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Exchange Mechanisms for Hydrogen Bonding Protons of Cytidylic and Guanylic Acids†

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ABSTRACT: The pH dependence of buffer catalysis of exchange of the C-4 amino protons of cyclic cytosine 2′,3′-monophosphate (cCMP) and the N-1 proton of cyclic guanosine 2′,3′-monophosphate (cGMP) conforms to an exchange mechanism, in which protonation of the nucleobases at C(N-3) and G(N-7) establishes the important intermediates at neutral to acidic pH. Rate constants for transfer of the G(N-1) proton to H_2O, OH^-, phosphate, acetate, chloroacetate, lactate, and cytosine (N-3) were obtained from ^1H nuclear magnetic resonance line width measurements at 360 MHz and were used to estimate the pK or acidity of the exchange site in both the protonated and unprotonated nucleobase. These estimates reveal an increase in acidity of the G(N-1) site corresponding to 2 to 3 pK units as the G(N-7) site is protonated: At neutral pH the G(N-1) site of the protonated purine would be ionized (pK = 6.3). Determinations of phosphate, imidazole, and methylimidazole rate constants for transfer of the amino protons of cCMP provide a more approximate estimate of pK = 7 to 9 for the amino of the protonated pyrimidine. A comparison of the intrinsic amino acidity in the neutral and protonated cytosine is vitiated by the observation that OH^- catalyzed exchange in the neutral base is not diffusion limited. This leads to the conclusion that protonation of the nucleobase effects a *qualitative* increase in the ability of the amino protons to form hydrogen bonds: from very poor in the neutral base to "normal" in the conjugate acid.

A dominant characteristic of the biological function of monomeric and polymeric nucleotides is the ability of their nucleobase protons to participate in hydrogen bonding with a high degree of specificity that is crucial to their biological effect. Therefore, one might expect that the evolution of the purine and pyrimidine structures in biomolecules contained a selective element that would govern the characteristics of hydrogen bond formation peculiar to nucleic acids. This element would be perceived through the mechanism of hydrogen exchange, which describes two related aspects important for hydrogen bonding: proton lifetime and acidity.

For this reason, it was of particular interest to find that exchange of the $-NH_2$ protons of aqueous adenine, cytosine, and guanine nucleotides were accessible to direct study by NMR (McConnell & Seawell, 1972, 1973). Adenylic acid-NH_2 proton exchange at neutral and acidic pH occurs almost exclusively by a two-step mechanism, in which transfer of the $-NH_2$ proton to solvent is preceded by protonation of the basic endocyclic nitrogen of the purine (N-1) (McConnell, 1974). Confirmation of this mechanism in the adenine mononucleotide was achieved by stopped-flow methods (Cross et al., 1975). Although there is $-NH_2$ exchange from the unprotonated form of the nucleotide as well, this becomes significant only well above neutrality. It appears that the important mechanism at physiological pH involves the ability of the conjugated purine structure to communicate the effects of ring protonation as a regulatory aspect of proton exchange at a separate site on the molecule, the exocyclic $-NH_2$. This two-step exchange mechanism accounts for properties of hydrogen exchange in

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A-U double helical structures and has been deduced from data on polymeric G-C as well (Teitlebaum & Englander, 1975a,b). A matter of interest here is that, unlike the A-U and A-T base pairs, the G-C pair of the double helix contains an endocyclic basic nitrogen that is exposed to solvent. An exchange mechanism similar to that of adenine would provide a means for communication from solvent to the helix interior with consequent effects on Watson–Crick H bonding.

This study is an examination of the extension of the exchange characteristics documented for the $-NH_2$ protons of adenylic acid to the (N-1) proton of guanylic acid and the $-NH_2$ protons of cytidylic acid. Like adenine, guanine and cytosine contain weakly basic ring nitrogens in addition to weakly acidic groups. No confirming evidence has been published thus far for a similar exchange mechanism in G and C, although the suggestion of such a mechanism in the mononucleotides is supported by preliminary observations of exchange catalysis in the cyclic 2′,3′-nucleotides (McConnell & Seawell, 1972, 1973).

The results of this study indicate that the predominant $-NH_2$ proton exchange mechanism in cytosine at neutral pH is similar to that established for adenine in the requirement for a protonated intermediate. This mechanism applies also to the exchange of the (N-1) proton of guanine. Below pH 7.5 measurable transfer of these protons to a solvent acceptor (a buffer, water or the nucleotide itself) requires protonation of the basic ring nitrogen (N-7 of G or N-3 of C). Exchange of the $-NH_2$ protons of guanylic acid is treated in a separate study, due to the presence of possible ionized exchange intermediates in addition to the protonated nucleobase form (McConnell, in preparation).

A quantitative comparison of the neutral and protonated nucleobases as exchange intermediates leads to the suggestion that the G(N-1) group of (N-7)-protonated guanine would be ionized at physiological pH. In addition, nucleobase protonation may greatly increase the ability of the amino protons of cytosine to participate in "normal" H bonding.

Materials and Methods

The cyclic 2′,3′-nucleotides, guanylic and cytidylic acids (Na^+) (Sigma), were used as supplied and weighed on the basis of hydrated molecular weights. Imidazole and 2-methylimidazole (Sigma) were recrystallized from benzene. Other exchange catalysts, monochloroacetic acid (Allied Chemical Reagent), lactic acid (Aldrich, 99%), acetic acid, and sodium phosphate (Baker Reagent), and Tris (Sigma) were used as supplied. In addition to its use to determine concentrations of catalyst stock solutions, pH titration was used routinely to measure (1) pK_a values of participating functional groups in experimental mixtures, (2) hydrolysis of the nucleotide cyclic phosphates, and (3) the catalytic purity of the buffer catalysts. All pH measurements were made at 25 °C with a Beckman Model 4500 pH meter and a combined glass electrode having a KCl-AgCl reference. Although many experiments were performed at 5 °C all pH values are presented as measured at 25 °C. The pH change in lowering the temperature from 25 to 5 °C is between 0.2 and 0.25 unit (McConnell, 1974). Sample pH adjustment was both sequential and random and all series of experiments with pH as variable were repeated. Following sequential experiments that involved buffer catalysts, pH adjustments were repeated at the initial pH to verify that sample history had no effect on the reproducibility of line width measurements. Line width measurements were the same for solutions containing 2 and 4 mM EDTA or 0.1 M NaCl.

