1955) and 1.6 at a concentration of 0.025 M (potentiometric titration) (Levene and Simms, 1925). Both results are consistent with the results of this investigation, $pK' = 2.19 - 17.9c$, at 25° (Figure 3), where c is the molar concentration of guanosine. From this, a pK' of 1.7 would be estimated at 0.025 M, in fair agreement with the result of Levene and Simms (1925) of 1.6.

Discussion

The introduction of a ribose group has only a small effect on the ionic equilibria of cytosine and adenine, the heats of ionization being altered only slightly (Rawitscher and Sturtevant, 1960), and the pK values decreasing by 0.2 and 0.5 unit, respectively (Jordan, 1955). In the case of guanine, however, the pK_{1a} decreases from 3.2 to 2.2, whereas the pK_{2a} (9.6) decreases by only 0.1 unit. In the case of cytosine and cytidine, heats of ionization from spectrophotometric and calorimetric observations are in substantial agreement (Rawitscher and Sturtevant, 1960). For guanosine, however, the heat of ionization deduced from spectrophotometric measurements, at high dilution, as reported here, is approximately twice as large as the calorimetrically determined value (Rawitscher and Sturtevant, 1960). This discrepancy may be the result of intermolecular association (see below).

The isotopic effect on the pK for the ionization of guanosine, $\Delta pK(pK_D - pK_H) = 0.42$, is "normal" (Figure 4) in the sense that it is consistent with observed values for acids of similar pK (Bell, 1959); MacDougall and Long, 1962). Exceptions to the generally observed variation of ΔpK_H are seen in salicylic acid (MacDougall and Long, 1962) and maleic acid (Dahlgren and Long, 1960) in which there is substantial intramolecular hydrogen bonding. From the magnitude of the isotope effect on the pK of guanosine, it appears reasonable that other equilibria such as hydrogen bonding, in addition to the dissociation of a proton, may be excluded from consideration.

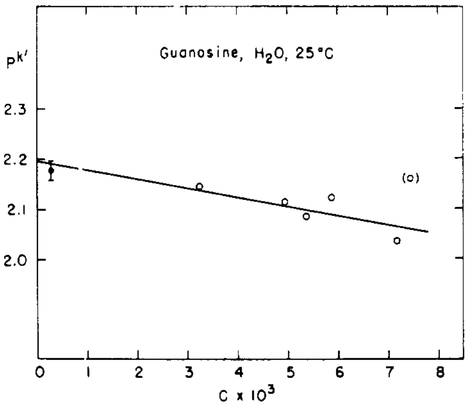

FIGURE 3: pK' of guanosine as a function of total concentration. ● spectrophotometric; ○, potentiometric titration at 25.2°; ionic strength, 0.100.

The thermodynamic quantities for the ionization of guanosine are summarized in Table II. It is seen that the heat of ionization, determined from spectrophotometric observations, of 2.22 ± 0.17 kcal/mole differs substantially from the calorimetrically determined value of 0.99 ± 0.25 kcal/mole (Rawitscher and Sturtevant, 1960). Although the latter result has been corrected for heat of dilution, the dependence of pK upon concentration is suggestive of an intermolecular association. Purine and various purine derivatives have been shown to be associated in aqueous solution (Ts'o *et al.*, 1963; Ts'o and Chan, 1964), and in the case of purine the diminished activity of the monomer leads to a decrease in the apparent dissociation constant, pK_{1a}, with increasing concentration (Read and Goldstein, 1965). Furthermore, guanosine monophosphate, in concentrated solutions, has been shown by X-ray diffraction to be extensively aggregated (Gellert *et al.*, 1962), indicating an intrinsic association constant of the guanine moiety of sufficient magnitude to overcome the positive contribution to the free energy of the negatively charged phosphate groups.

TABLE II: Thermodynamic Functions for the Ionization of Guanosine, 25°.[a]

	H_2O	D_2O
$\Delta F°$, kcal/mole	2.62	3.54
$\Delta H°$, kcal/mole	2.22 ± 0.17	2.08 ± 0.22
$\Delta S°$, eu	−2.5 ± 0.6	−4.9 ± 0.7

[a] Data of Table I.

