Proton Exchange of Nucleic Acids. Amino Protons of Mononucleotides†

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ABSTRACT: Exchange of the amino protons of 2′:3′-cAMP, 2′:3′-cGMP, 5′-AMP, 5′-GMP, 3′-AMP, and adenosine can be measured in aqueous solution with the aid of the 100-MHz nmr spectrometer. The variation in line width of the $-NH_2$ proton resonance with pH is used to estimate empirically the second-order rate constants for proton-exchange catalysis by H_3O^+ and OH^-, which are in the range 10^6–10^8 M^{-1} sec^{-1}. These estimates correspond to rates for intrinsic exchange that are several orders of magnitude less than rates for aliphatic and aromatic amines, and account largely for the fact that "outside" amino protons of helical polynucleotides can be measured by slow kinetic techniques. The pH–exchange profiles do not coincide for the adenine and guanine $-NH_2$ protons, which accounts for the different kinetic classes of exchangeable hydrogen from the DNA helix. Amino proton exchange is catalyzed by phosphate and imidazole and is highly temperature dependent.

The ways in which the exchangeable purine and pyrimidine protons of helical DNA can be hidden from contact solvent by secondary structure appear to be few and well defined. A space-filling model of the double helix would show one proton of each of the amino pairs sterically accessible to solvent in the helical grooves and free from participation in intramolecular hydrogen bonding. In contrast, the second amino proton and the imino protons of the guanine and thymine rings are much less accessible to solvent molecules because of their location and their involvement in hydrogen bonds between the complementary strands. Therefore, initial observations that exchange of the hydrogens of helical polynucleotides was slow and accessible to measurement by gel filtration techniques is significant for the use of hydrogen exchange as a general probe of macromolecular structure (Printz and von Hippel, 1965, 1968; Englander and Englander, 1965). The DNA helix might represent a relatively simple model for obtaining information, which could be applied not only to questions of its own structure, but to interpretation of HX data of the more complex proteins (Hvidt and Nielsen, 1966). Potentially, this information is of two kinds: (1) the *amount* of macromolecular structure, which is obtained by counting "slow" hydrogens and (2) the *motility* of the structure, which is derived from exchange rates (McConnell and von Hippel, 1971). Both types of information require a quantitative knowledge of the hydrogen-exchange rate in the absence of structure, which has not been available for nucleic acids.

Consideration of the general aspects of DNA and polynucleotide hydrogen-exchange data that has been gathered to date indicates that there is a need for measurements of the intrinsic exchange of the purine and pyrimidine protons before a mechanism can be elucidated for exchange in the macromolecule, especially for the purpose of describing the nature and position of the structure motility in the sequence of events that initiate exchange. A comprehensive list of these general features would include the following. (1) Structural and chemical processes related to exchange appear to be too complicated for the formulation of a simple kinetic model of exchange (von Hippel and Printz, 1965; McConnell and von Hippel, 1970a). (2) An important consideration in addition to the structural role is the negative atmosphere of the DNA sugar phosphate backbone, which regulates exchange by modifying structural stability and the approach of ionic catalysts (Printz and von Hippel, 1965). (3) In spite of the structural uniformity, exchange-out curves of DNA reveal several kinetic classes of inter-base protons, which may exhibit an interdependence in their exchange, even though they originate from chemically different sites (McConnell and von Hippel, 1970a). (4) Hydrogen exchange is not initiated by structural events that are to be associated with thermal stability, but appears to be related to conformational changes more subtle than the separation and destacking of the DNA bases (McConnell and von Hippel, 1970b; Bird *et al.*, 1970). (5) Finally, it has been observed that the hydrogen-bonded internucleotide protons are not the only protons that undergo slow exchange. The nonhydrogen-bonded amino protons that are sterically accessible to solvent in the helical grooves exchange only a few times faster than do the inter-strand protons (Hanson, 1971; Englander *et al.*, 1972; Englander and von Hippel, 1972). The issue involved in this observation is whether the intrinsic exchange of the amino protons is slow, or whether their intrinsic exchange is rapid (diffusion controlled), but slowed greatly by structural factors other than hydrogen bonding in the macromolecule. This latter alternative is difficult to rationalize in view of the fact that the most important factor seen to depress proton exchange of "normal" bases has been direct intramolecular or stabilized inter-species hydrogen bonding of the dissociable proton (Eigen and Kruse, 1963; Eigen, 1964).

We report approximate determinations of the exchange rates of the amino protons of aqueous adenine and guanine, which were obtained from 100-MHz proton magnetic resonance (pmr) spectra of adenylic and guanylic acids. Analysis of the amino proton line widths as a function of pH leads to two observations that bear directly upon the general features of DNA hydrogen exchange outlined above. The first is that the hydronium and hydroxyl rate constants are of the order of 10^6–10^8 M^{-1} sec^{-1}. The corresponding first-order rates are

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several orders of magnitude slower than rates for amino groups of the aliphatic and aromatic compounds, whose rate constants are diffusion controlled. These low intrinsic rates largely account for the slow exchange of the non-hydrogen-bonded amino protons of the helical grooves in DNA. The second observation is that the pH dependence is not the same for amino proton exchange of adenine and guanine which explains the distribution of different kinetic classes of exchangeable inter-base protons in DNA. These data are discussed in regard to the notion that proton transfer involving the outside amino protons or DNA base nitrogens might *precede* "structural" events such as strand separation in helical DNA. This does not conform to the sequence of events tacit in the kinetic models used thus far to describe exchange in the DNA double helix.

Materials and Methods

Solutions of 5′-AMP,[1] 5′-GMP, 2′:3′-cAMP, 2′:3′-cGMP (sodium salts), and adenosine (Sigma) were made with double-distilled water and if turbid, filtered on Millipore HA 0.45 μ before pH adjustment. As supplied, these compounds contain small, varying amounts of inorganic phosphate, which catalyzes exchange (see Results), but has little effect on line width at the extent of contamination in these samples (0–0.8% of the sample molarity according to product specification sheets). Analyses for P_i on all compounds indicated phosphate levels below detectable limits (Fiske and Subbarow, 1925) with the exception of 3′-AMP and some lots of 2′:3′-cAMP, which contained up to 1.2% phosphate; a concentration too low to affect line widths. The solution pH was measured with a Beckman Model 1019 research pH meter and was accurate to within ±0.05 pH unit. The pH was checked before and after the proton magnetic resonance (pmr) spectra were obtained and if it varied the value was obtained by interpolation and the limits are indicated in the data presentations. Sodium perchlorate solutions, used to increase the solubility of adenosine, were made from the anhydrous salt (G. F. Smith) and their pH adjustment was achieved by use of the agar reference electrode bridge described previously (McConnell and von Hippel, 1970b). Nucleotide concentrations were measured by A_{260} using P-L Biochemicals, Inc. Circular OR-10 or product data sheets as a source of extinction values.

Pmr Experimental Procedures

Pmr spectra were obtained in the Varian HA 100 spectrometer in frequency sweep mode locked on solvent water. Attempts at heteronuclear decoupling (^1H–^{14}N) were made with the HD-60A heteronuclear decoupler, Nuclear Magnetic Resonance Specialties, Inc. Temperature control was maintained with a Varian variable-temperature controller and monitored by chemical shift measurements of acidified methanol. Signal to noise ratios were improved for some samples below 0.2 M with the Varian C-1024 computer of average transients (CAT).