Two NMR spectrometers were used in continuous wave (CW)[1] mode to obtain the bulk of the line width data: a Varian HA-100 spectrometer of the Chemistry Department, University of Hawaii; and the Bruker HXS-360 NMR spectrometer at the Stanford Magnetic Resonance Laboratory, Stanford University.[2] Experiments performed on the latter instrument involved the technique of correlation spectroscopy (see Gibbons et al., 1975, and references therein) for the rapid accumulation of multiple scans necessary for accurate determination of line widths between 50 and 400 Hz. The combination of correlation capability with the high-field frequency extended the usable pH range over which lifetime-broadened resonances could be measured. All experiments were repeated at different sweep rates in consideration of slow passage conditions necessary for exchange measurements (McConnell, 1958) and to verify that line width was independent of sweep rate for the filtering conditions used. Further verification of time domain and exchange modes in relation to line broadening was obtained from the Varian XL-100 in CW-correlation mode.[3] Correlation spectroscopy in both the Bruker and Varian instruments was attained by the use of the Nicolet 1080 instrument computer system.

Kinetics. The experimental approach for detecting the mechanism involving initial protonation of the purine or pyrimidine ring nitrogens is based on the prediction that relatively large concentrations of a buffer of known pK_a would catalyze exchange exclusively through the action of the buffer base as an acceptor of the observed proton (McConnell, 1974). With this restriction the pH dependence of exchange is defined by the relative concentration changes of both protonated nucleotide and buffer conjugate base, whose respective pK_a values appear as terms in the kinetics.

A scheme showing two exchange routes for the H-bonding protons of G and C is presented in Figure 1. The exchange lifetime of the G(N-1) and C($-NH_2$) protons is governed by their rate of transfer to a proton acceptor, A, either from the unprotonated form of the nucleotide (U mechanism) or from the nucleotide protonated at a basic ring nitrogen (P mechanism).[4] The basic endocyclic nitrogen can be N-3 of cytosine ($pK \sim 4$) or N-7 of guanine ($pK \sim 2$). The rate equations for each exchange route are shown in Figure 1 in terms of W_U or W_P, the ^1H NMR line broadening of the C($-NH_2$) or of the G(N-1) resonances at half height induced by acceptor A. For the amino protons of cytosine and the imino (N-1) proton of guanine, the total line broadening associated with exchange catalysis by A would be a sum of the two separate pathway contributions, W_U and W_P.

$$W_A = W_U + W_P = \left(\frac{1}{\pi}\right) \sum_i k_{U_i}[A_i] + \frac{\left(\frac{1}{\pi}\right) a_{H^+}}{K_N + a_{H^+}} \sum_i k_{P_i}[A_i] \quad (1)$$

where W_A is the total broadening, k_U and k_P are the second-order rate constants for proton transfer to A in the U and P

[1] Abbreviations used: cCMP, cyclic cytosine 2′,3′-monophosphate; cGMP, cyclic guanosine 2′,3′-monophosphate; CW, continuous wave.

[2] The Bruker HXS-360 NMR spectrometer is part of a National Facility funded by National Science Foundation Grant GR23633 and National Institutes of Health Grant RR00711.

[3] Of the laboratory of Professor Oleg Jardetzky, Stanford Magnetic Resonance Laboratory, Stanford University.

[4] Exchange of the G-NH_2 protons involves consideration of additional intermediates and will be considered in a separate study (McConnell, B., in preparation).

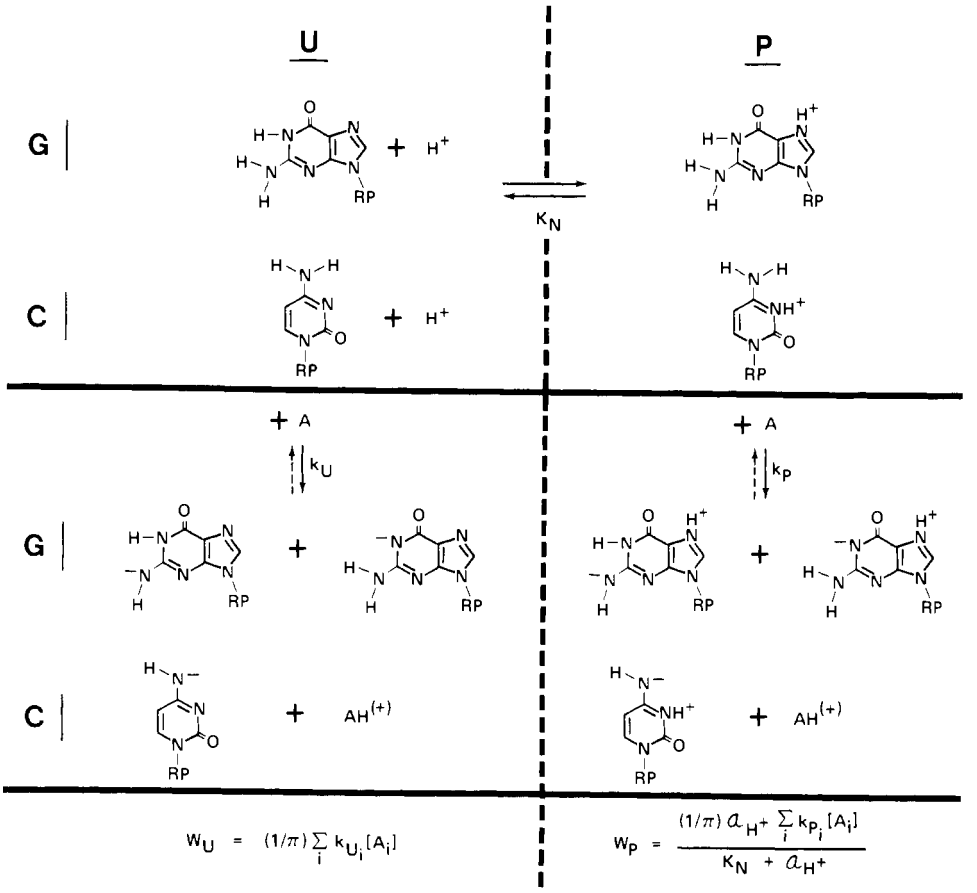

FIGURE 1: Two exchange routes for the H-bonding hydrogens of guanylic and cytidylic acids. Kinetic expressions (bottom of figure) for each route define the variation in additional experimental ^1H NMR line broadening (W_U and W_P) in terms of hydrogen ion activity ($a_{H_3O^+}$), dissociation constants of the endocyclic nitrogens (pK_N) and the second-order rate constants k_U and k_P for proton transfer to the acceptor (A). A is the conjugate base of any acid-base pair in solutions, whose concentration ([A]) is pH dependent.

mechanisms, respectively, a_{H^+} is the hydrogen ion activity measured by the pH electrode, and K_N is the acid dissociation constant of the basic ring nitrogen of the purine or pyrimidine.