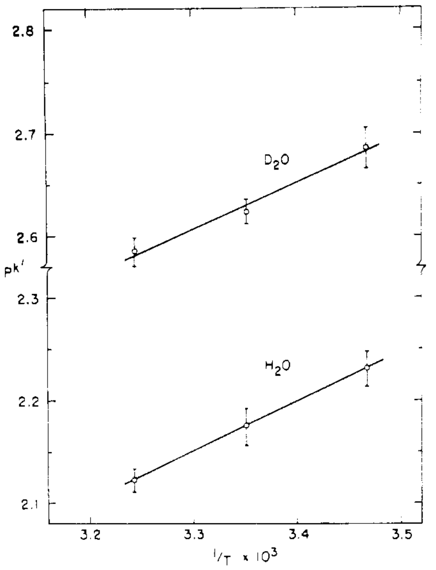

FIGURE 4: pK' of guanosine in H_2O and D_2O; spectrophotometric titration, ionic strength 0.100.

From the temperature dependence of the solubility of adenine in the presence of purine (Ts'o *et al.*, 1963), and of 9-methyladenine in the presence of 1-methylthymine, and conversely (Gill *et al.*, 1963), it may be inferred that this association is exothermic by approximately 7 kcal/mole.

If the dependence of the pK' of guanosine upon concentration (Figure 2) arises from the decrease in the activity of either monomeric species through association, then K_{app} for the over-all process

$$BH^+ \rightleftarrows B + H^+ \quad K_{1a} \tag{1a}$$

$$B + B \rightleftarrows B_2 \quad K_2 \tag{1b}$$

$$B_2H^+ \rightleftarrows B_2 + H^+ \quad K_{2a} \tag{1c}$$

will depend upon total guanosine concentration, B_0, as

$$K_{\text{app}} = \frac{1 + K_2B(2 + H^+/K_{2a})}{1 + K_2BK_{1a}/K_{2a}} \tag{2}$$

$$B_0 = B(1 + H^+/K_{1a}) + 2K_2B^2(1 + H^+/K_{2a}) \tag{3}$$

For $K_{2a} = K_{1a}$, the limiting value of the dependence of pK_{app} upon B_0 is $(\text{d}(\text{p}K_{\text{app}})/\text{d}B_0)_{B_0 = 0} = -0.434K_2$. From the observed dependence of pK_{app} on concentration at 25° K_2 would be estimated as 40 l./mole. Neglecting oligomers greater than the dimer is reasonable in view of the low concentrations employed; consideration of doubly charged dimers is unnecessary because of the large, positive electrostatic contribution to the free energy. Although the chemical shift of the proton resonance in neutral purine solutions is concentration dependent, that for solutions of the purine cation is not, indicating the absence of association of the cation. It should be noted that, although the results of Read and Goldstein (1965) show a decrease in pK with gross composition of the sample, pK is independent of pH at a given concentration, as a result of the rapid exchange of the various species and the consequent averaging of the various magnetic states of the protons (nonexchangeable) of purine. Since $\Delta H_{\text{assocn}}°$ is negative, determination of the temperature dependence of pK' for the ionization of guanosine at moderate concentrations will lead to an underestimate of the heat of ionization.

Interpreting the results from the spectrophotometric determination of pK as being representative of the dissociation of a proton from the guanosine cation (eq 1a), the results in D_2O indicate that the isotope effect on the free energy of dissociation is largely entropic in origin, $\Delta S_{\text{ionization}}°$ being more negative in D_2O by approximately 2 ± 1 eu.