At 29° the exchange broadened resonance signals for the amino protons of 5′-AMP, 5′-GMP and the corresponding 2′:3′-cyclic compounds reside about 2 ppm downfield of the water proton signal (lock) and appear on the shoulder

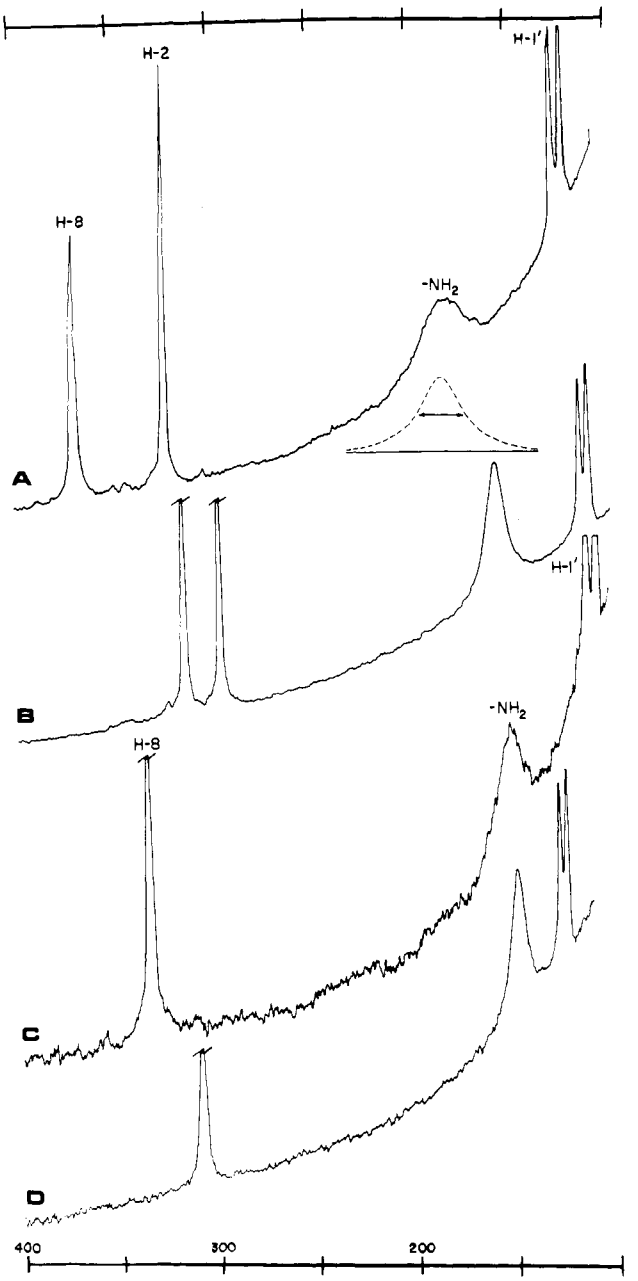

FIGURE 1: Pmr spectra (100 MHz) of aqueous adenylic and guanylic acids (sodium salts). All spectra were recorded at 0.2 M and 29 ± 1°. Curves top to bottom: (A) 5′-AMP, pH 7.5; (B) 2′: 3′-cAMP, pH 7.2; (C) 5′-GMP, pH 6.2; (D) 2′:3′-cGMP, pH 5.1.

of the much larger water line (Figure 1). Although the 5′-mononucleotide spectra were obtained with a combination of high filtering and slow sweep time to attain maximum signal enhancement, it is evident from Figure 1 that obtaining half-height widths from the 5′-mononucleotide spectra at this temperature is complicated by several factors. The full amino proton signal is buried to some extent by the large water background and the upfield half of the signal is obscured further by proximity to the H-1′ doublet. In addition, broadening of the water signal by factors such as changes in pH near pH 7 (Meiboom, 1961) leads to the false impression of line broadening of the amino peak upon casual inspection of the spectrum.

In spite of these drawbacks, sufficiently accurate estimates of line widths for the 5′-mononucleotides could be made at

[1] Abbreviations used are: 2′:3′-cAMP and 2′:3′-cGMP, the 2′:3′-cyclic phosphates of adenosine and guanosine, respectively; 5′-AMP and 5′-GMP, the 5′-monophosphates of adenosine and guanosine, respectively; 3′-AMP, 3′-adenosine monophosphates.

concentrations greater than 0.2 M. To do this the pmr spectrum was obtained after radiofrequency phase adjustment to maximize the symmetry of the resonance signals of the H-8 and H-2 protons (absorption mode). On the recorded spectrum the Lorenzian (water) base line of the amino proton signal was adjusted to yield an area for the downfield half of the signal equal to the areas of the H-8 or H-2 signals. The base line thus established provided a difference curve for the half-signal, from which the intensity was obtained to give a half-height width for the full signal. Under conditions outside the range of minimum exchange for the 5′-mononucleotides concentrations of 0.2 M were required, since the theoretical error rapidly increases when the signal intensity becomes less than half the line width at half-height. This problem was not encountered with the cyclic compounds and with 3′-AMP, whose signal to noise ratio for the amino protons are several times larger and the half-height widths are about 10 Hz. Assignment of this signal to the amino protons (with 5′-AMP) was verified with the use of the 300-MHz spectrometer,[2] which provided sufficient separation of the amino and water proton peaks (550 Hz) for accurate comparison of the signal area (2 protons) to that of the H-8 and H-2 single protons (3.7 and 3.3 ppm, respectively) (McConnell *et al.*, 1972). The similar assignment for the amino proton signal of 5′-GMP relative to H-8 and H-1′ is in agreement with the tentative assignment made by Miles *et al.* (1963) for this compound in aqueous solution.

The half-height widths of the 300-MHz amino proton signals were somewhat less than those of corresponding experiments at 100 MHz (McConnell *et al.*, 1972). However, we consider the line widths to be the same for the two magnetic fields, since the 5′-AMP samples used for the 300-MHz spectra were highly purified and were measured in a 2-mm capillary, which eliminates the water spinning side bands that contribute to the overall line width in the 100-MHz experiments. This independence of line width to changes in peak separation between $-NH_2$ and H_2O protons indicates that the exchange rate is at the slow exchange limit, a condition in which the specific exchange rate can be taken directly from the half-height width of the amino proton signal (Johnson, 1965):

$$(\Delta\nu_{1/2})_{obsd} = \frac{1}{\pi T_2} + \frac{(1 - P)}{\pi}\left(\frac{1}{\tau}\right)_{-NH_2} \quad (1)$$

where $(\Delta\nu_{1/2})_{obsd}$ is the observed half-height width (hertz) of the amino proton line, T_2 is the transverse relaxation time, P is the mole fraction of amino protons, *i.e.*, $P = [-NH_2]/([-NH_2] + [H_2O])$ and $1 - P \equiv 1$, and $(1/\tau)_{-NH_2}$ is the reciprocal of the proton lifetime on the amino nitrogen (sec), *i.e.*, the specific exchange rate or pseudo-first-order rate constant ($1/[-NH_2] \times$ exchange velocity) (Meiboom, 1960).

The nitrogen quadrupole does not appear to be a large factor in line broadening, since in the case of 5′-AMP and 2′:3′-cAMP the signal is not sharpened by application of strong radiofrequency at 7.223 MHz varied in the audiorange over ±4 kHz (Grunwald and Price, 1964) and the amount of ionized amino group is negligible in the pH range used in these experiments (McConnell and von Hippel, 1970a; Hertz, 1960). Therefore, the relaxation and exchange contributions are considered independent, which leads to the treatment of the data in two ways. The first is an estimate of the specific exchange rate according to eq 1, which involves the assumption that $(1/\pi)(1/T_2)$ is a small correction representing the natural line width of the amino protons in the absence of exchange

$$\left(\frac{1}{\tau}\right)_{-NH_2} = \pi(\Delta\nu_{1/2})_{obsd} - \pi(\Delta\nu_{1/2})_{C-H} \quad (2)$$

where $(\Delta\nu_{1/2})_{C-H}$ is the observed line width of a nonexchanging carbon proton obtained from the same spectrum; from the H-2 signal in the case of the adenosine compounds and H-8 for the guanosine derivatives (Figure 1). This method of estimation leads to an upper limit or *overestimated* value of the true exchange rate, since the natural line width of the amino proton in the absence of exchange could be larger than the correction used, especially in the case of the 5′-nucleotides where interaction of the phosphate with the purine could contribute to line broadening through relaxation effects (see Results).

The second use of the data is in the estimation of the H_3O^+ and OH^- rate constants by fitting assumed values to observed variations in $(\Delta\nu_{1/2})_{obsd}$ with changes in pH. Therefore, these rate constants are the empirically determined changes in exchange rate in response to changes in the concentration of H_3O^+ and OH^-. In this case the exchange contribution $(1/\pi)\cdot(1/\tau)_{-NH_2}$ of eq 1 is split into two separate exchange terms

$$(\Delta\nu_{1/2})_{obsd} = \frac{1}{\pi}\left(\frac{1}{T_2}\right) + \frac{1}{\pi}\left(\frac{1}{\tau}\right)_{ex} + \frac{1}{\pi}\left(\frac{1}{\tau}\right)_{pH} \quad (3)$$

where $(1/\tau)_{ex}$ is the specific exchange rate from catalysis by solution components other than H_3O^+ and OH^-. This term contains both pH-dependent and -independent contributions, especially in the case of the 3′- and 5′-mononucleotides, where the secondary phosphate ion catalyzes exchange (see Results). In the last term, $(1/\tau)_{PH}$ represents exchange initiated by H_3O^+ and OH^- and leads to the empirical evaluation of their rate constants by the relation

$$\left(\frac{1}{\tau}\right)_{pH} = k_{H_3O^+}(a_{H_3O^+}) + k_{OH^-}\left(\frac{K_w}{a_{H_3O^+}}\right) \quad (4)$$

where $k_{H_3O^+}$ and k_{OH^-} are the second-order rate constants for H_3O^+ and OH^- catalyses, respectively, $a_{H_3O^+}$ is the activity of hydronium ion obtained from pH readings without further correction and K_w is the ion product of water (10^{-14} at 30° and 10^{-15} at 0–5°).