The acceptor A is the conjugate base produced by acid dissociation of (1) a buffer, (2) functional groups on the nucleotide itself, or (3) the water species H_3O^+ and H_2O

$$AH \rightleftharpoons A + H^+$$

whose concentration is calculated from the uncorrected Henderson–Hasselbalch equation

$$A = \frac{A_{tot} 10^{pH-pK_A}}{1 + 10^{pH-pK_A}} \qquad (2)$$

where A_{tot} is the total acceptor concentration and pK_A is the negative logarithm of its acid dissociation constant. The broadening induced by catalyst A is related to the observed line width by

$$W_{obsd} = W_O + W_A \qquad (3)$$

where W_{obsd} is the experimental half-height width of the proton resonance and W_O is the contribution of all other line-broadening processes including solvent exchange in the absence of the acceptor.

Reversal of proton transfer from the exchange site to an acceptor occurs rapidly at the same rates for all exchange intermediates through several solvent-mediated pathways. Because of this, the rate constant for the forward reaction, k_P (eq 1), represents a comparative measure of acidity in the protonated and unprotonated form of the nucleotide. This acidity can be expressed in terms of pK_P from the relation (Eigen, 1964)

$$k_P = \frac{k_D \times 10^{(pK_A - pK_P)}}{1 + 10^{(pK_A - pK_P)}} \qquad (4)$$

where k_P and pK_A are as defined above, pK_P is the negative logarithm of the dissociation constant of the exchange site in the protonated nucleobase, and k_D is the rate constant for formation of the donor–acceptor encounter complex required for proton transfer in the thermodynamically favored direction (when $pK_A > pK_P$). Substitution of k_U for k_P provides the corresponding acidity constant, pK_U, for the neutral form of the nucleotide. Equation 4 provides a quantitative means for referral to the acidity of the exchangeable proton or for a measure of k_D for different acceptors of the G(N−1) proton (see Results). The application of this equation as a comparison of acidities of the C(−NH_2) and G(N−1) protons will be evaluated in the discussion.

Results

Cytosine-NH_2. Although W_A is the sum of two terms W_U and W_P (eq 1), the relative contribution of these terms changes drastically with pH and W_{obsd} becomes a separate measure of each. This separation in W_U and W_P can be seen in the pH dependence of W_{obsd} for the individual -NH_2 ^1H NMR signals of cCMP (Figure 2). For each -NH_2 resonance there is a broad minimum in W_{obsd} at approximately pH 6, with W_U representing line broadening above pH 7 and W_P accounting for the

TABLE I: Kinetic Constants of Catalysis of $-NH_2$ Exchange for Cyclic 2′,3′-Cytidylic Acid[a].

kinetic constants	catalyst (acceptor)				
	C(N-3)	phosphate	imidazole	methyl-imidazole	OH^-
molarity of acceptor	0.4	0.05	0.05	0.05	10^{-6}
pK_N[b]	4.0	4.0	4.0	4.0	4.0
pK_A[b]	3.9	6.8	7.1	8.0	15.7
k_P (M^{-1} s^{-1})	1.2×10^3 (H_u)[c] 8×10^2 (H_d)	1.5×10^6 (H_u) 1.2×10^6 (H_d)	3.0×10^6 (H_u) 1.9×10^6 (H_d)	1.3×10^7 (H_u) 0.9×10^7 (H_d)	
k_U[d] (M^{-1} s^{-1})					3×10^7 (H_u) 3×10^8 (H_d)

[a] Catalysts or acceptors are defined in eq 1 of text. [b] All pK_a values were obtained independently by extraction from the kinetic data and by potentiometric titration of the reaction mixtures. [c] H_u and H_d are the separate upfield and downfield NH_2 proton resonances of cCMP, respectively. [d] To calculate $[OH^-]$, $K_w = 14.8$ at 3 °C and 14.0 at 25 °C.

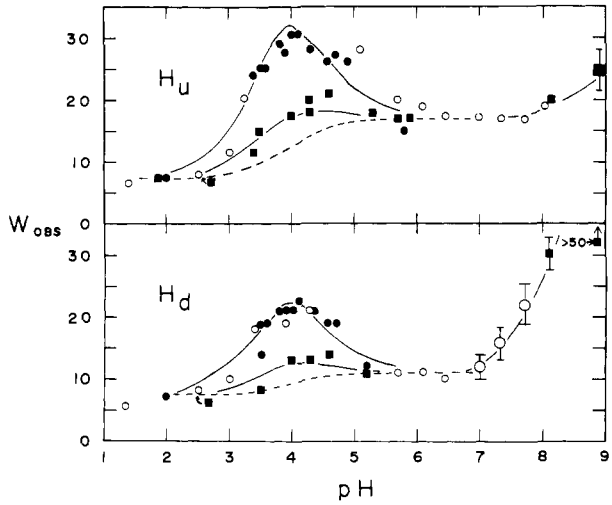

FIGURE 2: The effect of pH on ^1H NMR line width of the $-NH_2$ protons of cCMP. Observed line widths for the separate upfield (H_u) (top) and downfield (H_d) (bottom) resonances obtained for nucleotide concentrations of 0.4 M (circles) and 0.1 M (squares). Data were obtained at 360 MHz (closed figures) and 100 MHz (open figures) at probe temperatures of 3 ± 1 °C. Solid curves are theoretical line widths from eq 1-3. The dashed curves trace the calculated values of W_O (eq 4) (see text).

