Amino Protons of Cytosine. Chemical Exchange, Rotational Exchange, and Salt-Induced Proton Magnetic Resonance Chemical Shifts of Aqueous 2′,3′-Cyclic Cytidine Monophosphate†

Bruce McConnell* and Patricia C. Seawell

ABSTRACT: Low rates of rotation and solvent exchange establish separate well-resolved proton magnetic resonance (pmr) absorbances of the amino protons of aqueous 2′,3′-cCMP. Signal intensities and line widths for these protons become equal and temperature independent in neutral salt solutions at temperatures between −15 and −26°. Accordingly, all modes of exchange in the temperature range −26 to −5° can be reduced to permit measurement of environmental influences on the individual chemical shifts. Although temperature-induced upfield shifts of their pmr absorbances are the same, the two amino protons are quite different in their response, both in their tendency for solvent exchange and in their perception of the environmental effects of chaotropic salts. These results are viewed as a reflection of intermolecular association of the nucleotide, which involves only one of the amino protons in the hydrogen-bonded intermolecular complex. The other proton is hydrogen bonded to free solvent. At 0° the rate constant for OH^- catalysis is about 10^9 M^{-1} sec^{-1} for the downfield proton and much less for the upfield proton.

Recent independent reports have shown that the amino protons of biologically active heterocyclic bases provide observable proton magnetic resonance (pmr) signals in aqueous solution because of unexpectedly slow exchange of these protons with water hydrogens. Exchange rates measured for the amino protons of thiamine (Suchy *et al.*, 1972) and for adenosine and guanosine monophosphates (McConnell and Seawell, 1972) correspond to catalytic rate constants that are several orders of magnitude lower than diffusion-controlled constants typical of aliphatic and aromatic amines.

Results with the purine compounds have a direct bearing on extensive hydrogen exchange data accumulated for double helical polynucleotides. Slow intrinsic exchange of the amino protons of adenine and guanine could account largely for the fact that the non-hydrogen-bonded amino protons of polynucleotides can be measured by rather slow kinetic methods (Hanson, 1971; Englander *et al.*, 1972; Englander and von Hippel, 1972). The exchange of the less accessible protons between the helical strands does not appear to be greatly reduced from intrinsic exchange of the monomeric units (McConnell and von Hippel, 1970a,b). This conclusion has led to a reconsideration of possible structural and chemical events that initiate exchange in the macromolecule (McConnell and Seawell, 1972). The formulation of a mechanism for hydrogen exchange in the double helical polynucleotide will require further data on the intrinsic exchange of the imino protons of thymine and guanine and on the amino protons of cytosine. Measurements of the latter would account for the remaining amino protons of DNA.

In this study we report pmr observations on the amino protons of 2′,3′-cyclic cytidine monophosphate in aqueous solution. Because of slow rotation of the C-4–amino nitrogen bond, each amino proton provides its separate, well-resolved pmr resonance at 100 MHz below 0°. This resolution was advantageous for determining that each amino proton was different from the other with regard to (1) catalysis of solvent exchange by OH^- and (2) upfield shifts induced by salts that are general denaturants of macromolecules and perturbants of water structure (von Hippel and Schleich, 1969). The salt effects lead to the conclusion that one of the amino protons is involved in intermolecular association of the nucleotide. The other proton is not involved in the association, but is hydrogen bonded to solvent.

Methods and Materials

Solutions of 2′,3′-cCMP[1] (sodium salts, Sigma) were made with double distilled water and filtered on Millipore HA 0.45μ before pH adjustment. Nucleotide concentrations were determined by A_{260} with product data sheets as a source of extinction values. The solution pH was measured at 0° or at room temperature with a Beckman Model 1019 Research pH Meter before and after the nuclear magnetic resonance (nmr) spectra were obtained. Most of the experiments were performed on solutions containing high concentrations of neutral salts, which contain varying amounts of metal ion contaminants, notably Fe. Some metal contents were as follows: sodium perchlorate (G. F. Smith Anhydrous Reagent), 0.001% Fe; sodium thiocyanate (Baker Reagent), 0.0002% Fe; sodium bromide (Baker Reagent), 0.001% Fe, 0.001% Ba; sodium chloride (Mallinckrodt AR), 0.0002% Fe, 0.001% Ba. Although these salts were used without further purification, their concentration effects (see text) were not dependent upon the contaminating ion concentrations. For example, the addition of 10^{-4} M Fe and 10^{-5} M Cu to a nucleotide solution containing 3 M sodium perchlorate did not change values of chemical shift or line width for any of the protons observed. Also, passage of 2′,3′-cCMP[1] solutions through columns of Dowex

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† From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii 96822. *Received June 16, 1972.* This investigation was supported by National Science Foundation Research Grant No. GB-20164.

[1] Abbreviations used are: 2′,3′-cCMP, 2′,3′-cyclic cytidine monophosphate; $\Delta\nu_{1/2}$, half-height width (hertz) of the proton resonances; $\delta\nu_e$, peak separation (hertz) of the separate 100-MHz absorbances of the amino protons of 2′,3′-cCMP; $\delta\nu$, the limiting, low-temperature value of $\delta\nu_e$.

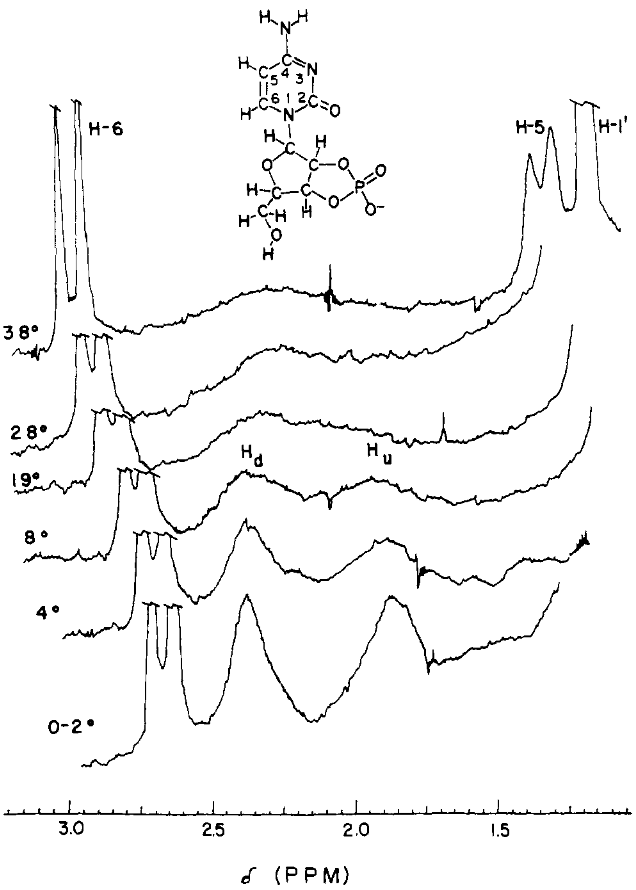

FIGURE 1: The effect of temperature on the 100-MHz pmr spectrum of aqueous 2′,3′-cCMP. The solution was 0.36 ± 0.03 M nucleotide (pH 6.18 ± 0.05) (measured at room temperature). Signal intensities are not exactly in the same scale. Resonance positions are relative to water protons (lock) at 0 ppm.