Since we do not assume knowledge of the relative line-broadening contributions of the first two terms on the right of eq 3, these are combined and substitution of eq 4 for the last term of this equation gives

$$(\Delta\nu_{1/2})_{obsd} = (\Delta\nu_{1/2})_{T_2+ex} + \frac{1}{\pi}\left(k_{H_3O^+}a_{H_3O^+} + K_{OH^-}\frac{K_w}{a_{H_3O^+}}\right) \quad (5)$$

where

$$(\Delta\nu_{1/2})_{T_2+ex} = \frac{1}{\pi}\left(\frac{1}{T_2}\right) + \frac{1}{\pi}\left(\frac{1}{\tau}\right)_{ex}$$

The application of eq 5 for the evaluation of rate constants

[2] The 300-NHz pmr spectra were kindly provided by Mr. L. H. Johnson and Mr. L. Cary from the HR300 nmr spectrometer, Varian Associates, Palo Alto, Calif.

TABLE I: Assignments for pH Dependence of Amino Proton Line Width.[a]

Compound	Soln Condn	$(\Delta\nu_{1/2})_{T_2+ex}$ (Hz)	$k_{H_3O^+}$ (M^{-1} sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})	pH min
2′:3′-cAMP	0.1 M, 30°	9	2×10^6	4×10^7	6.2
2′:3′-cAMP	0.1 M, 0°	5	7×10^5	1×10^7	6.2
2′:3′-cGMP	0.1 M, 30°	2	2×10^6	6×10^8	5.7
5′-AMP	0.6 M, 30°	32	3×10^8	1.2×10^7	7.7
5′-GMP	0.1 M, 30°	12		5×10^8	5–6
3′-AMP	0.2 M, 30°	10	1×10^8	4.5×10^7	7.1
Aliphatic amines[b]	20°		$2–4 \times 10^{10}$		
Aniline[b]	20°		10^{10}		
Adenine[b]			1×10^{10}		
Purine	0.04–0.85 M, 20.4°		$2–3 \times 10^{10}$		
N-Methylacetamide[d]	1 M, 24°		3×10^5	3×10^9	

[a] Values used in eq 5 to generate theoretical profiles in Figures 2 and 3. [b] Obtained from Eigen (1964) for the reaction

$$-NH_2 + H^+ \underset{k_{OH^-}}{\overset{k_{H_3O^+}}{\rightleftharpoons}} -NH_3^+$$

where k_{OH^-} for this dissociation is 3×10^{10} M^{-1} sec^{-1}. [c] Obtained from Marshall and Grunwald (1969). [d] These values are catalytic constants for D_3O^+ and OD^- obtained from measurements in D_2O ($K_w = 2 \times 10^{-15}$) taken from Klotz and Frank (1965).

is not strictly rigorous, since $(\Delta\nu_{1/2})_{T_2+ex}$ is not pH independent. This is particularly true for the 3′- and 5′-phosphates and the utility of this approach will be discussed in Results.

Results

Initial pmr studies of amino proton exchange of mononucleotides were made on 5′-AMP (McConnell *et al.*, 1971, 1972), in which it was observed that proton-exchange catalysts have a distinct influence on the observed line width. In this study, data on the guanosine derivatives are included and the primary emphasis is on the 2′:3′-cyclic compounds, since the use of the cyclic derivatives provides advantages over that of the 5′-phosphates. First, the cyclic derivatives represent better models than the 5′- (or 3′-) phosphates in relation to polynucleotide exchange, since the phosphate is restricted from intramolecular interaction by its two P–O bonds and is singly charged throughout the pH range studied (as in polynucleotides). Secondly, the observed amino proton resonance lines are much narrower for the cyclic phosphates (see Experimental Section) and, in contrast to the 5′-mononucleotides, solutions of the cyclic phosphates do not form gels at low pH.

The influence of pH on the observed amino proton line width for 2′:3′-cyclic phosphates of adenosine and guanosine is shown in Figure 2. For each compound both hydroxyl and hydronium ions are exchange (line broadening) factors, but the pH profiles for each are separated on the alkaline side by over a full pH unit. The pH of minimum line width (pH min) is more acid for the guanosine compound.[3] The dashed lines through each set of points are theoretical profiles obtained by assigning numerical values for $(\Delta\nu_{1/2})_{T_2+ex}$, $k_{H_3O^+}$, and k_{OH^-} in eq 5 for the best fit of the data. These assignments are listed in Table I, which includes for comparison the second-order rate constants for amides (Klotz and Frank, 1965), for aromatic and aliphatic amines (Eigen, 1964) and for the ring proton of purine (Marshall and Grunwald, 1969). It is noteworthy that the constants for amines and for purine represent values associated with diffusion controlled reactions (Eigen, 1964) and are up to four orders of magnitude higher than the rate constants for the amino protons of the cyclic

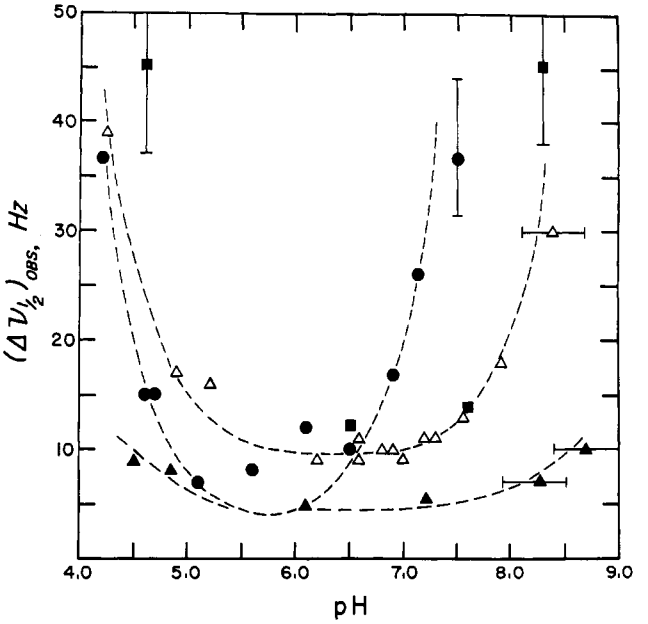

FIGURE 2: The effect of pH on line width $(\Delta\nu_{1/2})_{obsd}$ of the amino protons of 2′:3′-cAMP and 2′:3′-cGMP. All solutions were sodium salts and 0.1 ± 0.03 M. (△) 2′:3′-cAMP, 29 ± 1°; (▲) 2′:3′-cAMP, 0°; (■) 2′:3′-cAMP, 29 ± 1°, 0.01 M sodium phosphate; (●) 2′:3′-cGMP, 29 ± 1°. Points with large vertical bars represent upper limits of line widths, since the corresponding resonance lines were too broad for rate estimation.

[3] For 2′:3′-cGMP the deviation of experimental points from the theoretical line near pH_{min} may be due to nonexchange broadening, since line widths track quite well if corrected by subtraction of the H-8 line width, which is also broadened in this range.

TABLE II: Pmr Line Widths (100 MHz) and Specific Exchange Rates of Amino Protons.[a]

Compound	Soln Condn	pH	$(\Delta\nu_{1/2})_{obsd}$ (Hz)	$(\Delta\nu_{1/2})_{C-H}$[b] (Hz)	$(1/\tau)_{-NH_2}$ (sec^{-1})	$(1/\tau)_{pH}$ (sec^{-1})
2′:3′-cAMP	0.1 M, 30°	7.0–7.5	9–12	2	22–31	4–12
2′:3′-cAMP	0.1 M, 0°	7.0–7.5	8–9	2	19–22	0.1–0.3
2′:3′-cAMP	0.15 M, 29°, 0.2 M PO_4	6.5	>50	2	>150	
2′:3′-cAMP	0.15 M, 1°, 0.2 M PO_4	6.5	11	2	27	
2′:3′-cGMP	0.1 M, 30°	5.7	8	5	9	7
2′:3′-cGMP	0.1 M, 30°	7.0–7.5	21–50	2	60–150	60–180
5′-AMP	0.7 M, 30°	7.5	34	3	97	13
5′-AMP	0.2 M, 30°	7.0	28	2	82	33
5′-AMP	0.2 M, 30°, 4 M NaCl	7.0	24	3	66	
5′-AMP	0.2 M, 30°, 4 M NaCl	7.3–7.5	22	3	60	
5′-AMP	0.2 M, 30°, 0.02 M imidazole	7.5	40	2	120	
3′-AMP	0.2 M, 30°	8.0	21	2	60	46
3′-AMP	0.2 M, 0°	8.0	10	2	25	
5′-GMP	0.1 M, 30°	6.5	18	3	47	15
5′-GMP	0.1 M, 30°	7.0	28	2	82	50
Adenosine	0.02 M, 30°	7.5	40	15	78	
Adenosine	0.1 M, 30°, 4.6 M $NaClO_4$	7.5	23	2	66	
5′-dAMP	0.1 M, 30°	6.5	26	2	75	
5′-dAMP	0.1 M, 30°	7.0	18	2	50	