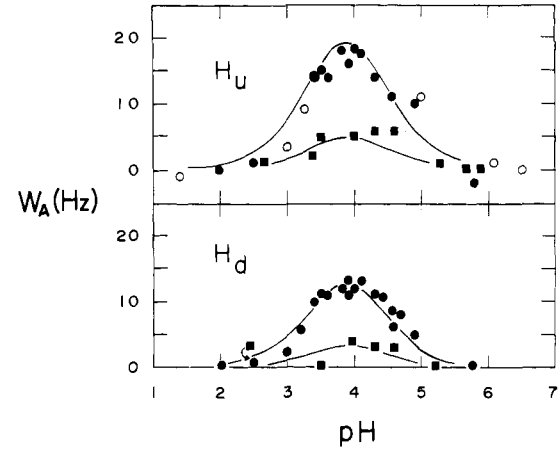

FIGURE 3: The effect of pH on ^1H NMR line broadening, W_A, due to the (N-3) of cCMP. Data taken from Figure 2 were corrected by eq 3 for the pH variation of W_O (eq 5) to show the position of the maximum with respect to pH.

broadening below pH 5 (which exhibits a maximum at pH 4). The alkaline broadening (W_U) begins well above neutrality, indicating that the only effective acceptor from the neutral nucleobase is hydroxyl ion (pK = 15.7). From eq 1 k_U is 3 ± 1×10^8 and 3 ± 1×10^7 M^{-1} s^{-1} for the downfield (H_d) and upfield (H_u) resonances,[5] respectively (Table I).

The pronounced maximum in the acid pH region (Figure 2) lies at pH = pK_N for C(N-3) and conforms very well with the broadening expected from the W_P portion of eq 1, where the acceptor A = C(N-3). The line width is dependent on nucleotide concentration only in the pH region of this maximum, where the concentration dependence is first order. Treatment of the data for variable concentrations first involves an estimate of W_O (eq 3), which increases from about 10 Hz at low pH to larger values as the pH = pK_N region is traversed. The variation in W_O is not concentration dependent and is due mainly to an increase in rotational broadening (McConnell & Seawell, 1973). By assuming rapid exchange between the slowly rotating (low pH) and more rapidly rotating (high pH) forms, intermediate values of W_O were calculated from the relation

$$W_{O_{pH}} = \frac{W_{O_a} + w_{O_b} \times 10^{(pH-pK_N)}}{1 + 10^{(pH-pK_N)}} \quad (5)$$

where $W_{O_{pH}}$ is the estimated broadening determined from the low pH (W_{O_a}) and pH 6 (W_{O_b}) experimental values. The use of eq 3 and 5 for the determination of $W_A = W_P$ at several pH values provided data symmetrically disposed with a maximum shown at pH 4.0, the value of pK_N (Figure 3). Justification of this method of correction is based on the observation that (1) an erroneously high pK_N value would be extracted from the uncorrected data and (2) the pH profiles for different concentrations of nucleotide converge to the same values of W_{O_a} and W_{O_b} at pH 2.5 and pH 6. In Figure 3 the corrected data follow closely the theoretical pH profiles at two nucleotide concentrations, which were calculated with k_P = 400 and 600 M^{-1} s^{-1} for the downfield and upfield resonances, respectively (Table I).

Additional evidence for the P mechanism for the $-NH_2$ protons of cytosine (Figure 1) can be seen in the line broadening induced by the addition of phosphate as the acceptor. In this case, eq 1 predicts a maximum in W_A midway between pK_N and the pK of the acceptor (pK_A).[6] This prediction is

[5] Spectral assignments of the rotationally separate $-NH_2$ proton resonances of cCMP and their different responses to OH^- catalysis are discussed in McConnell & Seawell (1973).

[6] Theoretical pH profiles of catalysis for several combinations of pK_A and pK_N are presented elsewhere (McConnell, 1974).

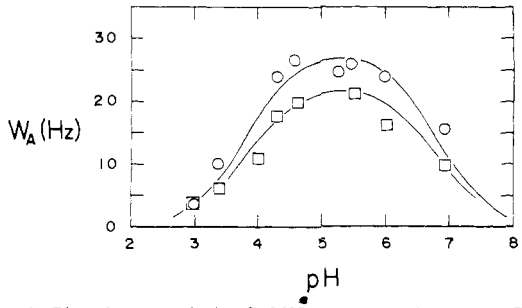

FIGURE 4: Phosphate catalysis of $-NH_2$ proton exchange in cCMP as a function of pH. Additional ^1H NMR line broadening, W_A, following the addition of 0.055 M sodium phosphate was measured for 0.1 M nucleotide at 3 ± 1 °C in the 360-MHz spectrometer for both the H_u (circles) and H_d (squares) resonances. Solid curves are calculated from eq 1-3 with W_O in this case identical with W_{obsd} of Figure 2.

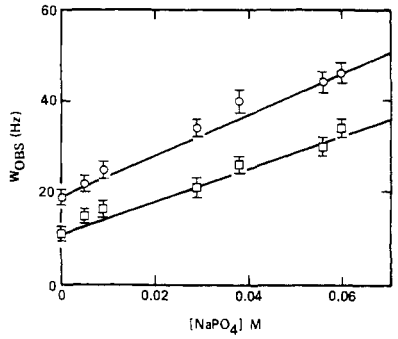

FIGURE 5: The effect of phosphate concentration on the line width for the $-NH_2$ protons of cCMP. Data were collected at pH 5.50 ± 0.05, 3 ± 1 °C in the 360-MHz spectrometer at a nucleotide concentration of 0.1 M. Solid lines are calculated from eq 1 and 2 (see text) with k_P values obtained for the separate upfield (circles) and downfield (squares) $-NH_2$ resonances (Table I).

borne out (Figure 4) and shows that the positions of the maximum and of the ascending limbs flanking it provide a sensitive, independent measure of pK_A and pK_N that does not depend on other terms in eq 1. The values of $pK_N = 4.0$ and $pK_A = 6.8$ obtained from Figure 4 agree with values obtained by pH titrations of the same mixture (Table I). These constants and k_P values for phosphate catalysis of 1.2×10^6 M^{-1} s^{-1} (downfield resonance) and 1.5×10^6 M^{-1} s^{-1} (upfield resonance) provide the theoretical plots seen in Figure 4 and Figure 5. In the latter figure, the linear concentration dependence for phosphate catalysis supports the premise (see Kinetics) that phosphate functions in the P mechanism exclusively as an acceptor of the $-NH_2$ proton and that higher order effects arising, for example, from an additional role as a proton donor, are absent (see McConnell, 1974; Cross et al., 1975; and Discussion).

The expected pH dependence of W_A shown for phosphate in Figure 4 is produced also by the addition of imidazole and 2-methylimidazole with appropriate displacements that conform to their pK_A values (not shown). Rate constants for all catalysts are listed in Table I and would fit a Brönsted slope of 1 with no more than 80% deviation in k_P, indicating good correspondence between pK_a and k_P. The exception is the cytosine N-3 of cCMP, whose k_P value is low by a factor of 2 or 3 from this correspondence.

