# Intermolecular Proton Transfer Reaction between Base and Phosphate Moieties of Mononucleotides in Solution

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Abstract: The ultrasonic relaxation method has been used to demonstrate and to study a rapid intermolecular proton transfer reaction between 5'-AMP molecules in the vicinity of pH 5. The transfer occurs between the protonated N-1 of one molecule and the anionic phosphate group of another molecule. A similar reaction also appears to occur between 5'-CMP molecules. Rate constants $k_1 = 3 \times 10^9\ M^{-1}\ \mathrm{sec}^{-1}$ and $k_{-1} = 1 \times 10^7\ M^{-1}\ \mathrm{sec}^{-1}$ were determined for reaction 3, where the superscript and subscript on AMP denote the charge on the base and phosphate, respectively. These observations confirm the occurrence in solution of the same kind of facile base–phosphate interaction seen in crystal structures of adenine and cytosine nucleotides, which show a hydrogen bond between the protonated base of one molecule and the anionic phosphate of a neighbor.

The crystal structures of adenine nucleotides show a strong hydrogen bond between the protonated N-1 atom of one molecule and the anionic phosphate group of a neighbor.[1-3] This bonding scheme is also found for crystalline cytosine nucleotides,[4-6] where N-3 is the ring protonation site. Sundaralingam and

5'-AMP

5'-CMP
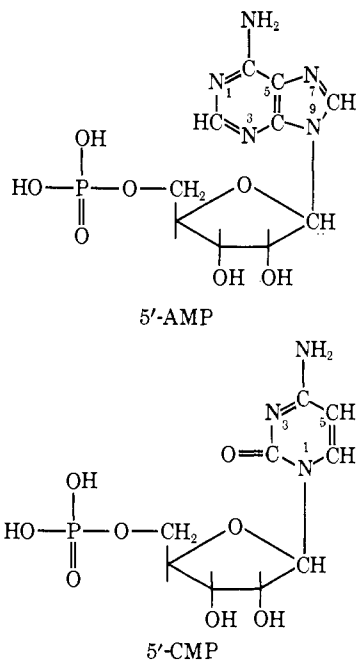
Abola have pointed out that this interaction may be of significance in, for example, stabilizing the tertiary structure of nucleic acids such as transfer RNA.[2] We report here a study of this interaction in aqueous solution by the ultrasonic relaxation method. Using this technique, it has been possible to demonstrate and to study the proton transfer between N-1 of one 5'-AMP molecule and the dianionic phosphate group of another molecule.

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Table I. Relaxation Parameters for 5'-AMP Solutions

AMP concn, m	pH	Temp, °C	$10^8 A/m$, sec/m cm	$10^{17}B$,[c] sec²/cm	$10^8\tau$, sec	$10^{-5}v$, cm/sec
0.085	5.00[a]	25	1.70	24.0	3.18	1.52
0.135	5.00[a]	25	1.76	28.0	1.99	1.53
0.20	5.00[a]	25	1.71	36.0	1.45	1.54
0.235	5.00[a]	25	1.74	34.0	1.27	1.54
0.14	4.30[b]	40	0.67	20.6	1.00	1.56
	4.90[b]	40	1.37	20.6	1.99	1.56
	5.50[b]	40	0.93	20.6	1.38	1.56

[a] Solvent was aqueous 0.2 M NaCl and contained additional Na⁺ resulting from titration of the acid form of AMP to pH 5 with NaOH. [b] Solvent was H_2O and contained Na⁺ resulting from titration of acid form of AMP to desired pH. [c] Accurate B values were difficult to determine because of the suggestion of a second relaxation effect of small amplitude at higher frequencies (see text). However, the relaxation parameters tabulated here are fairly insensitive to the exact choice of B, since the amplitude of the major relaxation is so large. For example, if values of $B = 22.0\ \mathrm{sec^2/cm}$ (at 25°) and 14.6 sec²/cm (at 40°) are used, which correspond to background absorption of H_2O at 25 and 40°, respectively, then the values of τ and A/m change less than 10%, in most cases.