[a] Specific exchange rates: $(1/\tau)_{-NH_2}$ obtained from tabulated line widths and eq 2; $(1/\tau)_{pH}$ obtained from $k_{H_3O^+}$, k_{OH^-} (Table I) and eq 4. [b] H-2 and H-8 resonance lines for adenosine and guanosine compounds, respectively.

nucleotides. Lowering the experimental temperature from 30 to 0° produces even sharper amino proton resonance signals for 2′:3′-cAMP and the pH dependence indicates that the decrease in the rate constants is three to fourfold (Table I). The estimates of pH min (Table I) are calculated values (eq 4), which are acid to neutrality for both compounds and differ by less than one pH unit due to the similarity of their hydronium ion rate constants. The difference in the pH profiles is accounted for only by the higher hydroxyl ion rate constant for the amino protons of cGMP, which is over an order of magnitude larger than that of cAMP.

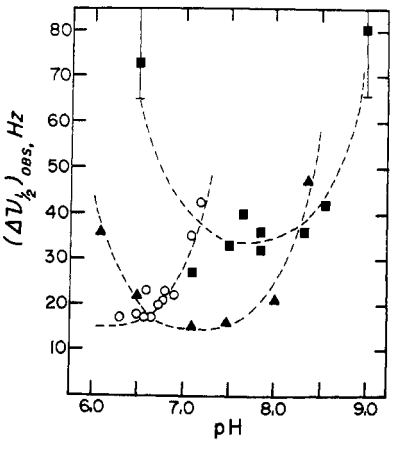

FIGURE 3: The effect of pH on line width $(\Delta\nu_{1/2})_{obsd}$ of the amino protons of 5′-AMP, 3′-AMP, and 5′-GMP. All solutions were sodium salts adjusted to the desired pH with HCl or NaOH. (■) 5′-AMP, 0.65 M, 29 ± 1°; (▲) 3′-AMP, 0.2 M, 29 ± 1°; (○) 5′-GMP, 0.1 M, 29 ± 1°. Points with large vertical bars represent upper limits to line widths, since the corresponding resonance lines were too broad for extraction of rates.

The pH dependence of the amino proton line width of the 5′-mononucleotides and 3′-AMP agrees with the observation on the cyclic compounds (Figure 3). The hydroxyl ion rate constant for 5′-GMP is about 50 times k_{OH^-} for 5′-AMP and ten times that of 3′-AMP, which contributes to the much lower pH min for the guanosine amino protons (Table I). Implications regarding the magnitude of $k_{H_3O^+}$ are not clear for 5′-GMP, since solutions of this compound form gels below pH 6.5 at the experimental temperature and concentration. Gel formation could result in sufficient change in the correlation time to produce broadening of all the proton resonance signals. However, this does not appear to contribute to line broadening above pH 5, since the H-8 proton is not excessively broadened in this range.[4] The value of 3×10^8 M^{-1} sec^{-1} for $k_{H_3O^+}$ of the amino protons of 5′-AMP must be considered approximate and could be less, since the line widths in the region of minimum exchange are as large as the signal intensity and are difficult to estimate accurately. The hydronium ion rate constant for 5′-AMP at lower concentration, however, has been estimated to be about 3×10^8 M^{-1} sec^{-1} (McConnell *et al.*, 1972) at the same temperature.

In view of the higher value of $k_{H_3O^+}$ for 5′-AMP it is noteworthy that the line widths for the amino protons of the 5′-mononucleotides, especially of 5′-AMP, are generally broader than those of the other compounds (*e.g.*, compare values of $(\Delta\nu_{1/2})_{T_2+ex}$, Table I). This might be accounted for by the influence of the sugar phosphate, which carries a doubly negative charge (pK = 6.7, Phillips *et al.*, 1965) variable in concentration in the pH range studied and could produce line broadening by catalysis of proton exchange, *e.g.*, by acting as a proton acceptor to initiate exchange. This is borne out

[4] Preliminary observations on the pH dependence of the 5′-GMP amino proton line widths extended to lower pH values indicate H_3O^+ catalysis (Dr. M. Raszka, personal communication).

by the fact that sodium phosphate added to cAMP produced a dramatic broadening of the amino proton signal at 0.2 M (Table II). In addition, the pH dependence of the amino proton exchange of 2′:3′-cAMP in the presence of 0.01 M phosphate differs from the pH dependence of cAMP alone by an increase in $k_{H_3O^+}$. This is evident from the fact that the resonance line measurable in the absence of phosphate at pH 4.2 is "wiped out" at pH 4.6 in the presence of sodium phosphate (Figure 2) in spite of the fact that there is only a small amount of broadening by phosphate in the vicinity of pH min.

The suggestion that the sugar phosphate may catalyze exchange is demonstrated also in Figure 4, which shows that $(1/\tau)_{-NH_2}$ (eq 2) is not constant for 5′-AMP or for 3′-AMP, but increases with concentration of these compounds. Therefore, for completeness we can write

$$\left(\frac{1}{\tau}\right)_{-NH_2} = k_0 + k_{H_3O^+}(a_{H_3O^+}) + k_{OH^-}\left(\frac{K_w}{a_{H_3O^+}}\right) + k_{HA^{+0}}[HA^{+0}]^n + k_{A^{-0}}[A^{-0}]^m \quad (6)$$

where k_0 is catalysis by pH-independent processes and $k_{HA^{+0}}$ and $k_{A^{-0}}$ are second-order rate constants for catalysis of $-NH_2$ exchange by the conjugate acid and base of added catalyst, respectively. The HA^{+0} and A^{-0} terms would be equivalent to $(1/\tau)_{ex}$ in eq 3 when added to k_0 and represent catalysis of exchange from several proton-transfer agents including the mononucleotides themselves. If the A^{-0} term in eq 6 is assumed to represent intra-species catalysis by the sugar phosphate and if $m = 1$, then a theoretical pH profile for the 5′-mononucleotides can be calculated by combining eq 5 and 6. However, experimental error for the 5′-mononucleotide data does not permit an accurate evaluation of $k_{PO_4^{2-}}$, which probably does not exceed 500 M^{-1} sec^{-1}. Moreover, it is not clear whether the increased line widths for 5′-AMP amino protons arise from the sugar phosphate solely by catalysis or if it occurs also by a separate nonexchange influence on line width. The phosphate contribution to line width may account for 80% of lowering of $(1/\tau)_{-NH_2}$ when the temperature is lowered (Figure 4), since the contribution of $k_{H_3O^+}$ and k_{OH^-} is relatively small at pH 7.5. One would expect, therefore, that if the phosphate term in eq 6 were due to the sugar–phosphate catalysis exclusively, then the two concentration curves for 5′-AMP at different temperatures might converge at low concentration. The fact that they do not may be a reflection of the ability of the 5′-ribose phosphate to increase exchange or to affect relaxation times of the amino proton through its ability to interact with the purine ring (Ts'o *et al.*, 1969). Although 3′-AMP shows a similar concentration dependence (Figure 4) the resonance lines of its amino protons are narrower at the same temperature. The sugar phosphate of this compound is much less capable of interaction with the purine, which is indicated by the narrower resonance line for the H-8 proton (compared to H-8 for 5′-AMP) and a line separation for H-8 and H-2 similar to that of 2′:3′-cAMP) (Figure 1).