Kinetic constants for the U mechanism of exchange derived from the separate cCMP amino proton resonances (Table I) show that, while k_U for the separate $-NH_2$ resonances differ by an order of magnitude, the corresponding k_P values are

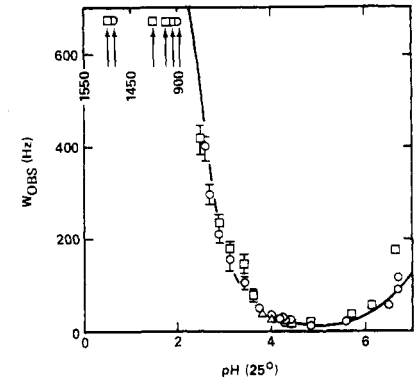

FIGURE 6: Observed ^1H NMR line width as a function of pH for the G(N-1) proton of cGMP. Experimental line widths were obtained by accumulation of multiple scans of the G(N-1) proton to the G(C-8) proton spectral region at 360 MHz and at a probe temperature of 4 ± 2 °C. Concentrations of cGMP were 0.04 M (circles) and 0.1 M (squares). The solid curve through the data locates calculated line widths according to eq 1-3 with W_O = 15 Hz, a nucleotide concentration of 0.04 M, and A = H_2O only (55 M) (see text). Kinetic constants are listed in Table I. Arrows at top left of figure represent unsuccessful attempts to measure the very broad resonance at the pH values indicated. Numbers are calculated values (see text).

quite close and may even be slightly reversed in relative magnitude.

Guanine(N-1). The pH dependence of the experimental line width for the G(N-1) proton resonance (Figure 6) exhibits a broad minimum at pH 5, which allows a separate evaluation of the W_U and W_P contributions to the total broadening (eq 1). The pronounced broadening above pH 6 conforms as expected to the U mechanism, where A = OH^- and eq 4 reduces to $k_U = k_D$, owing to the greater pK of OH^- ($pK_A = 15.7$; $pK_U = 9.4$). The value of k_U is $2 \pm 1 \times 10^{10}$ M^{-1} s^{-1} obtained from an exponential least-squares fit of the alkaline data (not completely shown) with a coefficient of determination, r^2 = 0.98. Measurements of this broadening are of limited accuracy because of the minimum buffer capacity in this neutral pH range. Effective buffers such as phosphate cannot be used, due to their large catalytic effect (see below). Also, for this reason, it is preferable not to attempt an identification of the source of the marked concentration dependence of alkaline broadening seen in Figure 6. This increased broadening could be accounted for by about 10% contamination of the cyclic nucleotides by secondary phosphate hydrolysis products. Although such contamination was not observed by pH titration, it cannot be ruled out in these experiments.

The line broadening below pH 5 (Figure 6) can be accounted for exclusively by the P mechanism (Figure 1), which provides a satisfactory fit of the experimental data. In this case pK_N = 2.1, the pK of G(N-7) and A = H_2O ($pK_A = -1.7$). The use of water as the only acceptor is based on the assumption that the other two acceptors present, G(N-7) and the primary cyclic phosphate of the nucleotide ribose, would have much lower k_D values (eq 4) and would make a negligible contribution (see below). This is justified by the observation that the low pH broadening goes to indeterminately large values between pH 0 and 1 (Figure 6). A theoretical curve based on equal k_D values for these acceptors and water would fit all the experimental data above pH 2.5, but would exhibit a maximum at pH 1.5 with measurable line widths between pH 0 and 1 (i.e., less than 500 Hz). Several attempts to observe the G(N-1) proton resonance at low pH (arrows at top left of Figure 6) were unsuccessful after several hundred accumulated scans, which should allow detection of a resonance exceeding

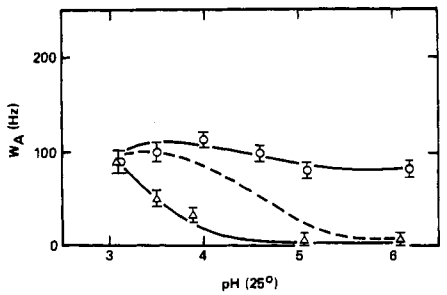

FIGURE 7: The effect of pH on exchange catalysis by acetate and chloroacetate. Exchange broadening, W_A, of the G(N-1) proton induced by 0.1 M acetate (circles) and 0.1 M chloroacetate (triangles) was obtained from eq 3 for 0.02 M cGMP at 3 ± 1 °C at 360 MHz. Solid curves through each set of data are calculated from eq 1-3 for pK_A values of 4.7 (upper) and 2.8 (lower) for acetate and chloroacetate, respectively. Kinetic constants for theoretical curves are listed in Table I. The dashed curve represents the calculated contribution of the W_P mechanism alone to W_A for acetate. To obtain W_A, W_O was obtained under the same conditions as in Figure 6 in the absence of catalyst for each pH value indicated.

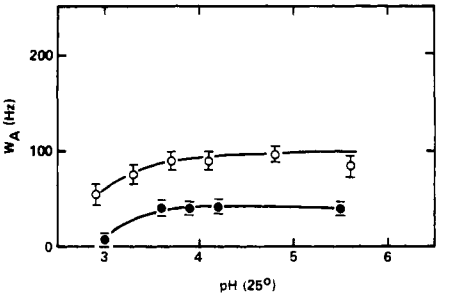

FIGURE 8: pH dependence of exchange catalysis of the G(N-1) proton by N-3 of cytidylic acid. W_A, the line broadening of the G(N-1) resonance of 0.025 M cGMP, was measured for cCMP at concentrations of 0.2 M (open circles) and 0.05 M (closed circles). To obtain W_A (eq 2), $W_O = W_{obsd}$ without catalyst in separate, identical experiments. Kinetic constants for the calculated curves are listed in Table II. Line width measurements were made at 360 MHz with a 3 ± 2 °C probe temperature.

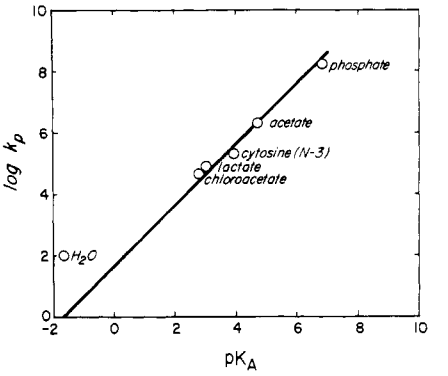

FIGURE 9: Brönsted plot for several catalysts in P mechanism exchange of the G(N-1) proton. The ordinate, log k_P, was obtained from k_P values listed in Table II for each acceptor having the listed pK_A values.

