onances of N7, N9, and N6′ shifted downfield by 2, 11.7, and 10 ppm, respectively. The N3 and N1 resonances were identified by protonation of adenine specifically labeled at N3 (see Figure 5). On protonation of adenine labeled at N3 with ^{15}N, the N3 shift changed to 161.0 ppm. This allows the 164.3 ppm resonance to be assigned to N3 in the natural-abundance spectrum of the protonated form. The discrepancy of chemical shifts for N3 in these samples of 3.3 ppm can be reasonably attributed to the large differences in the concentrations.

Protonation of N^6,N^6-diethyladenine and N^6,N^6-dimethyladenine was found to occur principally on N1, N3, and N7, all resonances of which moved upfield, while those of N9 and N6′ shifted downfield. The degree of protonation decreased in the order N3, N1, and N7. Apparently, substitution on the N6′ nitrogen by *N*,*N*-alkyl groups has considerable influence on the preferred site of protonation. The predominance of protonation at N3 over N1 may be the result of the steric interactions between *N*,*N*-dialkyl group and the proton attached to N1.

The behavior of the ^{15}N resonances of adenine in aqueous solution (Figure 6) is worthy of additional comment. On the acid side, the pattern of protonation shifts is generally similar to that observed for dimethyl sulfoxide with upfield shifts of N1 and N3 of 51 and 11 ppm, respectively, and a downfield shift of N9 of 10.5 ppm, the resonance of N7 moving slightly upfield rather than slightly downfield.

Comparison of these results with those for adenosine phosphates[2] indicates some degree of protonation of N3 and N7 as well as of N1. Regardless of the partial protonation of N7, which causes the N9 resonance to move somewhat downfield, there can be no question that in acid solution, as in neutral solution, the N9-H tautomer predominates.

In basic solution, the N9 resonance of adenine undergoes a dramatic downfield shift of 56 ppm on formation of the corresponding anion. Curiously, at about pH 11, all of the ring-nitrogen resonances fall within a 5 ppm range. Large downfield shifts are customary on removal of a σ-bonded proton attached to an aromatic system through enhancement of the second-order paramagnetic effect associated with the presence of σ unshared pairs,[15] and it is interesting that the downfield shift increment on removal of the N9-H proton is comparable to that which occurs on the removal of the proton from N1 of adenine's conjugate acid. A downfield ^{15}N shift has also been reported for the ionization of imidazole in basic solution.[24]

Registry No. **1**, 4059-12-5; **2**, 84133-05-1; **3**, 84133-06-2; **4**, 84133-07-3; **5**, 84133-08-4; **6**, 118-70-7; **7**, 84133-09-5; **8**, 84133-10-8; trimethyl orthopentanoate, 13820-09-2; pentanoic anhydride, 2082-59-9; adenine, 73-24-5; adenosine, 58-61-7; N^6-benzyladenosine, 4294-16-0; 9-ethyladenine, 2715-68-6; 7-ethyladenine, 24309-36-2; N^6,N^6-dimethyladenine, 938-55-6; N^6,N^6-diethyladenine, 6284-24-8; [1-^{15}N]adenine, 79364-53-7; [3-^{15}N]adenine, 79364-54-8; [7-^{15}N]adenine, 79364-55-9; [9-^{15}N]-adenine, 56777-22-1; [6′-^{15}N]adenine, 19713-11-2; ^{15}N, 14390-96-6.

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Estimation of the Energy Barrier to Syn–Anti Interconversion in a Pyrimidine Nucleotide: Ultrasonic Investigation of Syn–Anti Glycosyl Isomerization in Cytidine Cyclic 2′,3′-Monophosphate with Bound Ethidium Bromide[1]

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Abstract: Ultrasonic relaxation studies were performed on aqueous solutions of cytidine cyclic 2′,3′-monophosphate in the absence and presence of ethidium bromide in the 15–95 MHz frequency range. The relaxation observed in the presence of ethidium bromide (but not in its absence or in aqueous solutions of cytosine–ethidium bromide) could be assigned to a unimolecular conformational isomerization. Consistent with earlier results, this relaxation could be assigned to the syn–anti glycosyl isomerization process. The findings strongly suggest that the barrier to syn–anti interconversion is diminished in cytidine cyclic 2′,3′-monophosphate upon addition of ethidium bromide and that even in the absence of this dye the barrier cannot be very large, although it is larger than in purine nucleotides. The heterostacking constant between the nucleotide and the dye was determined to be 100 M^{-1} according to both ultrasonic and ^1H NMR methods.