It can be inferred from Figure 4, also, that stacking does not appear to operate by *supressing* exchange, which is in agreement with the findings of Marshall and Grunwald (1969), who showed that proton exchange at the ring nitrogen of purine is not decreased by molecular stacking. In the case of aqueous 5′-AMP stacking effects measured by changes in chemical shift of the H-2 proton are saturated at concentrations of about 0.2 M AMP (Ts'o *et al.*, 1969). Thus far, linewidth estimates of the amino proton signals at 100 MHz have

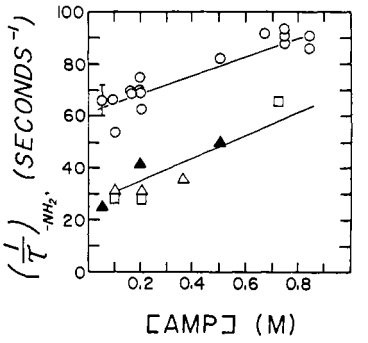

FIGURE 4: The effect of concentration on specific exchange rate, $(1/\tau)_{-NH_2}$, of the amino protons. (○) 5′-AMP, 29 ± 1°, pH 7.5; (□) 5′-AMP, 5 ± 1°, pH 7.5; (△) 2′:3′-cAMP, 29 ± 1°, pH 7.1; (▲) 3′-AMP, 29 ± 1°, pH 7.1.

not been accurate enough below 0.2 M to allow observation of a corresponding pattern for amino proton exchange for testing the possibility that stacking might affect exchange.

Listed in Table II are values of $(1/\tau)_{-NH_2}$ calculated from eq 3 for all compounds tested, including adenosine, which provides the nonphosphate control and 5′-dAMP, which demonstrates the same results for a DNA monomeric unit. The data for adenosine (Table II) require comment. Because of the low solubility of this compound (0.02 M, pH 7.5) linewidth estimation required 177 CAT scans for signal enhancement. Since this number of scans required about 10 hr, the resonance signals of nonexchanging protons were broadened due to field (homogeneity and phase) shifts over this period of time. Therefore, the half-height width of H-2 subtracted from the corresponding width of the amino proton was about 15 Hz. The summation of all these spectra produced an amino proton signal at the same frequency as that for the AMP amino protons and was visible as a signal, whose half-height width was about twice its intensity. Therefore, we assume that the amino and water signals are not coalescing: $(1/\tau)_{-NH_2}$ must be less than the separation between the amino and water proton lines (180 Hz) and the calculated rate less than 90 sec^{-1}. Another estimate of $(1/\tau)_{-NH_2}$ from the adenosine $-NH_2$ line was obtained from an adenosine solution containing 4.6 M sodium perchlorate. The use of this salt provided the double advantage of increasing the solubility of adenosine to detectable levels in the pmr (Robinson and Grant, 1966) and of shifting the water background further upfield for better separation, which derives from the ability of this salt to break hydrogen bonds between water molecules (Emsley *et al.*, 1965). At this point the observed $(1/\tau)_{-NH_2}$ for adenosine of 70 sec^{-1} must be conditionally accepted as high since the extent of the ability of perchlorate anion to act as a proton acceptor at this high concentration, its effect on relaxation times and the kinetic implications arising from possible ion–dipole interaction between the large numbers of sodium ion and N-1 (and $-NH_2$) of the purine is not known. The effect of sodium ion may not be important, however, since the line widths in the presence of 4 M NaCl are slightly, but significantly narrowed for 5′-AMP (Table II). A small reduction in line width would be expected if phosphate catalyzes exchange, since the sodium counterion effect would reduce the negative potential of the phosphate ion. In any event these effects of sodium perchlorate are not large enough to postulate a much higher specific rate value than observed, since cAMP, whose singly ionized phosphate is restricted from intramolecular interaction with the purine ring, exhibits a remarkably

low rate of exchange of its amino protons. In this case the estimate of 25 sec^{-1} for $(1/\tau)_{-NH_2}$ (Table II) is quite accurate (as obtained from eq 2), since the narrow (10 Hz) resonance signal is well resolved from the background. It is apparent from these values of $(1/\tau)_{-NH_2}$ in Table II that none of the compounds tested contain amino protons whose exchange approaches the rate of 10^3 sec^{-1} expected from H_3O^+ and OH^- diffusion controlled rate constants of 10^{10} M^{-1} sec^{-1} (eq 4). The value of $(1/\tau)_{-NH_2}$ is the upper limit, since the line width from which it is estimated may include line broadening influences not related to exchange (*e.g.*, water spinning side bands) and the T_2 correction may be too small, as approximated from the H-2 resonance. Calculated values of $(1/\tau)_{pH}$ for each compound from the hydroxyl and hydronium ion rate constants according to eq 4 are listed in Table II and in all likelihood represent more closely the rates to be expected for amino proton exchange of the bases involved in polymeric structure. The similarity of the line widths and rate estimates for 5′-dAMP indicates that similar values of $k_{H_3O^+}$ and k_{OH^-} apply to this DNA control. Measurements of $(\Delta\nu_{1/2})_{obsd}$ for this compound are complicated by proximity of the H-1′ resonance triplet.

The effects of sodium phosphate on line width of the amino protons of 2′:3′-cAMP and of imidazole on that of 5′-AMP are listed in Table II to indicate the dramatic broadening, which was too large for accurate measurement. For the present, we tentatively assume that this reflects exchange catalysis, since changes in relaxation times associated with direct interaction between these compounds and the amino would not be expected to bring about such large signal broadening. It is noteworthy that these broadenings are greatly decreased by lowering the temperature.

Discussion

The use of eq 5 for the determination of hydronium and hydroxyl rate constants depends upon the fact that pH-induced line broadening can be realized, which is greater than line broadening from other factors including pH-independent exchange. Therefore, after lumping the latter and T_2 broadening into one term, the rate constants determined from the pH-dependent term by curve fitting are not as inaccurate as one might suppose from inspection of the data. This arises from the fact that the $-NH_2$ resonance broadens from the minimum width to an unobservably wide absorbance well within the span of one pH unit and increases to large, measurable widths within a half-pH unit. This is predicted from eq 5 in which the changes in concentration of H_3O^+ and OH^- are large in these pH ranges and the separate terms of the equation are additive. Even with considerable manipulation of the assignment for the pH-independent term, a twofold difference in the assignment of the rate constants would clearly displace the calculated curves from the data.

A second assumption in the use of eq 5 is that the various contributions to the pH-independent term remain pH independent throughout the pH range. Of chief concern here is the T_2 term, which shows some pH dependence as judged from the H-2 and H-8 resonances. We assume this dependence remains relatively small and contributes little to the assignments for $k_{H_3O^+}$ and k_{OH^-}, since specific values for this contribution were not included in line-fitting calculations. It is noteworthy that if such a correction of the data were made using the line width of H-8, the calculated curve for 2′:3′-cGMP would track exactly with the corrected data in the region of minimum line width (Figure 2).

Nonexchange broadening of $-NH_2$ may arise through intramolecular interactions, which include the phosphate–C-8 proton interaction in 5′-AMP (Ts'o *et al.*, 1969) and possible salt bridge formation between the phosphate and N-1 of 5′-AMP (Phillips *et al.*, 1965). Also, the phosphate and imidazole effects may reflect a direct nonexchange interaction of the amino proton with the phosphorus atom and with a quadrupole (imidazole nitrogen), each of which might produce line broadening in a manner similar to proton line broadening associated with interaction of paramagnetic ions (Yamane, 1971). These effects, to be considered in detail in a later publication (B. McConnell and P. Seawell, 1972), do not obscure the fact that $(1/\tau)_{-NH_2}$ must represent only upper possible limits of exchange, since none of these interactions would operate to produce narrower lines.

The third consideration related to the natural line width to be found in these experimental conditions is whether pH-dependent exchange is the dominant expression, or whether pH-independent exchange (k_0 in eq 6) contributes significantly to the observed line width. It is evident from Table II that rates calculated from the total line width (corrected for that of H-2 or H-8) are greater than rates obtained from the H_3O^+ and OH^- rate constants alone (eq 4). However, it is not clear that these larger values for $(1/\tau)_{-NH_2}$ are real, since the T_2 contribution is not really known. The H-8 contribution for 2′:3′-cGMP is large so that $(1/\tau)_{pH}$ is as large as $(1/\tau)_{-NH_2}$ for this compound and may be the more valid than the H-2 correction for 2′:3′-cAMP. Therefore, for present discussion of possible exchange mechanisms, we restrict ourselves to those involving hydronium and hydroxyl ions only and use $(1/\tau)_{pH}$ for the cyclic phosphates as the working value relevant to these mechanisms.