Additional information concerning the perturbation of solvent–solute interactions that accompany the transfer of guanosine from H_2O to D_2O may be obtained from the difference spectrum of the nucleoside in D_2O (Figure 2).[1] Electronic transitions involving lone pair electrons on nitrogen and oxygen are easily perturbed by interactions with the solvent, and in hydrogen-bonding solvents undergo shifts to higher energies (lower wavelengths). Such shifts arise from stabilization of the ground state through hydrogen-bond formation. In the case of pyridazine, Brealy and Kasha (1955) have estimated the energy of the hydrogen bond between solute and solvent water molecules. Similarly, if the decrease in extinction coefficient of guanosine in D_2O is identified as resulting from an increased stabilization of the ground state by enhanced hydrogen bonding, the difference in energies of the H^2–H^1 bond may be estimated as

$$\Delta\epsilon = 285.7 \times 10^3\left(\frac{\lambda_2 - \lambda_1}{\lambda_1\lambda_2}\right) =$$

$$285.7 \times 10^3\frac{\Delta\lambda}{\lambda^2}\text{ kcal/mole} \tag{4}$$

$$\Delta\lambda = -\frac{\Delta\epsilon}{(\text{d}\epsilon/\text{d}\lambda)}$$

where, at the wavelength λ (A), Δϵ is the extinction coefficient difference and (dϵ/dλ) the first derivative of the absorption spectrum (Donovan *et al.*, 1961). For guanosine at 2900 A, these quantities are −355 l./mole and −54.2 l./mole A, respectively. Accordingly, it is estimated that the ground-state energy of guanosine

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[1] The spectra of DNA in D_2O and H_2O are reported to be identical (Falk, 1964); however, the spectral changes accompanying the change of solvent would be expected to be small, and thus undetectable in the comparison of absolute spectra, as reported by Falk (1964), or obscured by small differences in concentrations of the two samples.

is lowered by approximately 0.19 kcal/mole in D_2O relative to H_2O. This decrease in energy of the ground state is very similar to the excess enthalpy of solution of methanol-*d* in D_2O, relative to that of methanol in H_2O, of 0.140 kcal/mole reported by Benjamin and Benson (1963). Because of the numerous possibilities for H-bond formation involving guanosine as both donor and acceptor, the observed decrease in extinction coefficient reflects only the net difference in stabilization of the ground state in D_2O.

From the dependence of the difference spectrum upon the composition of the solvent, atom fraction deuterium, *n* (Figure 5), an equilibrium constant for the exchange of hydrogen-bonded H_2O and D_2O may be estimated as

$$\underset{a_1}{\mathrm{B} + \mathrm{HOH}} \rightleftarrows \underset{f_1}{\mathrm{B}\cdots\mathrm{HOH}} \quad k_1 \qquad (5a)$$

$$\underset{a_2}{\mathrm{B} + \mathrm{HOD}} \rightleftarrows \underset{f_2}{\mathrm{B}\cdots\mathrm{HOD}} \quad k_2 \qquad (5b)$$

$$\underset{a_2}{\mathrm{B} + \mathrm{HOD}} \rightleftarrows \underset{f_3}{\mathrm{B}\cdots\mathrm{DOH}} \quad k_3 \qquad (5c)$$

$$\underset{a_4}{\mathrm{B} + \mathrm{DOD}} \rightleftarrows \underset{f_4}{\mathrm{B}\cdots\mathrm{DOD}} \quad k_4 \qquad (5d)$$

for which an apparent equilibrium constant can be defined as

$$K_{\mathrm{app}} = \frac{k_4a_4\left(1 + \dfrac{k_3a_2}{k_4a_4}\right)}{k_1a_1\left(1 + \dfrac{k_2a_2}{k_1a_1}\right)} = \frac{k_4}{k_1}\left(\frac{n}{1-n}\right)^2\left[\frac{1 + 2\dfrac{k_3}{k_4}\left(\dfrac{1-n}{n}\right)}{1 + 2\dfrac{k_2}{k_1}\left(\dfrac{1-n}{n}\right)}\right]$$

where the activities of the various isotopic species of water have been taken as $a_1 = (1 - n)^2$, $a_2 = 2n(1 - n)$, $a_4 = n^2$ (Purlee, 1959). For the case of $n = 0.5$ and $k_3/k_4 \simeq k_2/k_1$, K_{app} corresponds to that for the exchange equilibrium

$$\mathrm{B}\cdots\mathrm{HOH} + \mathrm{DOD} \rightleftarrows \mathrm{B}\cdots\mathrm{DOD} + \mathrm{HOH} \qquad K = k_4/k_1 \quad (6)$$

The data of Figure 5 indicate that one-half the total absorbance change occurs at an atom fraction of deuterium of 0.5, or $K \approx K_{\mathrm{app}} = 1$, and $\Delta F° = 0$. From the estimated decrease in the energy of the ground state in D_2O of 0.19 kcal/mole, the entropy change accompanying the transfer would be $-0.19/298$, or -0.6 eu.