50-X2 (Bio-Rad) resulted in pmr spectra identical with that of the untreated nucleotide, which were obtained both in the presence and absence of neutral salt. Solutions containing sodium perchlorate were adjusted to the desired pH with the use of agar reference electrode bridge (McConnell and von Hippel, 1970b). The concentrations of sodium chloride, sodium thiocyanate, and sodium perchlorate were obtained by direct weighing. Measurements of pmr chemical shifts and of line widths were insensitive to pH and concentration at the respective values used throughout this study, *i.e.*, pH 6.2 ± 0.01 and 0.36 ± 0.03 M. Pmr spectra from the Varian HA 100 nmr spectrometer were obtained in frequency sweep mode and locked on solvent water. Temperature control was maintained with a Varian variable temperature controller and was monitored by a modified V-6040 Varian temperature control unit that provided a digital readout from a probe thermocouple and bridge. Isothermal measurements of the water proton chemical shifts in different salt solutions were determined with a Wilmad precision bore concentric tube (5 mm o.d.) containing Me_4Si as external standard in the inner sealed tube and the salt solution in the annular space. Chemical-shift measurements of the cytosine and adenine protons in salt solutions were taken relative to water.

Results

The 100-MHz pmr absorbance of the amino protons of 2′,3′-cCMP at 28° is quite broad (Figure 1). This is in marked contrast to the much narrower absorbances obtained for the corresponding protons of 2′,3′-cyclic phosphate derivatives of adenine and guanine (McConnell and Seawell, 1972). As the temperature is lowered, this broad resonance resolves into two separate absorbances that become narrower at 4° and integrate into approximate one-proton areas separated by 0.5 ppm at 0°. Such resolution of the amino protons is a manifestation of different magnetic environments for each proton due to slow rotation about the N–C-4 bond and has been observed by other workers for 1-methylcytosine in Me_2SO and dimethylformamide (Miles *et al.*, 1963; Shoup *et al.*, 1971), and recently for 5′-CMP in aqueous solution (Raszka and Kaplan, 1972).

It is apparent from the 0° spectrum of Figure 1 that both rotational and solvent exchange may be close to the slow exchange limits. The ratio of peak intensity to central minimum intensity is well over ten, which indicates that rotation at or below this temperature may be too slow to seriously affect separation of the absorbances (Allerhand *et al.*, 1966; Jackman, 1970). Solvent exchange must be at the slow lifetime-broadening limit, also; otherwise, absorbances separated by 2 ppm from the water proton absorbance would be broadened to much larger line widths before moving toward the water resonance (Johnson, 1965).

At least three different processes other than transverse relaxation govern the line shape of the amino proton absorbances: restricted rotation, proton exchange with solvent, and tautomeric equilibrium involving a residence time for one of the amino protons on the N-3 of the pyrimidine ring (Lee *et al.*, 1971, 1972). For the moment we defer the spectral assignment of the amino protons (see Discussion) and identify each as H_d, the proton corresponding to the downfield resonance, and H_u, the proton that provides the upfield absorbance (Figure 1). If all exchange processes at lower temperatures are sufficiently slow to exert minimal broadening of the H_d and H_u resonances, then the greater line width of the latter may reflect a shorter lifetime due to chemical exchange. Therefore, it appears feasible to assess and compare approximate lifetimes associated with rotational and chemical exchange separately and to study their relationship by direct observation of the amino proton resonances.

Factors Affecting Rotation. Spectra obtained at temperatures above 0° do not provide sufficient data for estimation of rotation rates for two reasons. First, there is no definite indication that the temperature effect on the spectral position of the separate amino protons is due to rotation alone. It is possible that temperature-induced shifts that might be environmentally related are not equal. Second, limiting equations (Jackman, 1970) that deal with narrow lines would not apply, since the necessary parameters include line widths, which are large in this case (Gutowsky and Holm, 1956). Both these problems also obscure the required estimate of the peak separation in the absence of rotation.

To surmount these problems and obtain a more favorable ratio of peak separation $(\delta\nu_0)$[1] to line width $(\Delta\nu_{1/2})$, spectra were obtained at temperatures as low as −26°, which was made possible by the addition of neutral salts. Typical of these are spectra of 2′,3′-cCMP in 4 M NaCl obtained between 0 and −23° (Figure 2). It is noteworthy that the line width of H_u becomes narrower and equal to that of H_d at −18 and −23°, which indicates that the greater width of the H_u resonance at the higher temperatures derives from a chemical exchange process (see below). Both the separate line widths are sufficiently reduced to exert little effect on peak separation or rotational coalescence, *i.e.*, $\delta\nu_0/\Delta\nu_{1/2} \approx 5$ (Allerhand *et al.*, 1966). Throughout the temperature range where this condi-