The simplest view of the pH-dependent exchange process for the amino protons is a direct reaction, in which the initial rate-limiting event is a removal or addition of a proton to the amino nitrogen by added catalyst as well as by H_3O^+ and OH^-

$$B\text{–}NH_2 + HA^{+0} \underset{}{\overset{k_{HA^+}}{\rightleftharpoons}} B\text{–}NH_3^+ + A^{-0} \qquad (7)$$

$$B\text{–}NH_2 + H_3O^+ \overset{k_{H_3O^+}}{\rightleftharpoons} B\text{–}NH_3^+ + HOH \qquad (8)$$

$$B\text{–}NH_2 + A^{-0} \overset{k_{A^-}}{\rightleftharpoons} B\text{–}NH^- + HA^{+0} \qquad (9)$$

$$B\text{–}NH_2 + OH^- \overset{k_{OH^-}}{\rightleftharpoons} B\text{–}NH^- + HOH \qquad (10)$$

where B is the nucleotide base (adenine or guanine) and HA^{+0} and A^{-0} are the conjugate species of the exchange catalyst. If, as we have seen, the H_3O^+ and OH^- rate constants for the forward reactions of eq 8 and 10 are *not* diffusion controlled ($k_{H_3O^+}$ and $k_{OH^-} \ll 10^{10}$ M^{-1} sec^{-1}), then this direct mechanism leads to the prediction that added catalyst such as phosphate or imidazole would *not* show line broadening through increases in exchange rate. The argument is as follows. The rate constants, $k_{H_3O^+}$ and k_{OH^-} can be viewed in this scheme as the product of the bimolecular collision rate and a factor inversely related to the number of collisions required for the reaction, *i.e.*,

$$k_{H_3O^+} \text{ (or } k_{OH^-}) = k_d \times 10^{(pK_A - pK_D)} \qquad (11)$$

where k_d is the diffusion-limited collision rate (10^{10} M^{-1} sec^{-1}) and $10^{(pK_A - pK_D)}$ is the factor, which embodies the idea that the number of collisions required for the reaction is greater than

one if the pK of the donor (pK_D) is higher than the pK of the acceptor (pK_A) (Eigen, 1964). Using 10^7 M^{-1} sec^{-1} for $k_{H_3O^+}$ and k_{OH^-} (Table I), a value for this factor is obtained from eq 11, from which we can estimate a "pK" for the amino groups in each of the reactions listed in eq 8 and 10.

For eq 8 this value (pK_A) is -5 and for eq 10 it is $+20$ (pK_D), assuming pK_D = -2 and pK_A = 16 for H_3O^+ and OH^-, respectively.

By defining $k_{HA^{+0}}$ and $k_{A^{-0}}$ the same way as was done for $k_{H_3O^+}$ and k_{OH^-} (eq 11) and using literature pK values for the catalyst, the separate catalytic contributions of HA^{+0} and A^{-0} can be calculated from the amino "pK's" and eq 6. If this is done it immediately becomes apparent that the HA^{+0} and A^{-0} terms are much smaller than the H_3O^+ and OH^- terms at moderate catalyst concentrations. Even if the B–NH_2 pK_A = 13 and pK_D = 0 for the appropriate reactions the effect of 0.1 M catalyst estimated from eq 6 would be too small to be seen above and in addition to the contribution of hydronium and hydroxyl ions. A situation in accord with this prediction is found in proton exchange of peptide model compounds, where initiation of exchange of the amide hydrogen is not diffusion controlled (Klotz and Frank, 1965). In this case, imidazole catalysis of *N*-methylacetamide in an aqueous system is not seen unless a_{OH^-} is drastically reduced by the addition of dioxane, which decreases K_w by three or four orders of magnitude. On the other hand, it is easy to show by the same process that if $k_{H_3O^+}$ and k_{OH^-} were diffusion controlled, *i.e.*, $10^{pK_A - pK_D}$ = 1 for eq 8 and 10, then the catalyst terms would be as large as or larger than the pH terms. In this case catalysis by added imidazole or phosphate should be visible at moderately low concentrations (see McConnell and von Hippel, 1970a, for the actual calculations).

Therefore, according to this argument our data are contradictory to the "direct" mechanism outlined in eq 7–10. We find that catalysis by phosphate and imidazole can be seen even though the hydronium and hydroxyl rate constants are not diffusion controlled. Indeed, without data on the effect of pH, which provides the estimates for the hydronium and hydroxyl rate constants, diffusion-controlled (or high) values for these constants would be erroneously implied by the "direct" mechanism and by the observation of catalysis by an organic acid or base (McConnell *et al.*, 1971). None of the estimates made here for $k_{H_3O^+}$ and k_{OH^-} (Table I) approach the values of 1×10^{10} to 4×10^{10} M^{-1} sec^{-1} reported for aliphatic and aromatic amines (Eigen, 1964) or 2.4×10^{10} M^{-1} sec^{-1} for the ring nitrogen of purine (Marshall and Grunwald, 1969).

We would conclude from this that the effects of phosphate and imidazole reflect an indirect, more complicated mechanism for exchange of the amino protons. Complicated mechanisms involving interposition of solvent molecules to account for high proton transfer rates are known for similar systems (Grunwald and Puar, 1967). However, a possible mechanism that eliminates rather neatly the dichotomy of catalysis in the face of low hydronium and hydroxyl rate constants is one in which the initial proton transfer occurs at a separate diffusion-controlled site and not at the amino group. For example

$$\text{B–NH}_2 + \text{H}_3\text{O}^+ \text{ (or HA}^{+0}\text{)} \underset{k_{-1}}{\overset{k'_{H_3O^+}}{\rightleftharpoons}} \text{H}^+\text{–B–NH}_2 + \text{HOH} \quad (12)$$

$$\text{H}^+\text{–B–NH}_2 + \text{OH}^- \text{ (or A}^{-0}\text{)} \xrightarrow{k'_{OH^-}} [\text{H}^+\text{–B–NH}^-] \longrightarrow \text{B–NH}_2 + \text{HOH} \quad (13)$$

where H^+–B–NH_2 is the protonated species formed by proton transfer to a ring nitrogen of the base. In this case the exchange reaction represented by removal of a proton from the amino group (eq 13) takes place rapidly in a fast biomolecular reaction from the small amount of nucleotide base that is protonated at the experimental pH.[5] The site of protonation would be a ring nitrogen (N-1 for adenine and N-7 for guanine), since, unlike the amino groups, protonation of these sites is preferred at measurable pH values in aqueous solution (Christensen *et al.*, 1970).

Of these two equations, the first (eq 12) represents a rapid equilibrium that establishes the concentration of H^+–B–NH_2, which can be determined from the pK_a' of the ring nitrogen. If the forward rate constant, $k'_{H_3O^+}$ is diffusion controlled (10^{10} M^{-1} sec^{-1}) then the reverse rate constant, k_{-1}, calculated from this pK'_a (Christensen *et al.*, 1970) would be of the order of 10^6 sec^{-1}. Therefore, the velocity of this reverse reaction would be greater than the velocity of deprotonation (eq 13), since the latter contains the concentration of OH^- even though k'_{OH^-} might be diffusion controlled. This condition, in which the reverse reaction in eq 12 is faster than the deprotonation (eq 13), is appropriate for calculating the rate of exchange by the following relationship

$$\text{Rate (M sec}^{-1}\text{)} = [\text{H}^+\text{–B–NH}_2]\frac{K_w k'_{OH^-}}{a_{H_3O^+}} \quad (14)$$

where k'_{OH^-} is the rate constant for removal of the –NH_2 proton from H^+–B–NH_2 and $K_w/a_{H_3O^+}$ gives the concentration of OH^-. If k'_{OH^-} is close to the diffusion rate, then eq 14 provides an estimate of the exchange rate that agrees with the rate calculated from the observed rate constants ($k_{H_3O^+}$ and k_{OH^-}) and eq 4, *i.e.*

$$\frac{\text{Rate (M sec}^{-1}\text{)}}{[\text{B–NH}_2]} \simeq \left(\frac{1}{\tau}\right)_{pH} \quad (15)$$

In this mechanism the addition of catalyst (HA^{+0}) would not alter the equilibrium concentration of H^+–B–NH_2, since this is fixed at constant pH. However, the reaction of eq 13, which represents the decrease in lifetime of the proton on the amino group, would depend on the concentration of A^{-0}. A similar "indirect" mechanism can be visualized, in which the opposite events occur, *i.e.*, protonation of the amino nitrogen of guanine by HA^{+0} or H_3O^+ after initial deprotonation of N-1 of this base.

A notable feature of this "indirect" mechanism is that amino proton exchange should be independent of pH, since a pH change would represent equal and opposite changes in the concentration of hydronium and hydroxyl ions, which are *both* necessary for exchange by this mechanism. Although the pH profiles are broad, a pH dependence is quite apparent from our data (Figures 2 and 3).