700 Hz in width. The calculated line widths shown in Figure 6 for A = H_2O are expressed as numbers under the arrows.

Although other sources of the acidic line broadening can be excluded (see Discussion), estimation of the contribution of the P mechanism requires the resolution of two aspects: (1) confirmation of the influence of the G(N-7) proton dissociation on the exchange kinetics; and (2) determination of rate constants for other buffer acceptors that catalyze exchange in this pH region. Both of these aspects are resolved by the application of homologous buffers of different pK_A. If a mechanism of the P type did not contribute to W_{obsd} (eq 1 and 2), then appropriate concentrations of acetate (pK_A = 4.7) should broaden the G(N-1) proton resonance at pH ≥4, but not at pH 3, while similar amounts of monochloroacetate (pK_A = 2.8) should broaden at neither pH. Conversely, both buffers would increase line width at pH 3, but only the stronger base would broaden at pH 5 (via the U mechanism), if the P mechanism did contribute. The choice in favor of the W_P contribution (eq 1) is borne out by the observed broadening (W_A) by these two catalysts as a function of pH (Figure 7). Theoretical fits of the data in this figure corresponded to pK_N = 2.1 and k_P values of 1×10^6 and 4.8×10^4 M^{-1} s^{-1} for acetate and monochloroacetate, respectively. The calculated contribution of W_P to acetate catalysis as a function of pH is shown as the dashed line in Figure 7 and shows that the broadening by the acetate at pH 5.5 is due only to W_U. The consequent determination of k_U at this pH leads to an estimate of k_D, since this is the only unknown in eq 4 for the U mechanism. The value $k_D = 2 \times 10^8$ M^{-1} s^{-1} is an expected one for solute-solute proton transfers (Bell, 1973).

The data of Figure 8 are a demonstration that the N-3 site of cytosine can function as an acceptor for the G(N-1) proton *in the P mechanism.* The pH dependence of exchange catalysis by cCMP is not the same as that of the acetates. First, the W_P contributions at low pH are lower than expected on the basis of a simple Brönsted relationship with the acetates (Figure 9). Second, there is a strong indication that theoretical curves could not be obtained to accommodate simultaneously highly accurate data below and above a pH region between pH 3.7 and 4.0 with constant values of k_P or k_U. The low k_P value for C(N-3) catalysis is not accounted for by appropriate changes in pK_N as judged from the pH variance of the G(C-8) proton chemical shifts, but may be related to the fact that the charge on the C(N-3) buffer goes through a transition at pH 4. Better fits of the data are obtained clearly by imposing a 10% increase in k_P and a 20% decrease in k_U as the pH 4 region is traversed from low to high pH. However, the data for catalysis by cCMP (Figure 8) were fitted by single, invariant values of k_P and k_U (Table II).

The kinetic constants for several buffer catalysts are listed in Table II with a summary of pK_N and pK_A values determined independently from titration and exchange data. The estimates of k_D for the U mechanism were obtained from eq 4, since the value of pK_U is a known quantity. However, for the P mechanism both k_D and pK_P are unknowns. An estimate of pK_P = 6.3 can be obtained from the measured k_P for water catalysis and the assumption that the corresponding k_D is 10^{10} M^{-1} s^{-1}. This is the recombination rate constant for solvent water in a variety of systems of the Eigen type (Eigen, 1964) and is applied in this case, since the G(N-1) proton of the protonated base appears to be a "normal" acid. Support for this is shown in a Brönsted plot (Figure 9) for all of the acceptors in the P mechanism listed in Table II. Points for all catalysts except H_2O and cytosine (N-3) fall close to a straight line of unit slope. Accepting unit slope and valid pK_A values, the upward displacement of the rate constant for water reflects a 100-fold greater value of k_D. If this value is placed at 10^{10} M^{-1} s^{-1}, then the corresponding k_D values for the other acceptors fall at 10^8 M^{-1} s^{-1}. These values fall in the range expected for solute-solute proton transfer in "normal" acids (Bell, 1973; Crooks, 1975) and are little different from the k_D values obtained in the U mechanism. On the basis of this, eq 4 for each mechanism can be solved simultaneously to give log k_U − log k_P =

TABLE II: Kinetic Constants for Exchange[a] Catalysis of the G(N-1) Proton of cGMP.

acceptor	acceptor concn (M)	pK_A[b]	pK_N	exp pH	k_U (M^{-1} s^{-1})	k_P (M^{-1} s^{-1})	k_D[d] (M^{-1} s^{-1}) U mechanism	k_D[d] (M^{-1} s^{-1}) P mechanism	$pK_U - pK_P$
H_2O	55	−1.7	2.1[c]	3.1[e]		100		1×10^{10}	3[e]
OH^-	variable	15.7		6-7.5	$2 \pm 1 \times 10^{10}$		$2 \pm 1 \times 10^{10}$	10^8	
phosphate	variable	6.8	2.1	3.75	5×10^5	$2 \pm 0.5 \times 10^8$	2×10^8	10^8	2
phosphate	variable	6.8	2.1	5.50	2×10^5	2×10^8	7×10^7	10^8	2
lactate	0.12	3.0	2.1	3.8		7×10^4		10^8	
lactate	0.12	3.0	2.1	4.6		7×10^4		10^8	
acetate	0.1	4.7	2.1[c]	3-6	2.5×10^3	1.2×10^6	2×10^8	10^8	3
chloroacetate	0.1	2.8	2.1[c]	3-6	50	48×10^4	$\leq 2 \times 10^8$	10^8	3
cCMP (N-3)	0.05	4.0	2.1	3-6	2.6×10^3	$9 \pm 1 \times 10^4$	$7 \pm 1 \times 10^8$	10^8	2
cCMP (N-3)	0.2	4.0	2.1	3-6	1.6×10^3	$9 \pm 1 \times 10^4$	$5 \pm 1 \times 10^8$	10^8	≥ 2

[a] All rate constants are for proton transfer from G(N-1) to the acceptor according to eq 1-3 of text. Experiments were performed at 3 ± 2 °C. [b] Literature values except for cCMP, which was obtained from McConnell (1977) and acetate, which was obtained by direct pH titration. pK_A for H_2O = −log 55; pK_A for OH^- = −log ($K_{w(t)}$/55), at temperature t. [c] Determined by ^1H NMR titration under conditions of experiment. [d] Obtained from eq 4 based on an assumed value of 1×10^{10} for H_2O and Figure 6. See text for U mechanism k_D determinations. [e] pK_P = 6.3 was obtained from k_P for water, eq 4 and the assumed k_D value for water. pK_U = 9.4 by pH titration. All othe $pK_U - pK_P$ were determined from eq 4 solved simultaneously for the U and P mechanisms having equal k_D values.