Ultrasonic relaxation experiments[7,8] were carried out over the frequency range of ca. 10–200 MHz. Aqueous solutions of 5'-AMP ($\sim 0.1\ M$) show a relaxation effect around 10 MHz which has a large amplitude at pH 5, 25°. The amplitude has its maximal value at approximately pH 5, while the relaxation time τ is longest at this pH; on either side of pH 5, the time constant shortens and the amplitude diminishes to the point where the process largely disappears at more than 1 pH unit away from pH 5.[9]

The concentration dependence of the relaxation parameters at 25°, and the pH dependence at 40°, are given in Table I. The data were analyzed in accordance with the expression for the single relaxation time[7]

$$\alpha/f^2 = [A\tau/[1 + (\omega\tau)^2]] + B \qquad (1)$$

where α is the pressure amplitude absorption coefficient, f is the frequency and $\omega = 2\pi f$, τ is the relaxa-

(7) M. Eigen and L. de Maeyer in "Technique of Organic Chemistry," Vol. VIII, Part 2, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience, New York, N. Y., 1963, p 895.
(8) L. M. Rhodes and P. R. Schimmel, *Biochemistry*, 10, 4426 (1971).
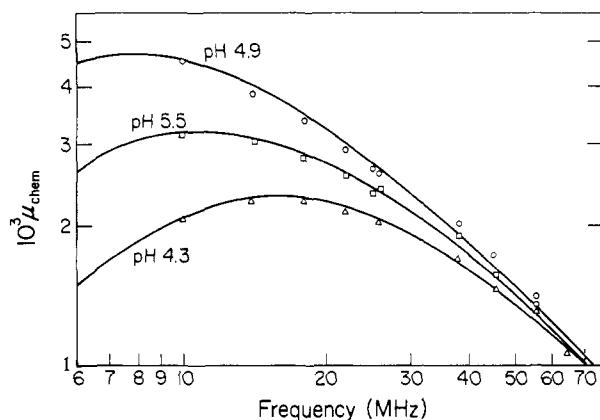(9) Detailed data are recorded in L. M. Rhodes, Ph.D. Thesis, M.I.T., 1972.

Figure 1. Log scale plots of μ_{chem} *vs.* frequency for solutions containing 0.14 *m* AMP at different pH values at 40°. The points are experimental values and the curves are calculated with the parameters given in Table I.

tion time, *A* is an amplitude parameter, and *B* is a constant. The estimated error in *A* and in τ is $\pm 10\%$. The concentration range studied at 25° was limited by the fact that below 0.1 *m* AMP, the time constant falls at too low a frequency to be accurately measured, while significantly above 0.25 *m* AMP the amount of material required is impractically large and also necessitates a large amount of counterion for charge neutralization.

An alternate representation of the data is in terms of the parameter μ_{chem}, the "absorption per wavelength" λ due to chemical relaxation effects[7]

$$\mu_{chem} = 2vf(\alpha/f^2 - B) \qquad (2)$$

where *v* is the sound velocity. Plots of μ_{chem} *vs. f* are given in Figure 1, for three different pH values at 40°. The curves are calculated with the parameters given in Table I, and the points are experimental values. It is clear that the points conform well to the calculated curve. The choice of 40° as a temperature for some experiments was done with the hope of displacing the relaxation process to higher frequencies, where it can be more easily studied with our apparatus. However, the relaxation time is not very temperature dependent between 25 and 40°.

The fact that the process is concentration dependent clearly excludes unimolecular or pseudounimolecular mechanisms from consideration. One alternative is base stacking, which is known to occur readily between AMP molecules.[10-12] This is ruled out on at least three grounds, however. First, the observed concentration dependence of τ does not fit that predicted for a simple dimerization reaction. Second, addition of 7 *M* urea does not abolish nor even greatly modify the relaxation parameters. Urea is known to be a nucleic acid denaturant,[13] and presumably causes unstacking. Third, a relaxation process similar to that observed in AMP solutions was found in solutions of 5'-CMP. The latter nucleotide is known to have a significantly weaker propensity to stack.[12]

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(11) I. Feldman and R. P. Agarwal, *J. Amer. Chem. Soc.*, **90**, 7329 (1968).
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(13) L. Levine, J. A. Gordon, and W. P. Jencks, *Biochemistry*, **2**, 168 (1963).