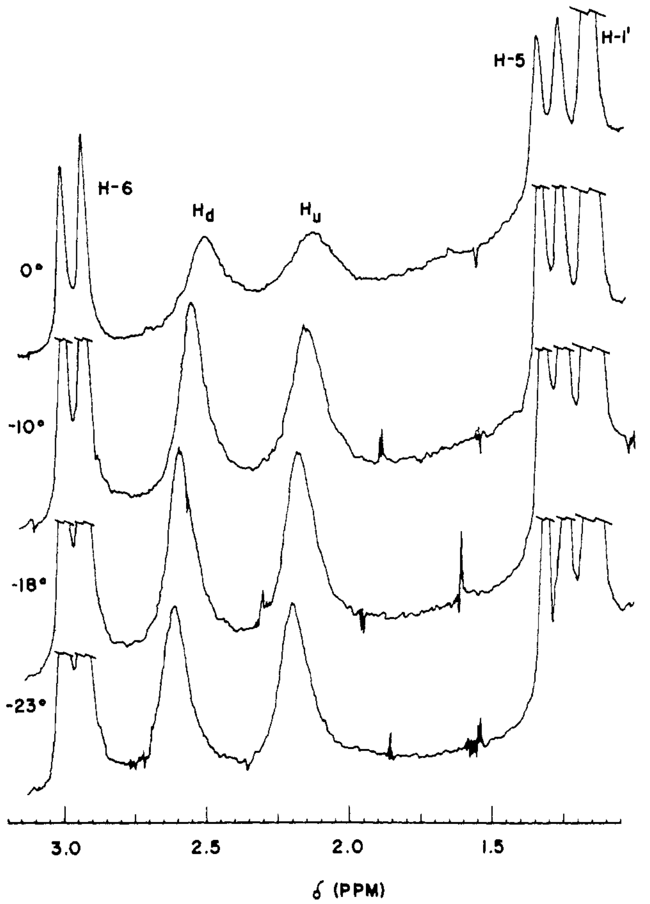

FIGURE 2: Low-temperature pmr spectra of aqueous 2′,3′-cCMP in the presence of 4 M NaCl. The solution contained 0.4 M nucleotide–0.01 M sodium cacodylate and was adjusted to pH 6.1 at 25°. Signal intensities are not exactly on the same scale. Resonance positions are relative to the water proton resonance (lock), which is at 0 ppm, for the 0° spectrum only.

tion holds, the temperature-induced chemical shifts are equal for H_d and H_u. This is illustrated in Figure 3, in which chemical shift is plotted against temperature for 2′,3′-cCMP in 4 M NaCl. Plots of the chemical shifts of H_u and H_d are parallel below 0° and begin to converge above this temperature because of rotational coalescence.

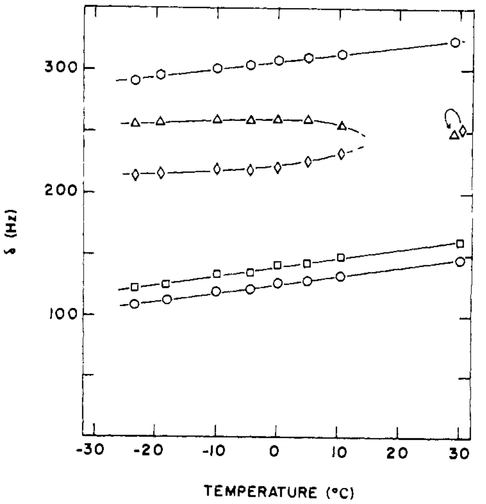

FIGURE 3: The effect of temperature on the chemical shift, δ, of protons of 2′,3′-cCMP in 4 M NaCl. Increasing chemical-shift values on the oridinate are expressed in hertz downfield of the water proton signal (lock). Conditions are the same as those used for Figure 2: (○) H-6; (△) H_d (amino); (◇) H_u (amino); (□) H-5; (○) H-1′.

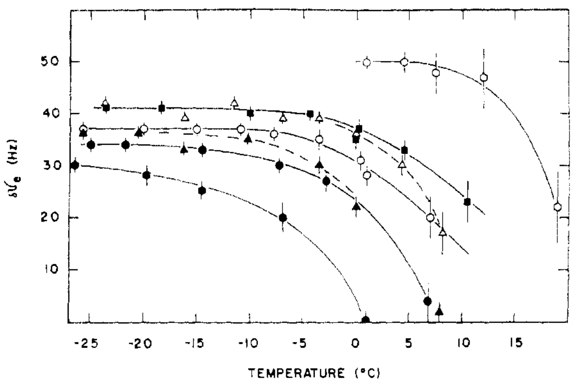

FIGURE 4: The effect of temperature on peak separation ($\delta\nu_e$) of the amino proton pmr signals for different salts. All solutions contained 0.4 M 2′,3′-cCMP–0.01 M sodium cacodylate and were adjusted to pH 6.1 at 25°: (○) no added salt; (△) 3 M NaSCN; (■) 4 M NaCl; (○) 3 M $NaClO_4$; (▲) 5 M NaSCN; (●) 4 M $NaClO_4$; (●) 6 M $NaClO_4$.

Figure 3 shows an approximately equal apparent downfield shift for the resonances of the H-6, H-5, and H-1′ protons of cCMP. As will be seen in other plots of this type, this downfield trend is not real, since it reflects the temperature-induced upfield shift of the water proton resonance, which is always adjusted to the same frequency to provide the lock signal. At pH ≥ 6 the actual shift response of the H-6 resonance is negligible for our purposes. Consequently, the comparatively small downfield trend for both the H_u and H_d resonances is actually a marked upfield trend induced by increases in temperature. This upfield shift for the amino protons is consistent with the notion that, like the water protons, the amino protons are involved in hydrogen bonding, which decreases with increasing temperature to give upfield shifts (Shoolery and Alder, 1955). The constancy of $\delta\nu_e$ in the low-temperature range suggests that the hydrogen-bond breakage induced by temperature is equal for H_u and H_d. That their equal response is not an artifact arising from saturation of the shift response of the water protons is supported by the linearity of the apparent shift for H-6 from −23 to +10°.

It is apparent that a specific salt effect is expressed upon the amino protons of the pyrimidine, since the largest value of $\delta\nu_e$ obtained in the 4 M NaCl solution is considerably smaller than the peak separation obtained even at higher temperature without added salt (Figure 1). It can be shown from a plot of $\delta\nu_e$ *vs.* temperature for a variety of sodium salts that this effect is a function of the nature and concentration of the anionic component of the salt (Figure 4). The limiting low-temperature value of $\delta\nu_e$ ($\delta\nu$) is 50 Hz for no added salt, 41 Hz for 4 M NaCl, 42 Hz for 3 M NaSCN, 34 Hz for 4 M $NaClO_4$, and 30 Hz for 6 M $NaClO_4$.

The decrease in $\delta\nu_e$ that results from the addition of salt could be imagined as arising from alterations in rate processes such as an increase in rotation rate. Although this is conceivable, the possibility is excluded largely by the constant value of peak separation in the low-temperature range −25 to −15° (Figure 4). According to the analysis of Gutowsky and Holm (1956), a decrease in $\delta\nu$ from 41 (4 M NaCl) to 34 Hz (4 M $NaClO_4$) due to increased rotation would be followed by a sharply decreasing value of $\delta\nu_e$ for perchlorate at these temperatures. This is not the case; $\delta\nu_e$ is fairly constant for 4 M $NaClO_4$ between −25 and −15°. Since rotational coalescence

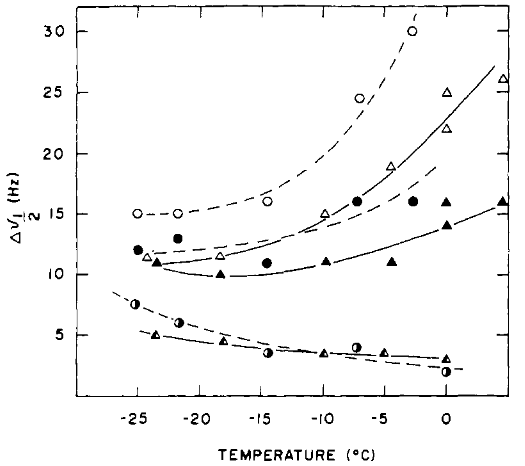

FIGURE 5: The effect of temperature on line width, $\Delta\nu_{1/2}$, of 100-MHz proton resonances of 2′,3′-cCMP for two salt conditions. Solutions were prepared as described for Figure 4: (○) H_u, 4 M $NaClO_4$; (●) H_d, 4 M $NaClO_4$; (◑) H-6, 4 M $NaClO_4$; (△) H_u, 4 M NaCl; (▲) H_d, 4 M NaCl; (◭) H-6, 4 M NaCl.