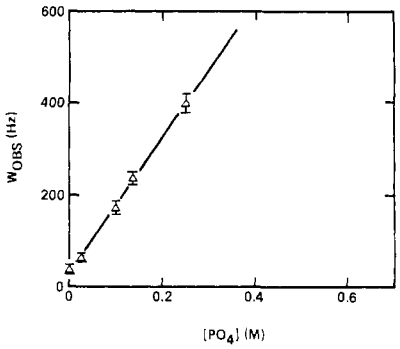

FIGURE 10: The effect of phosphate concentration of the observed ^1H NMR line width of the G(N-1) proton of cGMP. Line width measurements were taken from 360-MHz spectra obtained at 3 ± 2 °C probe temperature. Nucleotide concentration was 0.04 M and phosphate concentrations ranged from 0.025 to 0.25 M. All solutions were adjusted to pH 3.75 ± 0.02 at 25 °C.

$pK_U - pK_P$ for the other acceptors. As in the case of solvent acceptors (H_2O and OH^-), the solute acceptors provide estimates of $pK_U - pK_P$ of between two and three pK units. These are shown in Table II.

The contribution of W_P to W_A in eq 3 for phosphate can only be assumed in the absence of detailed pH dependence data. The k_P for phosphate is therefore tentative, but it is justified by (1) its placement in the Brönsted plot (Figure 9) and (2) the fact that k_D for phosphate would not exceed 10^8 M^{-1} s^{-1}. In support of the second point, similar rate constants for both mechanisms in phosphate catalysis at pH 5.5 are obtained, where $k_D = 7 \times 10^7$ M^{-1} s^{-1}. On the basis of the pH 5.5 data, the linear dependence of W_A with phosphate concentration seen at pH 3.75 (Figure 10) can be attributed to W_P alone. The linearity is consistent with the experimental premise that buffers function only as acceptors in this system and produce no higher order effects from their additional function as proton donors (McConnell, 1974). The k_P for phosphate in Table I and Figure 10 was obtained from this data.

Discussion

The experiments reported in this study are concerned primarily with an examination of the P mechanism as a candidate for the major exchange route of the H-bonding protons of cytosine and guanine at neutral to acid pH. The required demonstration of a protonated exchange intermediate was obtained by (1) agreement between independent titration and kinetic estimates of the endocyclic nitrogen pK, (2) conformance of experimental and calculated pH profiles for buffer catalysis, and (3) concentration requirements of buffer catalysis appropriate to the magnitude of pK_N (eq 1).

Sources of broadening of the G(N-1) proton other than exchange with water are ruled out as factors in low pH line width. Transfer of saturation from water protons to the G(N-1) proton is demonstrable in gated decoupling experiments. Consideration of a possible quadrupolar effect to account for G(N-1) broadening would require a limiting line width of ≥1400 Hz for this proton resonance for the fully protonated purine. However, acidification of cGMP in Me_2SO-H_2SO_4 mixtures is sufficient to completely protonate G(N-7) as measured by the G(C-8) proton chemical shift, but does not produce dramatic broadening of the G(N-1) proton (McConnell, unpublished observations). The observation of a sharp G(N-1) proton resonance in acidified Me_2SO testifies, also, to the role of H_2O as an acceptor in the P mechanism. It is of interest that the line width of the thymidylic acid proton at T(N-3) is relatively unaffected by large increases in proton concentration (McConnell, unpublished observations). Although the T(N-3) and G(N-1) sites are of quite similar acidity, the pyrimidine ring of thymidylic acid contains no basic nitrogen accessible to protonation (Christensen et al., 1970). However, the dissociable proton of the tryptophan pyrrole nitrogen shows low pH exchange behavior quite similar to that of the G(N-1) proton (Waelder et al., 1975). As a secondary amine, this nitrogen could accept a second proton to produce an exchange intermediate analogous to the protonated nucleotide, but now at the same site from which exchange is observed. Similar ^1H NMR broadening at very low pH is seen in $-NH_2$ protons of 7-methylguanosine (Rice, Uchima, & McConnell, in preparation) and may reflect protonation of the amide-like $-NH_2$ itself at pH <0 (Homer & Johnson, 1970).

Although alternative mechanisms cannot be ruled out on the basis of the above listed exchange characteristics alone, it is difficult to argue in favor of other possibilities. The most plausible alternative is that the buffer catalyst acts as a proton donor, rather than as an acceptor in this system, which would

account superficially for the loss of catalysis above the buffer pK_a value. The most pertinent argument against the proton donating function of added buffers is derived from the necessity of invoking dual roles as both acceptors and donors, since an acceptor function must be present in any case and it can originate only from the buffer in the pH range of maximum catalysis (see McConnell, 1974). This dual role is ruled out by the linear relationship between buffer concentration and exchange seen for adenine-NH_2 (imidazole) (McConnell, 1974), adenine-NH_2 (phosphate) (Cross et al., 1975), cytosine-NH_2 (cytosine and phosphate), and G(N-1) proton (phosphate) (this study). Additional evidence in support of the exclusive proton acceptor function of buffers is seen in the expected conformance between catalysis and buffer basicity for homologous catalysts, i.e., imidazoles–adenine (McConnell, 1974), acetate–G(N-1) (this work), and the Brönsted relationship for G(N-1) exchange (Figure 10). If acetate and chloroacetate acted as proton donors in catalysis of G(N-1) proton exchange, their contributions to W_A would not be equal at pH 3 (as seen in Figure 7), but would differ by a factor of 15. Experiments to rule out a fortuitous difference of this magnitude between the diffusion rates for these catalysts require accurate line width data below pH 3. These data are not available in the presence of the catalysts at the present time, due to low solubility and extensive association of the cyclic nucleotide in this pH region. Finally, the observed loss of buffer catalysis above the buffer pH is precisely what is expected from eq 1, in which the buffer acts solely as a proton acceptor. As increasing pH traverses the buffer pK region, the tenfold decrease in protonated nucleobase intermediate is no longer offset by the attenuated increase in buffer conjugate base. These considerations support the conclusion that all of the hydrogen bonding protons adenine, guanine and cytosine are transferred to buffer and solvent acceptors much more easily (are more acidic) when the basic endocyclic nitrogen of the nucleobase is protonated. Exchange involving protonated intermediates is known in other systems (Grunwald & Ralph, 1975).