The pK's of N-1 and of the phosphate group on AMP are *ca.* 3.7 and 6.1, respectively.[14,15] Therefore, a possible mechanism for the relaxation process is the ionization of one or both of these groups. At the AMP concentrations employed, however, neither ionization process should make a significant contribution to the sound absorption at pH 5, where the observed relaxation amplitude is very large.[16]

A plausible mechanism is proton transfer between the protonated N-1 of one AMP molecule and the dianionic phosphate of another molecule. This reaction is
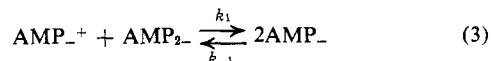
$$AMP_-^+ + AMP_{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2AMP_- \qquad (3)$$

where the superscript and subscript on AMP denote the charge on the base and phosphate, respectively. To test this possibility, the nucleoside, adenosine, was mixed with ribose 5'-phosphate and a relaxation effect was observed which was similar to that found in 5'-AMP solutions. Further, although an analogous process was observed in 5'-CMP solutions, this process was not found in 5'-UMP. The latter nucleotide lacks the appropriate ring nitrogen for protonation around pH 4. These facts all point to a specific reaction between the base nitrogen on AMP (or CMP) and the phosphate as being responsible for the observed effect.

The reciprocal relaxation time for eq 3 is

$$1/\tau = k_1(\overline{(AMP_-^+)} + \overline{(AMP_{2-})}) + 4k_{-1}\overline{(AMP_-)} \qquad (4)$$

or

$$(\tau\overline{(AMP_-)})^{-1} = k_1(\overline{(AMP_-^+)} + \\ \overline{(AMP_{2-})})\overline{(AMP_-)}^{-1} + 4k_{-1} \qquad (5)$$

or

$$1/\tau = k_1(AMP_-^+)(AMP_{2-}) \times \\ \left(\frac{1}{\overline{(AMP_-^+)}} + \frac{1}{\overline{(AMP_{2-})}} + \frac{4}{\overline{(AMP_-)}}\right) \qquad (6)$$

where the overbars denote equilibrium concentrations and the relationship $k_1\overline{(AMP_-^+)}\overline{(AMP_{2-})} = k_{-1}\overline{(AMP_-)}^2$ has been used in obtaining eq 6.[17] Amounts of AMP species may be calculated with the appropriate pK values. Literature values of the N-1 and phosphate pK's were used, as well as small variations around these values.[14] (Titration of AMP at the concentrations used in these experiments is not feasible owing to incipient precipitation of the species AMP_-^+ at acid pH values.) Rate constants may be obtained from plots of $(\tau\overline{(AMP_-)})^{-1}$ *vs.* $(\overline{(AMP_-^+)} + \overline{(AMP_{2-})})\overline{(AMP_-)}^{-1}$ in the case of the pH-dependent data (see eq 5), or by calculating k_1 (and subsequently k_{-1}) for each of the experiments at different concentrations at pH 5, by using eq 6.

All of the data on AMP conform well to the mechanism of eq 3. Values of $k_1 = 3 \times 10^9$ M^{-1} sec^{-1}

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(16) M. Hussey and P. D. Edmonds, *J. Phys. Chem.*, **75**, 4012 (1971).
(17) A more rigorous treatment of the relaxation kinetics would include the coupling of the rapid ionizations of the protonated ring and the phosphate group. This more exact analysis gives rate parameters not significantly different than the simplified treatment, however.

and $k_{-1} = 1 \times 10^7$ M^{-1} sec^{-1} are obtained from the data at both temperatures, with an experimental uncertainty of ca. $\pm 35\%$. The best pK values for the data appear to be ca. 3.8 and 6.2 for the base and phosphate, respectively. The pK values must agree, of course, with the constraint that log k_1/k_{-1} is equal to the ratio of the phosphate to the base pK. The fact that similar pK's can be used to represent the data at both temperatures is not surprising in view of the small temperature dependence of these pK's.[14] In addition, the fact that $k_1 \gg k_{-1}$ is in accordance with the expectation that eq 1 should be approximately diffusion limited in the forward direction, since (p$K_{acceptor}$ − pK_{donor}) > 2.[18]