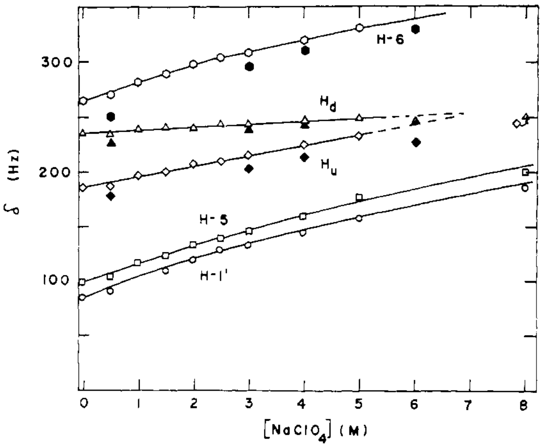

FIGURE 6: The effects of $NaClO_4$ concentration on 100-MHz pmr chemical shifts, δ, of 2′,3′-cCMP at two temperatures. All solutions were 0.36 ± 0.03 M 2′,3′-cCMP and 0.01 M sodium cacodylate, and were adjusted to pH 6.24 at 0°. Increasing values on the ordinate are expressed in hertz downfield of the water reference (lock). These were measured for the same solutions at two temperatures: 0° ± 1° (open symbols) and −7 ± 0.5° (closed symbols).

is defined by line width as well as by peak separation, support of the idea that rotation is not increased by salt also requires that, at a given temperature, calculated rotation rates are the same regardless of the amount of salt present. This would verify the fact that the remarkably large decrease in coalescence temperature (seen as $\delta\nu_c \rightarrow 0$, Figure 4) associated with the addition of salt is not due to increased rotation, but to the salt-induced decreases in $\delta\nu$ (and increases in $\Delta\nu_{1/2}$) at constant rotation.

Calculations were made by application of measured values of $\delta\nu$, $\delta\nu_c$, and $\Delta\nu_{1/2}$ to the numerical method of Gutowsky and Holm (1956) (G–H) by assuming that the signal intensities for H_u and H_d were sufficiently similar at the low temperatures to validate the use of the G–H method. The central problem in applying the G–H approximation is the selection of a single "natural" line width from the two, unequally broad H_d and H_u resonances. Our approach is to accept all line broadening processes other than rotation as those defining the "natural" line width. For H_d, the "natural" line width includes contributions from field inhomogeneity, solvent exchange (negligible at low temperatures, see below), dipolar broadening at low temperatures in high salt, and possible quadrupolar broadening. Non-exchange-broadening contributions were considered to be the same for H_u under all salt conditions. Therefore, the greater width of the H_u resonances must be due to extra exchange (see below), which is to be included in the "natural" line width for this proton. Accordingly, estimation of the G–H "natural" line width involved (1) selecting a "natural" line width of 6 Hz for the H_d resonance, (2) subtracting this value from the observed H_d line width to get rotational broadening for this absorbance, (3) subtracting the rotational broadening from the H_u line width to obtain its "natural" line width (which includes the extra nonrotational exchange contribution), and (4) averaging the "natural" line widths for the H_d and H_u resonances to obtain the required parameter for the G–H approximation.

Two assumptions required for this method of approximation are that the dipolar broadening was small and that the same "natural" H_d line width (6 Hz) applied for all salt conditions. These assumptions can be justified from inspection of Figure 5, in which the line widths of the H_u, H_d, and H-6 resonances are plotted against temperature for 4 M NaCl and 4 M $NaClO_4$. That the "natural" line width for H_d applies to all salts is supported by the similarity of the H_d line width at low temperature for both salt conditions. Regarding dipolar broadening, the increase in the H-6 resonance line width is less than 2 Hz from 0 to −20°. It is presumed to arise from the salt-induced field inhomogeneity at low temperatures and is reflected in all line widths.

The calculated rotation rates and the parameters used foı the G–H approximation are listed in Table I for three temperatures under different salt conditions. The selection of 6 Hz as the value for the "natural" line width of H_d and its applicability to all salt conditions are justified by (1) agreement between rotation rates calculated from the line broadening and from the G–H method, which is unique for the 6-Hz value (Table I), and (2) the fact that smaller assumed values would accentuate the difference in rotation rates between salt conditions at a given temperature. However, even with lower assumed values of "natural" line width it is clear that any difference between various salt conditions regarding rotation rates is marginal, if it exists at all. The rotational exchange rate for each proton is about 35 sec^{-1} for no salt at 0° and could be the same value for 4 M NaCl at −15°. Only a marginal trend toward higher rotation rates is indicated for higher temperatures. Therefore, we conclude that the profound effect of salt on $\delta\nu$ and on the coalescence temperature arises largely from the environmental effects imposed by salt that affect the chemical shifts of the two amino proton resonances independently. The order of salt effect as obtained from the values of $\delta\nu$ (Figure 4) is $ClO_4^- > SCN^- > Cl^- >$ no added salt.

The reason for the salt-induced decrease in peak separation for the H_d and H_u resonances is illustrated in Figure 6, in which chemical shift is plotted against sodium perchlorate concentration at constant temperature. Once again, the marked downfield trend of the H-6, H-5, and H-1′ shifts is essentially a reflection of the upfield trend of the water proton signal used for the lock. Upon correcting for the water shift, it becomes apparent that the H_d resonance shows a much stronger upfield shift than does the H_u resonance in response to salt. There is a linear approach to convergence of the two resonances, which is completed between 6 and 8 M perchlorate. Plots of this type are similar for other salts, except that the salt concentration at convergence of the H_u and H_d plots differs according to the salt.

TABLE I: Rotational Parameters for 2′,3′-cCMP[a] at −15, −8, and 0°.