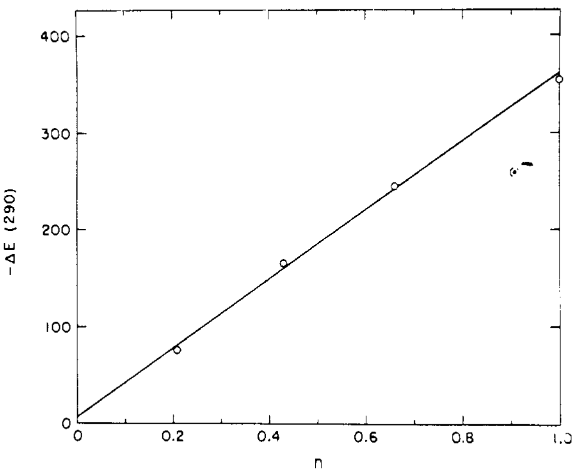

FIGURE 5: Difference spectrum of guanosine in D_2O–H_2O, as a function of atom fraction D, 25°.

Although no consideration has been given to the isotope effect of the six exchangeable hydrogens (two 2-amino-, 1-imino-, and 2′-, 3′-, and 5′-hydroxyls) on the thermodynamic and spectral properties of guanosine, similar effects will be present in the comparison of DNA in D_2O and H_2O. Thus, the isotope effect on the guanosine cation is seen to be predominantly entropic in origin. The entropy of ionization in D_2O is approximately 2 eu more negative in this solvent than in H_2O and approximately half of this difference is associated with the transfer of neutral guanosine from H_2O to D_2O.

The results for the various thermodynamic functions for the ionization of the guanosine cation, listed in Table II, in H_2O and D_2O, deserve some comment with regard to possible structures and solvent–solute interactions in the two systems. In the case of adenine, introduction of a methyl group at the nine position has no effect on the p*K* of the cation (Mason, 1957), and ribose decreases the p*K* for this dissociation by about 0.5 (Jordan, 1955). Introduction of a ribose group on guanine, however, decreases the p*K* for the dissociation of a proton from the cation by about 1.0, with almost no effect on the dissociation of a proton from the neutral base (Jordan, 1955). If protonation occurs at the N-3 position of the purine base, the effect of ribose is consistent with steric interference of hydration of the nucleoside cation, relatively less interference of hydration of the anion resulting from the dissociation of the N_1H proton.

Examination of the literature reveals only a few instances in which the temperature dependence of $pK_D - pK_H$ has been investigated, namely H_2O (Kirshenbaum, 1951), benzoic acid (Rule and LaMer, 1938), and acetic acid (Gary *et al.*, 1965). Thus, only limited information is available on the relative contribution of enthalpy and entropy changes to the free energy of ionization in the two solvents. The enthalpic and entropic contributions to the over-all isotope effect on the free energy of ionization at 25° are summarized in Table III for these three acids and for the guanosine

TABLE III: Enthalpic and Entropic Contributions to Ionization in D_2O.[a]

	$\Delta(\Delta H°)^f$	$-T\Delta(\Delta S°)^f$
H_2O^b	940	0
CH_3COOH^c	373	388
$C_6H_5COOH^d$	180	510
Guanosine·H^{+e}	0	570

[a] 25°. [b] Kirshenbaum (1951) [c] Gary *et al.* (1965). [d] Rule and LaMer (1938). [e] This work. $-T\Delta(\Delta S°) = 4.575T(\Delta pK)$. [f] In calories/mole.

cation. If the observed increase in the entropy of *protonation* of guanosine in D_2O arises from different degrees of order in the two solvents, then it is plausible that such solvent isotope effects might be more pronounced in the isothermal acid-induced helix–coil transition of protonated DNA than in the thermally induced transition of unprotonated DNA.

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