The behavior of the relaxation amplitude also conforms well to that predicted for the mechanism of eq 1. The amplitude parameter A in eq 1 is given by[7]

$$A = \frac{2\pi^2 \rho v}{RT} \Gamma_m [\Delta V - \frac{\alpha_p}{\rho c_p} \Delta H]^2 \qquad (7)$$

where ρ is the solvent density (assumed to be 1 g/cm^3), c_p and α_p are the isobaric specific heat and coefficient of thermal expansion, respectively, and ΔV and ΔH are the volume and enthalpy change of the reaction. The parameter Γ_m is given by[7]

$$\Gamma_m = ((\overline{AMP_-^+})^{-1} + (\overline{AMP_2-})^{-1} + 4(\overline{AMP_-})^{-1})^{-1} \qquad (8)$$

$$= ((f_-^+)^{-1} + (f_2-)^{-1} + 4(f_-)^{-1})^{-1} m \qquad (9)$$

where m is the total molal AMP concentration, f_-^+ is the fraction of $\overline{AMP_-^+}$, and etc. for f_- and f_2-. According to eq 7 and 9, A should be a linear function of m at any fixed pH. This agrees with the data in Table I, which shows that A/m is constant at pH 5 for various concentrations. Experimental values of $[\Delta V - (\alpha_p/\rho c_p)\Delta H]^2$ are tabulated in Table II. Since a ΔH of 10

Table II. Amplitude Parameters for 5'-AMP Solutions

AMP concn, m	pH	Temp, °C	$[\Delta V - \alpha_p/(\rho c_p)\Delta H]^2$, (cm^3/mol^2)	$|\Delta V|$, cm^3/mol
0.085	5.00	25	567	23.8
0.135	5.00	25	584	24.2
0.20	5.00	25	564	23.7
0.235	5.00	25	571	23.9
0.14	4.30	40	666	25.8
	4.90	40	479	21.9
	5.50	40	569	23.9

kcal mol^{-1} contributes ca. 18% or less to this quantity, it is reasonable to expect that the ΔV contribution dominates. In fact, ΔH must be extremely small since both the base and phosphate pK's are only slightly temperature dependent.[14] Under the assumption that the ΔH contribution is negligible, values of $|\Delta V|$ are also tabulated in Table II. A value of $|\Delta V| = 24 \pm 2.4$ cm^3/mol is obtained. This is not an unreasonable

(18) M. Eigen, *Angew. Chem., Int. Ed. Engl.*, 3, 1 (1964).

volume change for a charge neutralization reaction.[19]

We conclude that the interaction between the protonated N-1 and ribose phosphate seen in the crystal phase is also found in solution,[20] but as an association which is rendered transient because of the actual transmission of the bonding hydrogen from one molecule to another. This is a consequence of the strong solvation by water of isolated charges in solution. In special nonaqueous environments, provided by the internal milieu of a cell or even a folded macromolecule, this specific bonding might take place. In fact, under certain circumstances in aqueous solution it has been observed that pK's of A and C base residues in ordered nucleic acids are sharply elevated.[21–23] In two cases[22,24] it appears clear that the protonated base neutralizes the charged backbone, but not by direct base–phosphate bonding as is seen in crystal structures of AMP and CMP.[1–6] On the other hand, the abnormally high base pK's observed in tRNA under certain conditions[23] may result from direct base–phosphate bonding.

Finally, it should be mentioned that a second relaxation process, with a very small amplitude relative to the first one, was observed at high frequencies (50–100 MHz). In the pH range where the proton transfer process dominates, the contribution of the second process to the sound absorption is inconsequential. However, at pH 8 the transfer reaction is unobservable, and the fast relaxation process can be studied separately. Its relaxation parameters are roughly similar to those found for adenosine[8] and it appears possible, therefore, that it is associated with the syn–anti transition of the glycosidic bond.[8]

Experimental Section

Adenosine, 5'-AMP, and ribose 5'-phosphate were purchased from Calbiochem (A grade). Other nucleotides were obtained from Sigma Chemical Co. (Sigma grade). Urea was "Baker analyzed" reagent grade, and all other chemicals were also of reagent grade.

Nucleotide concentration determinations and pH measurements were generally performed both before and after each experiment, in order to check for sample deterioration. Information concerning the ultrasonic apparatus, and the method of obtaining and analyzing the absorption coefficients, is recorded elsewhere.[8,9]

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