	−15°				−8°				0°				
	4 M NaCl	4 M NaClO$_4$	5 M NaSCN	6 M Na-ClO$_4$	4 M NaCl	4 M Na-ClO$_4$	5 M Na-SCN	6 M Na-ClO$_4$	No Salt	4 M NaCl	4 M NaClO$_4$	5 M NaSCN	6 M NaClO$_4$
Obsd line width													
$(\Delta\nu_{1/2})_{\mathrm{H_d}}$	10	11	11	13	11	16	13	<17[b]	14	16	<18[b]	<20	
$(\Delta\nu_{1/2})_{\mathrm{H_u}}$	11	16	13	17	15	24	19	<26[b]	21	26	<30	<30	
$(1/\pi T_2)_{\mathrm{av}}$[c] (Hz)	7	8	7	8	8	10	9	11	13	11	12	11	15
$\delta\nu$[d] (Hz)	41	34	36	30	41	34	36	30	50	41	34	36	30
$\delta\nu_e$[d] (Hz)	>40	>32	>34	24	40	30	36	18	>48	37	23	24	0
$1/2\tau_r$[e] (G–H)	<19	<19	<22	34 ±2	<27	26 ±6	25 ±5	41 ±2	<35	29 ±6	42 ±3	48 ±3	>45
$1/2\tau_r$ $(\Delta\nu_{1/2})$	13	16	16	22	16	31	22	35	20	31	38	44	

[a] All samples were 0.36 ± 0.03 M 2′,3′-cCMP and 0.01 M sodium cacodylate (pH 6.2) in the salt solutions indicated. [b] Overlap of the H$_u$ and H$_d$ absorbances prevented accurate measurement of $(\Delta\nu_{1/2})$. Maximum values. [c] $(1/\pi T_2)_{\mathrm{av}} = (1/\pi T_2)_{\mathrm{H_u}} + (1/\pi T_2)_{\mathrm{H_d}}$, where $(1/\pi T_2)_{\mathrm{H_d}} = 6$ Hz (see text) and $(1/\pi T_2)_{\mathrm{H_u}} = (\Delta\nu_{1/2})_{\mathrm{H_u}} - [(\Delta\nu_{1/2})_{\mathrm{H_d}} - 6]$. The latter term in brackets represents the rotational broadening of H$_d$, which should be the same for H$_u$. [d] Values of $\delta\nu$ and $\delta\nu_e$ vary ±1 Hz, which represents the main source of error, since $\delta\nu_e/\delta\nu$ is the parameter used in the (G–H) method. The corresponding error in $1/2\tau_r$ is small when $\delta\nu_e/\delta\nu < 0.6$. [e] Rotation rate obtained graphically from the method of Gutowsky and Holm (1956) after conversion of $[1/\pi T_2]$, $\delta\nu$, and $\delta\nu_e$ into radians. The rate obtained is $1/\tau_r = (\tau_d + \tau_u)/\tau_d\tau_u$, where τ_d and τ_u are the rotational lifetimes of H$_d$ and H$_u$, respectively. Since they are equal, $1/2\tau_r$ is the rate for each. [f] Rotation rate calculated directly from the observed line width, *i.e.*, $1/2\tau_r = [(\Delta\nu_{1/2})_{\mathrm{H_d}} - 6]$, where 6 Hz is the "natural" line width of the H$_d$ resonance (see text).

TABLE II: Salt-Induced Changes[a] in Chemical Shift for the Pyrimidine Protons of 2′,3′-cCMP.

	Obsd (2–4 M Salt)		Corrected[d] (1–4 M Salt)				
Salt	H_2O[b]	H-6[c]	H-6	H$_d$	H$_u$	H-5	H-1′
NaCl	+14	−17	0	+14	+3	−2	−2
NaBr	+17	−17	0	+22	+7	−5	0
NaSCN	+14	−26	0	+25	+8	0	0
NaClO$_4$	+23	−22	0	+33	+9	−1	0

[a] Upfield and downfield shifts are signified by + and −, respectively. All solutions were 0.36 ± 0.03 M 2′,3′-cCMP–0.01 M sodium cacodylate (pH 6.22), 0°. Values were accurate to ±1 Hz. [b] Relative to Me$_4$Si from separate experiments (see Methods). The salt concentration range was 2–4 M, since data for 1 M were not available. [c] Relative to water (lock) for the salt concentration range 2–4 M. [d] Relative to H-6 for the salt concentration range 1–4 M.

Under certain circumstances these salt effects on the chemical shift of the amino proton resonances may be different than the effects of temperature. As we have seen in 4 M NaCl, the peak separation is constant with temperature, if the measurements are taken at the slow rotation limit (Figure 3, below −5°). Although the data in Figure 6 show that extrapolation to zero salt concentration provides the same (51 Hz) peak separation at both 0 and −7°, it does not follow that peak separation is constant with temperature in the absence of salt. In terms of water structure the range of salt concentration used here corresponds to much larger temperature ranges than are accessible to these studies (Gordon *et al.*, 1965). The temperature-induced shifts are too small to determine clearly that they are the same for each proton in the absence of salt.

It is possible that at the higher temperatures (>0°) the environmental effects of temperature on the amino protons of 2′,3′-cCMP are the same as the effect of salts that mimic temperature in their ability to disrupt water structure (Gordon *et al.*, 1965).

A comparison of the salt concentration effects at constant temperatures for different salts is summarized in Table II. Table II also contains a comparison of H-6 and water proton shifts to verify that the apparent downfield shift of the H-6 resonance seen in Figures 3 and 6 reflects the upfield shift for the water protons. This comparison was made by using a Me$_4$Si external reference to measure chemical shifts of the water protons in the same nucleotide-salt solutions. The notable exception to this quantitative similarity in the H-6 and water shifts is seen in the case of NaSCN. However, if the shifts for *all* salts are corrected for the apparent H-6 downfield trend, then the comparison of the anions follows the same order obtained from Figure 4, *i.e.*, NaClO$_4$ > NaSCN ≃ NaBr[2] > NaCl. This order of effect is reflected almost exclusively in the H$_d$ proton shift, since the magnitude of its response is much greater than that for the H$_u$ resonance (Table II).

Solvent Exchange. Solvent exchange can be separated conceptually from rotation, since, owing to the large reservoir of solvent protons, it would not represent a mechanism for the exchange of the amino protons with each other. Therefore, as a first approximation, rotational exchange would not be expected to be catalyzed by solvent exchange catalysts (*e.g.*, H_3O^+ and OH^-). This expectation must be qualified by the consideration that tautomeric exchange may link rotational exchange to solvent catalysts. Tautomeric exchange may respond to solvent catalysts (Lee *et al.*, 1971) and could be directly related to rotation under certain conditions. How-

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[2] Data for NaBr were obtained only in measurements of chemical shift as a function of salt concentration at 0°.