Therefore, it is conceivable that *both* the direct and indirect exchange mechanisms operate in the exchange of the amino protons of the free monomeric units of DNA in aqueous solution. The direct mechanism (eq 7–11) would be dominant at the extremes of pH, while the preequilibrium route (eq 12 and 13) would account for phosphate and imidazole catalysis in the pH-independent range (see Figures 2 and 3). The contributions of each route to the overall rate of proton exchange might be approximately equal in the vicinity of

[5] We are indebted to Drs. H. Teitelbaum and S. W. Englander for suggesting this type of mechanism to us.

neutrality (eq 15). Tests for the existence of these possible mechanisms and for assessing their relative contributions might be accessible through studies of the temperature dependence of exchange rate constants (in which K_w is varied) and of the pH dependence of exchange at different catalyst concentrations. These studies are presently underway (McConnell and Seawell, 1972).[6] Also, since neither the pH dependence nor the existence of H^+–B–NH_2 can be denied, it appears that both of these mechanisms are worthy of consideration and exploration as possible exchange mechanisms in the DNA double helix.

Double-Helical DNA and Polynucleotides. From these data we can account for one of the general features of DNA hydrogen exchange without reference to a specific exchange mechanism. It is that the slowly exchanging hydrogens of the DNA double helix fall into a distribution of kinetic classes having different exchange rates, which bears no clear relationship to structural considerations (McConnell and von Hippel, 1970a,b). Since the pH profiles for the amino protons of adenine and guanine do not coincide (Figures 2 and 3), we can follow the interpretations made by Woodward and Rosenberg (1971), who showed that different monomeric pH minima account for the distribution of different kinetic classes of hydrogens in denatured (and native) ribonuclease. A vertical line drawn through any given pH in Figures 2 or 3 intersects each pH profile at different exchange rates. We would expect, therefore, that single-stranded DNA (heteropolymer) would show a distribution of different classes for its amino protons, also. A homopolymer would exhibit a single kinetic class of amino protons, which appears to be borne out by studies on synthetic duplexes (Printz, 1970; Englander *et al.*, 1972). This adds support to the finding that the intrinsic exchange rate is reflected in the observed slow rates of exchange of hydrogens from the macromolecule (McConnell and von Hippel, 1970a).

The ability to detect the "outside" amino protons of the DNA helix through the application of the gel filtration method is largely, but not completely accounted for by the monomeric rates calculated from these data. Using our estimates of the hydroxyl and hydronium rate constants for the cyclic phosphates (Table I), which correspond to conditions that approximate those associated with the macromolecular studies (0°, pH 7), we estimate that the amino protons of 2′:3′-cAMP exchange at a rate of 0.3 sec^{-1} (eq 4). If the 2′:3′-cGMP amino protons show the same temperature dependence for k_{OH^-}, then their exchange is approximately 6 sec^{-1} for these same conditions. Half-times obtained from these first-order rates are 2 and 0.1 sec for the adenine and guanine derivative, respectively. We have found that rates for amino protons of cytosine would fall in this range, also (B. McConnell and P. Seawell, 1972).[7] This is outside the range of rates measurable by the gel filtration method, since these protons would be greater than 97% exchanged before the first (10 sec) point could be obtained by the "high-speed" technique. Therefore, in those molecules, in which the extra amino protons can be detected by the gel filtration method, the polymeric helical structure must impart some barrier to exchange of their "outside" amino protons. For native DNA the barrier is not as large as anticipated, since the monomeric–NH_2 rates are only 10–100 times faster than the helical DNA counterparts, whose half-times are measured at 15–25 sec (Hanson, 1971; Englander and von Hippel, 1972). If these rates apply to synthetic polynucleotides, then the barrier varies considerably with the nature of the homopolymeric strands; twice as large as that of DNA for poly[d(A-T)]·poly[d(T-A)], three times as large for poly(dG)·poly(dC), and perhaps 20 times as large for poly(rG)·poly(rC) (Englander *et al.*, 1972). Of course, the validity of this range of values as a measure of the extent to which the outer amino protons are slowed in the helix depends on the suitability of the cyclic phosphate derivatives as models for intrinsic exchange. The most appropriate control for estimates of the intrinsic rate would be the –NH_2 protons from the single-stranded polymer measured under conditions in which nonspecific hydrogen bonding is prevented. Since stacking might not be involved in exchange (McConnell and von Hippel, 1970b; Bird *et al.*, 1970), a stacked single strand might be the best choice for a given natural or synthetic polymer. These control criteria are approximated to a limited extent by the monomers, since the compounds tested here would not be expected to form intraspecies hydrogen bonds in aqueous solution and they would probably form stacked structures at these concentrations (Ts'o *et al.*, 1969). Although the proper control could exhibit slower exchange than that measured for the monomeric units, it might not differ greatly, since the high-speed gel filtration technique is *unable* to measure all the protons of poly(rA)·poly(rU) under similar conditions to those described above (Englander *et al.*, 1972). The exchange barrier for this homopolymer duplex could be quite small. Therefore, it is of interest to consider the possible nature of this barrier for the exposed amino protons of helical polynucleotides and to relate this to the possible sequence of events that lead to exchange of all the dissociable protons of the macromolecule.

The traditional formulation of hydrogen exchange of macromolecules is (Linderstrom-Lang, 1955)

$$\text{"closed" (nonexchangeable)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{"open" (exchangeable)} \xrightarrow{k_3} \text{exchange} \quad (16)$$

from which the general expression can be derived

$$k_{obsd} = \frac{k_1}{k_2 + k_3} \times k_3 \quad (17)$$

where k_1 and k_2 are the rate constants for respectively establishing and removing access of contact solvent to the exchangeable proton and k_3 is the intrinsic exchange rate, *i.e.*, $(1/\tau)_{pH}$. The use of this kinetic model for a description of exchange in native DNA has involved the identification of the closed $\rightleftharpoons$ open process as a "structural" event and is restricted to the tacit condition that the structural closed → open reaction must precede and never follow the chemical exchange events, open → exchange. For example, in a mechanism similar to the "indirect" scheme mentioned above, the complementary strands of DNA would first separate to allow proton transfer to (or from) a relatively hidden, hydrogen-bonded site on the purine or pyrimidine ring such as the N-1 of adenine (or the N-1 proton of guanine). Since exchange of the DNA protons seems to be interdependent (McConnell and von Hippel, 1970a; Englander *et al.*, 1972), these events would be followed by exchange of the amino protons. Such a mechanism involving the same "compulsory" structural distortion for exchange of all protons would not be an unreasonable postulate if it were assumed that the intrinsic exchange of the amino protons (k_3) was equal to that of the imino protons and much greater

[6] In preparation.

[7] Submitted for publication.

than the macromolecular rate (k_{obsd}) (Englander *et al.*, 1972).

On the other hand, it is possible to envision a mechanism for helical exchange that does not necessarily conform to a rigorous structural identification of the closed $\rightleftharpoons$ open equilibrium, and places the closed–open reaction in question as a preliminary, compulsory, rate-limiting event for all the protons.

For example, exchange of the outside amino protons of the helix might bear a *causal* relationship to subsequent structural events that initiate exchange of the inter-base protons. Initial exposure of the groove amino protons to solvent catalyst could involve a relatively subtle conformational change, but might also be governed to a large extent by local modification of the negative phosphate atmosphere. The exchange of these protons would constitute a nucleation event for sufficient structural distortion to allow exchange of the inter-base protons of the same base pair. The plausibility of this type of mechanism is based on the following considerations. (1) Changes in DNA hydrogen-exchange rates associated with manipulation of the phosphate charge by variations in salt concentration can be quite large (Printz and von Hippel, 1968; McConnell and von Hippel, 1970a), and thus could represent a significant portion of the barrier for DNA discussed above. (2) For DNA and for some (but not all) homopolymers the rates ascribed to the amino protons are faster than the rates for the inter-base protons, and with one exception (poly(rG)·poly(rC)) the former are not greatly different than rates for the monomeric $-NH_2$. (3) If the direct mechanism (eq 7–10) is a possibility, then solvent catalyst should choose the relatively exposed nonhydrogen-bonded amino protons over the inaccessible and hydrogen-bonded amino and imino hydrogens as initial candidates for exchange. (4) Proton transfer at the exposed part of the amino nitrogen in the helix, either by removal or addition of a proton would result in two changes in the base pair; a charge would be placed locally and the hydrogen bond of the other amino proton as well as that of the imino proton of the same base pair would be broken. Thus, the stage would be set for exchange of the inter-base protons of the same base pair. (5) Exchange of the inter-base protons is interdependent (McConnell and von Hippel, 1970a; Englander *et al.*, 1972) and this interdependence should extend in varying degrees to the outside $-NH_2$ proton of the same base pair. If this is true, then the observation of exchange of the outside amino protons as being *faster* or the *same* as that of the inter-base protons would depend on the rates associated with the *subsequent* structural event in relation to the intrinsic exchange rate of the inter-base protons. (6) The intrinsic exchange of the imino protons of guanine and thymine should be much faster than that of the amino protons. Their pK_a's are measurable in the monomer and polymer and, since they are less than that of OH^-, their exchange should be diffusion controlled (Eigen, 1964). However, in the helix their exchange is never faster, and sometimes slower than that of the outside $-NH_2$ protons. If the same structural opening were compulsory for all protons, the reverse would be expected.