In view of the relation between acidity and H-bond strength of a functional group (Allen, 1975), this common exchange route for the H-bonding protons of all amino containing nucleobases may represent a natural mechanism for regulating their acidity to accommodate some specific function of DNA in vivo. For example, an attempt to estimate the acidity increase in guanine following nucleobase protonation is useful in the consideration of factors that may affect G-C base pair stability in the double helix. A quantitative estimate can be approached by the comparison of pK_P and pK_U through the use of eq 4. This equation embodies a kinetic simplification, which arises from the assertion that of the three processes that describe proton transfer, i.e. (1) formation of an encounter complex through hydrogen bonding, (2) proton transfer within the complex, and (3) dissociation of the complex into products, only the first and third are rate limiting (see Crooks, 1975, and references therein). Therefore, this equation applies to proton donor groups that readily form H bonds of sufficient stability with solvent or solute acceptors to ensure completion of proton transfer before the hydrogen bridge is broken to dissociate the transition complex. These donor groups are "normal" acids with measurable pK values in aqueous solution, whose range would extend between the pK of H_3O^+ (−2) and the pK of OH^- (15.8) (Eigen, 1964). For these acids, k_D is about 10^{10} with solvent species as acceptors (H_2O or OH^-) (Eigen, 1964). Since the G(N-1) group exhibits both H_2O and OH^- catalysis and conforms to these criteria, a comparison of pK_P and pK_U can be made by assuming that k_D is the same in each case. As shown in Table II for a variety of acceptors k_D is the same (10^8 M^{-1} s^{-1}) and the acidity increase of G(N-1) upon G(N-7) protonation is about two or three orders of magnitude. This change would be largely a measure of the inductive effect of the proton at G(N-7). In the double helix at physiological pH, the G(N-1) group would be ionized as long as the proton remained at the G(N-7) site.

A similar calculation for the amino groups leads to a strikingly different result. The amino group of the protonated cytosine nucleobase qualifies as a "normal" acid through the use of eq 4 for a wide range of possible k_D values. A pK_P of 8 to 9 is obtained for any of the catalysts listed in Table I and an assumed $k_D = 10^8$ M^{-1} s^{-1}. However, a similar calculation for the neutral, unprotonated base provides a pK_U value 10 units greater than the above value of pK_P. Since the inductive effects of C(N-3) protonation would not produce changes of this magnitude in $-NH_2$ acidity, it is clear that eq 4 is not valid for estimation of pK_U, due to the absence of terms that modify k_D in this case. The amino group of the neutral base is not a "normal" acid and the low OH^- catalytic rate constants testify to the reluctance of this group to form an appropriate H-bonded proton transfer complex with the highly solvated hydroxyl (see Margolin & Long (1973) for relevant discussion). Thus, the chief effect of nucleobase protonation on the exocyclic amino group is its conversion from a very poor H-bond donor to a "normal" one. This is a qualitative change that can be applied to the exocyclic aminos of adenine and guanine as well as to cytosine, as evidenced by the low hydroxyl rate constants for these nucleobases and the accessibility of their protonated forms to catalysis by poor proton acceptors, including H_2O (McConnell & Seawell, 1972; McConnell, 1974; Cross et al., 1975).

In the double helix protonation of the solvent-accessible guanine N-7 would lead to ionization of the G(N-1) proton and a dramatic increase in H-bond strength of the amino protons. It is possible to view both these events as establishing a destablizing condition. Although the C(O-4)-to-G-NH_2 H-bond would be strengthened (Allen, 1975), the cytosine acceptor site may now compete poorly with water for the bond, due to the well established access of water to the helix interior (Teitlebaum & Englander, 1975a,b). The relation between existing stability data on helical DNA and predictions derived from a destabilization model based on this notion will be presented elsewhere (McConnell, 1978).

Acknowledgments

The willingness of Dr. W. W. Conover to provide expert advice and instrument modification on short notice was indispensable for this study. I am indebted, also, to Drs. N. Wade Jardetzky, S. L. Patt, and Oleg Jardetzky for their supportive activities during the author's sabbatical stay at Stanford University.

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CORRECTIONS

Correlation of Procollagen mRNA Levels in Normal and Transformed Chick Embryo Fibroblasts with Different Rates of Procollagen Synthesis, by David W. Rowe, Robert C. Moen, Jeffrey M. Davidson, Peter H. Byers, Paul Bornstein,* and Richard D. Palmiter,* Volume 17, Number 9, May 2, 1978, pages 1581–1590.

The formula on page 1583 should read:

$$\%\ \text{procollagen} = \frac{\text{collagenase-sensitive cpm} \times 100}{5.4(\text{collagenase-resistant cpm}) + 0.51(\text{collagenase-sensitive cpm})}$$

This formula results from a correction in the denominator of the formula developed for percent collagen that is required because procollagen contains collagenase-resistant regions. Thus, % procollagen can be expressed as:

$$\frac{\text{collagenase-sensitive cpm} \times 100}{5.4[\text{collagenase-resistant cpm} - 0.09(\text{collagenase-sensitive cpm})] + \text{collagenase-sensitive cpm}}$$

The two formulas above are arithmetically identical.

Gross Conformation of C1q: A Subcomponent of the First Component of Complement, by Paul A. Liberti* and Stephen M. Paul, Volume 17, Number 10, May 16, 1978, pages 1952–1958.

On page 1954, column 1, line 32, the sixth word should be asymmetric, not symmetric as printed.

Size and Shape of the Model Lipoprotein Complex Formed between Glucagon and Dimyristoylglycerophosphocholine, by Andrew J. S. Jones, Richard M. Epand,* K. Frank Lin, D. Walton, and W. J. Vail, Volume 17, Number 12, June 13, 1978, pages 2301–2307.

The equation on the last line of page 2305 should read:

$$(\bar{v}_2 + \delta_1 v_1^0)/\bar{v}_2$$