TABLE III: Effect of pH on the Pmr Line Widths (100 MHz) for the Amino Protons of 2′,3′-cCMP.[a]

Solution pH	Buffer	Temp (°C)	$\delta\nu_e$[b]	$\Delta\nu_{1/2}$[c] H_d (Hz)	$\Delta\nu_{1/2}$[c] H_u (Hz)
4.15	None	0 ± 1	54 ± 2[d]	18 ± 2	*d*
5.20	None	0 ± 1	51 + 2[d]	*d*	20 ± 2
6.25[a]	None	0 ± 1	49 ± 1	13 ± 2	18 ± 3
6.25	0.01 M sodium cacodylate	2 ± 1	50 ± 1	13 ± 2	21 ± 1
7.10[a]	None	0 ± 1	48 ± 1	12 ± 2	17 ± 3
7.70	None		51 ± 1	21 + 2	17 ± 3
4.91	None	28 ± 1		40[e]	
6.09	None	28 ± 1		35 ± 5	
6.08	0.01 M sodium cacodylate	28 ± 1		35 ± 5	
6.25	0.5 M sodium phosphate	0 ± 1		Too broad to detect	
6.05	0.5 M sodium phosphate	28 ± 1		Too broad to detect	

[a] All concentrations of 2′,3′-cCMP were 0.36 ± 0.03 M, except for samples at pH 6.25 and 7.10, whose cCMP concentrations were 0.2 M. [b] Peak separation of the H_d and H_u resonances (Hz). [c] Signal width at half-height. The signal intensity was determined by adjusting the base line for a one-proton area of the absorbance. [d] Hidden behind the H-6 absorbance due to a large selective downfield shift of *both* protons at pH values below 6. [e] A single, broad absorbance for the two coalesced amino protons at the limits of detectability because of the large width.

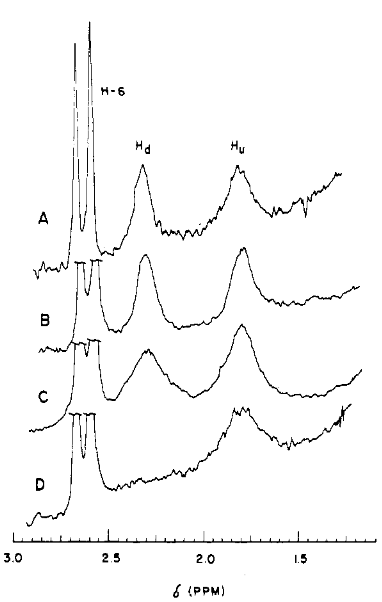

FIGURE 7: The effect of pH on the 100-MHz pmr spectrum of 2′,3′-cCMP. The nucleotide concentration was 0.35–0.4 M in unbuffered solutions. Spectra were obtained at 0° for pH values determined before and after the experiment: (A) pH 7.0 ± 0.1; (B) pH 7.3 ± 0.1; (C) pH 7.8 ± 0.1; (D) pH 8.2 ± 0.1.

ever, for purposes of assessing the contribution of solvent exchange to line shape at low temperature this qualification is not necessary, since it can be seen from Table III that the pH dependence of line width for the H_d and H_u resonances is not large. Between pH 4 and 7.7 some broadening of both protons does occur in response to increased H_3O^+ and OH^-. This indicates that the solvent exchange rates are in the slow lifetime broadening limit and can be estimated directly from the line width (Johnson, 1965). Although the pH-induced change in line width is larger than that seen in the case of cAMP and cGMP at the same temperatures (McConnell and Seawell, 1972), rate constants corresponding to this variation (about 10^9 M^{-1} sec^{-1}) would give a chemical exchange rate of ≤ 10 sec^{-1} under the conditions used to measure rotation (0°, pH 6.2 ± 0.1). Such a rate corresponds to a line broadening contribution of only a few hertz at 0° and essentially no contribution at lower temperatures. Therefore, line broadening observed for H_d from −25 to −5° must be due only to rotational exchange. Rotation does not appear to be affected by small changes in pH that may be coincident with changes in temperature and salt concentrations. Values of $\delta\nu_e$ and $\Delta\nu_{1/2}$ are constant from pH 5 to 7 (Table III). Below pH 5 increases in $\delta\nu_e$ reflect a larger downfield trend in the H_d chemical shift because of protonation of N-3 cytosine (B. McConnell and P. C. Seawell, manuscript in preparation).

Because of much smaller pH-induced broadening the larger width of the H_u resonance must *not* be due to larger H_3O^+ and OH^- catalytic rate constants for solvent exchange. Indeed, for H_u the hydroxyl ion rate constant is definitely smaller than for H_d. This is illustrated in a comparison of the spectra at 0° for increasing pH values up to pH 8.2 (Figure 7). While the H_u resonance is the broader of the two at pH 7.0, it actually becomes narrower at pH 7.3 and persists as a broadened absorbance above pH 8.2 in spite of the broadening of the H_d resonance to disappearance. It is clear that the different responses of the two amino protons to the addition of solvent structure-breaking salts are paralleled in a striking manner by their response to increases in the concentration of hydroxyl ions. The H_d proton is much more sensitive, both in its salt-induced shifts and its broadening by OH^-.

Chemical Exchange of H_u. We have noted that the larger line width for H_u must be due to an exchange process, since it decreases to a value equal to that for H_d at low temperatures (Figure 2). Also, its width appears to be affected specifically by high concentrations of sodium perchlorate, especially at the higher temperatures, where the dipolar contribution is minimal (Table I and Figure 5). Depending upon the spectral assignment of the amino protons there are two rate processes that might contribute to the extra exchange broadening of the H_u resonance, *i.e.*, tautomeric exchange and pH-*independent* solvent exchange. There would be no influence of tautomeric exchange if H_u were assigned as the proton nearest H-5 of the cytosine ring. Otherwise, since both may contribute to unknown extents we must consider them together as chemical exchange. In any event, in the temperature range −26 to −10° and at pH 6.2 the total line width for H_u can be expressed as

$$(\Delta\nu_{1/2})_{H_u} = 1/\pi(1/T_2 + 1/\tau_s + 1/2\tau_r + 1/\tau_c) \qquad (1)$$

where $1/T_2$ provides the natural line width, which is the same for H_d and H_u (see Figure 2, at −25°), and $1/2\tau_r$ is the rotation rate, which must be equal for the two protons. The pH-dependent solvent exchange contribution, $1/\tau_s$, may not be the same for both, but it is negligible. Therefore, the chemical rate, $1/\tau_c$, could be obtained merely by subtraction of the H_d and H_u line widths, provided that the amino site for the H_u proton is populated to a much larger extent than is the ring