A final consideration is that hydrogen exchange in the helical polynucleotide is associated with a lower activation energy than might be expected for thermal processes that separate the helical strands and produce unstacking of the bases (McConnell and von Hippel, 1970b; Bird *et al.*, 1970). This situation is similar to that of protein hydrogen exchange, in which exchange at moderate temperatures is not related to thermal folded $\rightleftharpoons$ unfolded reactions, but to events associated with much lower energy barriers (Woodward and Rosenberg, 1971). Data presented in this study indicate that catalysis of $-NH_2$ exchange by H_3O^+, OH^-, and phosphate may exhibit a very high-temperature dependence (Table II). Until the activation energy of these catalytic rate constants can be determined and compared with that of the hydrogen exchange of the helix, the possibility remains that the values for the monomer and the helix might be sufficiently similar to support the general mechanism cited above. After correction for changes in K_w, a high-temperature dependence would be expected for the direct mechanism (eq 7–10), but not for the indirect mechanism (eq 12–13), since diffusion-controlled reactions implied by the latter are of low activation energy (Eigen, 1964). The concentration of H^+–B–NH_2 would be temperature dependent, but not to the extent reflected by the rate decreases observed here (Figure 2).

In summary, there are two possible interpretations of DNA hydrogen exchange, whose selection awaits further study at the monomeric and macromolecular level before detailed exchange mechanisms can be postulated. The first and presently accepted one is that proton exchange in the helix requires an initial structural distortion that is sufficient for exchange of all protons in the helical segment. This conforms to the general aspects of the kinetic model (eq 16). The second interpretative approach offered here is that if an initial "structure" distortion exists, it is sufficient only for exchange of the nonhydrogen-bonded amino protons of the helical grooves. In this case the event that is compulsory for exchange of the interbase protons is not this initial structural deformation but exchange of these outside amino protons. Thus, the structural opening required for exchange of the hydrogen-bonded protons would *follow* an initial proton transfer. This approach is possible, also, by the indirect mechanism if the initial proton transfer involved the relatively accessible N-7 of guanine.

The main issue regarding these two mechanistic approaches as described here is the determination of *initial* events that lead to hydrogen exchange in the double helix, whose plausibility does not depend on a detailed argument pertaining to conceivable events that follow, but to further experimental evidence on the monomer and the polymer. These two mechanistic approaches are not mutually exclusive and at present there is no specific, unambiguous evidence to support or reject either possibility. It appears that an important part of this evidence can be obtained from the pmr spectrometer.

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References

Bird, R. E., Lark, K. G., Curnutte, B., and Maxfield, J. E. (1970), *Nature (London) 225*, 1043.

Christensen, J. J., Ryting, H. J., and Izatt, R. M. (1970), *Biochemistry 9*, 4907.

Eigen, M. (1964), *Angew. Chem., Int. Ed. 1*, 1.

Eigen, M., and Kruse, W. (1953), *Naturforschg. 18*, 857.

Emsley, J. W., Feeney, J., and Sutcliffe, L. H. (1965), High Resolution Nuclear Magnetic Resonance Spectroscopy, Vol. 1., New York, N. Y., Pergamon Press, p 512.

Englander, S. W., and Englander, J. J. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 370.
Englander, J. J., Kallenbach, N. R., and Englander, S. W. (1972), *J. Mol. Biol. 63*, 153.
Englander, J. J., and von Hippel, P. H. (1972), *J. Mol. Biol. 53*, 171.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Grunwald, E., and Price, E. J. (1964), *J. Amer. Chem. Soc. 86*, 2965.
Grunwald, E., and Puar, M. S. (1967), *J. Phys. Chem. 71*, 1842.
Hanson, C. V. (1971), *J. Mol. Biol. 58*, 847.
Hertz, H. G. (1960), *Z. Elektrochem. 64*, 53.
Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.
Johnson, C. S., Jr. (1965), *Advan. Magn. Resonance 1*, 39.
Klotz, I. M., and Frank, B. H. (1965), *J. Amer. Chem. Soc. 87*, 2721.
Linderstrøm-Lang, K. U. (1955), *Chem. Soc. Sp. Publ. 2*, 1.
Marshall, T. H., and Grunwald, E. (1969), *J. Amer. Chem. Soc. 91*, 4541.
McConnell, B., Raszka, M., and Mandel, M. (1971), *Biophys. Soc. Meeting, 15th, Abstr.*, M-9.
McConnell, B., Raszka, M., and Mandel, M. (1972), *Biochem. Biophys. Res. Commun. 47*, 692.
McConnell, B., and von Hippel, P. H. (1970a), *J. Mol. Biol. 50*, 297.
McConnell, B., and von Hippel, P. H. (1970b), *J. Mol. Biol. 50*, 317.
McConnell, B., and von Hippel, P. H. (1971), *Procedures Nucl. Acid Res. 2*, 389.
Meiboom, S. (1960), *Z. Elektrochem. 64*, 50.
Meiboom, S. (1961), *J. Chem. Phys. 34*, 375.
Miles, H. T., Howard, F. B., and Frazier, J. (1963), *Science 14*, 1458.
Phillips, R., Eisenberg, S. J. P., George, P., and Rutman, R. J. (1965), *J. Biol. Chem. 240*, 4393.
Printz, M. (1970), *Biochemistry 9*, 3077.
Printz, M. P., and von Hippel, P. H. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 363.
Printz, M. P., and von Hippel, P. H. (1968), *Biochemistry 7*, 3194.
Robinson, D. R., and Grant, M. E. (1966), *J. Biol. Chem. 241*, 4030.
Ts'o, P. O. P., Schweizer, M. P., and Hollis, D. P. (1969), *Ann. N. Y. Acad. Sci. 158, Suppl. 1*, 256.
von Hippel, P. H., and Printz, M. P. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 1458.
Woodward, C. K., and Rosenberg, A. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1067.
Woodward, C. K., and Rosenberg, A. (1971), *J. Biol. Chem. 246*, 4114.
Yamane, T. (1971), *Procedures Nucl. Acid Res. 2*, 262.

Selective Transfers of *trans*-Ethylenic Acids by Acyl Coenzyme A. Phospholipid Acyltransferases†

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ABSTRACT: *trans*-Octadecenoyl coenzyme esters are generally good substrates for acyl-CoA:phospholipid acyltransferase action to form phosphatidylethanolamine or choline derivatives. The CoA esters of the 9-, 10-, and 11-positional isomers, which are relatively common in processed dietary fats, have different activities in forming phospholipids. Comparison of the patterns of selectivity for the cis and trans series supports the concept that acyl transfer to the 1 position is quite sensitive to configurational differences in acids, whereas transfer to position 2 is not. The latter transfer, however, seems favorable for acids with π bonds at positions 5, 9, and 12.

The unsaturated fatty acids of glycerides in nature are almost always of the cis configuration; some exceptions being vaccenic acid (*trans*-11-octadecenoic acid) in animal fats (Bertram, 1928) and *trans*-3-hexadecenoic acids at the 2 position of phosphatidylglycerol in plants (Haverkate and Van-Deenen, 1965). Although rumen bacteria can convert cis to trans configuration (Shorland *et al.*, 1955), the effect of the trans fatty acids from these origins on our diet is probably not very significant. However, the presence of a large amount of positional isomers of trans fatty acids in margarine and shortening prepared from hydrogenated fats (up to 40% of total fatty acids by Mabrouk and Brown, 1956) led many investigators to examine the nutritional and biochemical features of trans fatty acids. When ingested, trans fatty acids were absorbed, oxidized, and transported across the placental membrane at rates comparable to saturated acids or cis isomers (Ono and Fredrickson, 1964; Coots, 1964). Trans fatty acids were also incorporated into lipids of most tissues, although they were partly excluded from the lipids of the brain and testes (Sinclair, 1935). Esterified trans isomers were shown to occupy mostly the 1(3) position of triglycerides (Raulin *et al.*, 1965) and both the 1 and 2 positions of phospholipids, with significant enrichment of the 1 position (Raulin *et al.*, 1963; Selinger and Holman, 1965). While the physiological significance of the displacement of saturated and un-

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