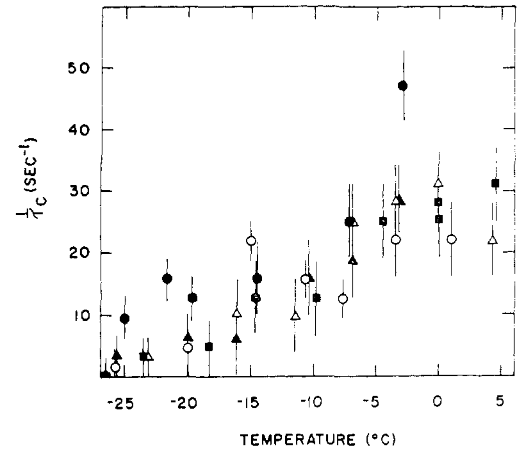

FIGURE 8: The effect of temperature on chemical exchange of H_u at different salt conditions. Chemical exchange is defined as tautomeric exchange or pH-independent exchange (see text). The rate constant, $1/\tau_c$, is defined as $\pi[(\Delta\nu_{1/2})_{H_u} - (\Delta\nu_{1/2})_{H_d}]$, where $(\Delta\nu_{1/2})_{H_u}$ and $(\Delta\nu_{1/2})_{H_d}$ are the line widths of the upfield and downfield protons, respectively. Conditions are as described for Figure 4: (⬢) 6 M $NaClO_4$; (●) 4 M $NaClO_4$; (○) 3 M $NaClO_4$; (△) 3 M NaSCN; (▲) 5 M NaSCN; (■) 4 M NaCl.

nitrogen site. Since the tautomer probably does not exceed 10% of the total (Lee *et al.*, 1972) and there appears to be no gross difference in the intensity of the two amino proton resonances, $1/\tau_c$ obtained by subtraction of the two line widths, *i.e.*, $\pi[(\Delta\nu_{1/2})_{H_u} - (\Delta\nu_{1/2})_{H_d}]$, should represent a fair approximation of chemical exchange between −25 and 0°. In Figure 8, these values of $1/\tau_c$ are plotted against temperature for a variety of salt conditions. The pseudo-first-order rate constant for chemical exchange, $1/\tau_c$, increases from less than 10 sec^{-1} at $<-15°$ to approximately 20 sec^{-1} at −5°, which is of the same approximate magnitude as the rotation rate (Table I). Above −5°, it is not possible to say whether there is further broadening.

As we have seen with rotation, there is marginal evidence that the chaotropic salts in high concentration may induce an increase in the rate of this extra exchange for H_u. In spite of the large error, the increase may be real, since there is about a twofold increase in rate in 6 M sodium perchlorate.

Discussion

A primary result of these studies is that salt effects on pmr chemical shifts of the amino proton resonances are selective and specific for the nature of the anion in a manner that corresponds to the ability of these salts to decrease water structure. In a comparison of concentration effects at 0° the order of effect is $NaClO_4 > NaSCN \simeq NaBr^2 > NaCl$ (Table II). This order of rank for the salts was obtained by using the H-6 shift as a correction for all direct and indirect salt effects other than hydrogen bonding. In all cases except NaSCN the H-6 shift largely reflected the salt-induced upfield shift of the water proton resonance. The shift to lower fields of the H-6 resonance induced by NaSCN was twice as large as that accounted for by the water proton shift and may arise from the large dipole effect possibly exerted in part through direct interaction of this particular salt with the nucleotide. In subtracting the H-6 chemical shift from that of the amino protons, we are assuming that this possible interaction affects all the protons of the molecule equally. Unequal proton chemical shifts due to stacking interaction are not observed with the pyrimidines (Schweizer *et al.*, 1965). There is no indication from our results that this salt produced selective broadening of particular protons, or that line widths are different from those measured in the presence of NaCl (Table I, Figure 5). We conclude that the use of the H-6 chemical shift as a correction for the amino proton shifts serves to isolate measurements of hydrogen bond breaking from other effects such as direct interaction between the salt and the nucleotide. This conclusion is supported by the fact that the same ranking[2] of the salts is obtained from independent measurements of the limiting low-temperature values of peak separation (Figure 4). This order of effect conforms to the ability of these salts to induce upfield shifts of the water protons (Schleich and von Hippel, 1970), but *not* to their ability to destabilize macromolecules (von Hippel and Schleich, 1969) or to increase solubility of the monomeric units of DNA (Robinson and Grant, 1966). In the latter cases the ranking of the salts is the same, except that NaSCN is more effective than $NaClO_4$.

What is the molecular basis of the salt-induced decrease in peak separation for the amino proton resonances? Since the influence of salt on exchange processes can be discounted, it is apparent that the two amino protons are in two different hydrogen bonding situations, one sensitive to salt and the other much more insensitive or resistant. That both are hydrogen bonded is evident from the similarity of their response to temperature (Figure 3). To some extent the effect of temperature would be nonspecific, since it is associated with increased translation of all molecules. Therefore, an explanation for the difference between the salt and temperature effects must be made within the context of intermolecular association of the nucleotide in aqueous solution.

The formation of an intermolecular complex would account for the fact that the two amino protons respond differently to the hydrogen bond breaking action of the salt. One of the protons participates in the complex and the other does not. If this is the case, then which of the protons is involved in the complex? The selection of H_u as the participant could account for its insensitivity to salt if presumptive upfield shifts induced by breaking of intermolecular H bonds could be nullified by their replacement by NH_2–H_2O bonds (see below). This choice would be consistent with the lower sensitivity of this proton to hydroxyl ion. The latter observation could be rationalized by the view that initiation of solvent exchange by the direct removal of a proton requires the formation of a hydrogen bond between the dissociable proton and its solvent acceptor, OH^- (Eigen, 1964). For H_u hydrogen bonding to OH^- would await the breaking of the bond between this proton and the complex, while H_d, already bonded to water, would respond more readily to increases in pH, as seen (Figure 7). The selection of H_u only as the proton hydrogen bonded to the complex could imply that the complex is stable during the extensive disruption of solvent structure induced by salt. This seems unlikely, since nonspecific intermolecular H bonds of aqueous polynucleotides are abolished by destabilizing salts (McConnell and von Hippel, 1970b). Implicit in this choice of roles for the amino protons is the reasonable expectation that the greater sensitivity of H_d to salt reflects a decrease in its hydrogen bonding to solvent.

Further arguments for the possibility that H_u is the proton involved exclusively in the intermolecular complex first require the spectral assignment of the amino protons. The assignment that appears to provide the most internally consistent and testable postulate is that H_u is the nontautomeric proton closest to H-5 of the cytosine ring and H_d is the proton closest to N-3 (see Figure 1). The reverse assignment could be argued on the basis that the upfield resonance would correspond to the proton shielded most by the extra electron density about N-3. However, our data show that the spectral position of H_d could be farthest upfield in a non-hydrogen-bonding solvent (Figure 6) and that its downfield position in water is a result of H-bond formation in opposition to the influence of its greater intramolecular shielding.

The assignment that the H_u proton is nearest H-5 and is the participant in the complex is in accord with the X-ray studies on the crystal of 2′,3′-cCMP (Coulter, 1973). In the crystal this proton can be hydrogen bonded to water within the complex, which, in turn, is involved in as many as four hydrogen bonds, all within the complex. A direct amino-NH–OP bond is formed by this proton, also. The extension of the crystal structure to aqueous solution is supported by the pmr data of Lavallee and Coulter (1973). Unlike the ribose portion of other pyrimidine nucleotides, the bicyclic ribose phosphate of 2′,3′-cCMP is locked in the syn conformation with respect to the pyrimidine ring. This would eliminate equilibrium conformations that would detract from the ordered structure necessary for such a marked segregation of the amino protons into their respective hydrogen bonding situations.

This assignment and intermolecular association involving H_u exclusive of H_d could account also for the greater line width of the H_u absorbance. It is reasonably clear that the extra line width of the H_u resonance is due to chemical exchange processes. The only alternative explanation for both its greater width and its narrowing at lower temperatures would be the interaction of this proton with the ^{14}N quadrupole, since the slower molecular motion at low temperatures would be accompanied by more rapid relaxation of the ^{14}N moment (Roberts, 1956). However, in this case the resultant low-temperature sharpening should be equal for the H_d resonance, which is of relatively constant width throughout the temperature range of -26 to $-5°$. If H_u is closest to H-5, then tautomeric exchange would not contribute to the extra width of its resonance. Since this proton can be hydrogen bonded directly to a phosphate acceptor in a neighboring cCMP molecule, we would expect a shorter proton lifetime reflecting a residence time for the proton at the –P–O site. Such exchange would be slow, since the proton acceptor is a much weaker base than the donor. This exchange would be pH independent at pH values far from the p*K* of the phosphate group. This pH-independent exchange is what we observe in the region of pH 4–8.

This postulate of the role and spectral assignment of the amino protons can be tested. Breaking the complex should (1) increase the sensitivity of H_u to salt-induced upfield shifts and (2) decrease the self-catalysis of H_u exchange. Studies in progress indicate that this may be the case. Protonation of N-3 renders both H_u and H_d sensitive to chaotropic anions (upfield shifts) and, because hydronium ion catalysis of solvent exchange is quite small, this protonation produces a dramatic sharpening of the H_u absorbance (B. McConnell and P. C. Seawell, manuscript in preparation). In view of this, the effect of perchlorate on line width is harder to explain. The specific broadening of the H_u resonance by high perchlorate concentrations (Figure 8) does not concur with the sharpening expected when the complex is disrupted. However, increased pH dependence of solvent exchange would be expected in this same circumstance. Studies to investigate this possibility are under way.

How is the chemical shift of the H_u proton resonance rendered insensitive to chaotropic salts by intermolecular hydrogen bonding? One possible explanation would be that disruption of the complex occurs in addition to general hydrogen bond breakage. The equilibrium shift to the dissociated form would bring about the replacement of intermolecular H bonds by NH_2–H_2O bonds. For example, the addition of perchlorate might eliminate one out of four water–hydrogen bonds (H_d–H_2O bonds). If, say, three out of four bonds at H_u are intermolecular, due to the complex, then breaking the complex by the addition of salt could result in three H_u–H_2O bonds (the same as for H_d) and one remaining H_u–cCMP bond, *i.e.*, no change. An assumption in this example is that the downfield shifts originally induced by the formation of H_u–cCMP bonds are equal to the shifts induced by the formation of H_u–H_2O bonds. This is reasonable, because the intermolecular H bonds involve water molecules (Coulter, 1973).

In any case the differential effects of destabilizing salts could be used in pmr experiments on polynucleotides, such as those reported for tRNA (Wong *et al.*, 1972a,b). The pmr absorbances of the amino protons that are hydrogen bonded in a nonspecific manner might be shifted selectively by the addition of salt for their identification, for simplification of overlapping spectra, and for verification of double helical regions. Solvent exchange can be virtually eliminated at low temperatures permitted by the addition of salt to aqueous solutions and rotational exchange can be reduced sufficiently to prevent its interference with chemical-shift measurements.

An objective of these pmr studies on aqueous cytosine that yet remains to be thoroughly elucidated is to determine whether or not the intrinsic solvent exchange of its amino protons is as slow as that of the corresponding purine protons. The demonstration of slow exchange of the amino protons of cytosine, as well as those of adenine and guanine, would establish that intrinsic exchange rates of *all* the amino protons of DNA did not greatly exceed the hydrogen exchange rates measured in the double helix (Englander *et al.*, 1972; Englander and von Hippel, 1972; Hanson, 1971). Thus far it appears that solvent exchange of the amino protons of 2′,3′-cCMP shows some of the same qualitative features exhibited by the amino protons of the corresponding adenine and guanine compounds. Exchange is catalyzed by phosphate and by OH^- (Table III). However, the broadening observed at pH 4.15 is *not* due to H_3O^+ directly, since $k_{H_3O^+}$ is quite small, *i.e.*, less than 10^{-3} M^{-1} sec^{-1} (B. McConnell and P. C. Seawell, manuscript in preparation). As seen in the case of the purines, the pH variation in line width indicates that catalytic rate constants for exchange are much lower than the diffusion-controlled constants typical of aliphatic and aromatic amines. On the other hand, the 0° pH variation of line width for the H_d proton is significantly greater than that of the purine amino protons. The selective broadening of the H_d resonance with pH above neutrality (Figure 7) would be consistent with a hydroxyl ion rate constant of about 10^9 M^{-1} sec^{-1}, an order of magnitude or more higher than that observed for the purines at this temperature (McConnell and Seawell, 1972).

The possibility of slow intrinsic exchange for the amino protons of all the DNA bases formed the basis for a mechanism postulated for the initiation of hydrogen exchange from the double helical polynucleotide. Its essential feature was that nucleation of helical strand separation necessary for exchange of the internucleotide protons might be initiated by removal of the non-hydrogen-bonded amino protons that are exposed to solvent in the helical grooves (McConnell and Seawell, 1972). Implicit in this notion was the assumption that the exchange of one amino proton would affect the other. However, our studies have shown that the separate amino protons can exchange at quite different rates and can respond differently to solvent exchange catalysis (Figure 7). It is apparent, therefore, that exchange of the groove amino protons of the double helix need not be communicated to the internucleotide protons between the helical strands.

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