Ultrasonic relaxation measurements have been demonstrated to be capable of monitoring the glycosyl syn–anti conformational equilibrium in nucleosides[4,5] and in a mononucleotide: adenosine cyclic 3′,5′-monophosphate (cyclic AMP).[6,7] It was demonstrated that stacking and the glycosyl conformational equilibrium are interrelated[7,8] and self-stacking can lead to an increase of the barrier to glycosyl interconversion.[7] On the other hand, heterostacking of 2′-deoxyadenosine to 3-indoleacetic acid or to ethidium bromide surprisingly led to a decrease in the conformational barrier.[9] To date most of the research was performed on purine

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(2) Rutgers University.
(3) Saga University. S.N. was on leave at Rutgers in 1979.
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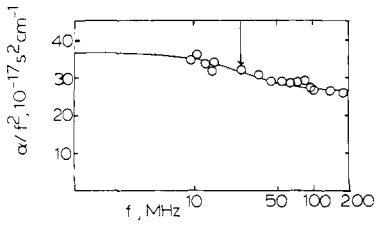

Figure 1. Ultrasonic absorption of 0.156 M cyclic CMP in the presence of 0.1 M ethidium bromide at 25 °C obtained on the Saga University instrument. The solid line is a theoretical one for f_r = 25 MHz, A = 10, and B = 26.5 according to eq 2 in the text.

substrates. On pyrimidine nucleosides and nucleotides neither the initial report[4] nor repeated attempts in this laboratory could detect a unimolecular relaxation in the ultrasonic range that could be attributed to the syn–anti glycosyl isomerization process. On the basis of the finding that the relaxation frequency, f_r, for 2′-deoxyadenosine was shifted to higher values upon addition of 3-indoleacetic acid or ethidium bromide, it was of interest to perform such "binding" experiments on a pyrimidine analogue. Since this substrate exhibits no measurable ultrasonic relaxation in H_2O, addition of ethidium bromide may shift the syn–anti equilibrium to high enough frequencies so as to make it observable in the conventional ultrasonic region. For these experiments cytidine cyclic 2′,3′-monophosphate (cyclic CMP) was selected for several reasons. First of all, this compound has been established as a kinetically competent intermediate in the mechanism of ribonuclease A catalyzed hydrolysis of dinucleotide monophosphates (e.g., CpC to cytidine 3′-monophosphate). Therefore, its conformational characteristics on and off a receptor are of importance. Second, the ready solubility and the relative conformational inflexibility as regards ribose puckering would simplify the assignment of a relaxation if observed. The ultrasonic results below indicate that only in the presence of ethidium bromide is a relaxation attributed to syn–anti isomerization of cyclic CMP visible and that the free energy barrier to this isomerization is not very large. The rate constants for interconversion of the glycosyl conformational isomers constitute the first such report for a pyrimidine nucleotide.

Experimental Section

Materials. Cyclic CMP sodium salt was from Sigma, St. Louis, MO, and ethidium bromide was from Sigma or from Wako Pure Chemical Industries. Both were used without further purification. A stock solution of cyclic CMP (0.2 mol dm^{-3}) was prepared in doubly distilled, degassed water, and ethidium bromide was added as a solid in the required amounts. The pH was adjusted and measured with a Radiometer pH meter, No. 26, or a Hitachi Horiba A-5 pH meter. The solution pH was 8 ± 0.2 throughout, far from either nucleic base or phosphodiester ionizations.

Methods. Two pulse methods were employed for the ultrasonic absorption measurements in the frequency range of 15–95 MHz[10,11] using a 5-MHz fundamental X-cut quartz transducer. In view of the small magnitude of the effect, it was necessary to check the accuracy and reproducibility of the measurements. To this end a number of experiments were also conducted on a different ultrasonic instrument located at Saga University, Japan. In that laboratory a 0.5-MHz crystal was used for the 9.56–14.5 MHz range and a 5-MHz crystal for the 15–95 MHz range while a 20-MHz crystal furnished data between 100 and 180 MHz. The data provided by that instrument were consistent with the data obtained by employing the previous method.[10,11] A typical result is shown in Figure 1. All measurements were performed at 25 ± 0.02 °C.

^{1}H NMR measurements were performed on a JEOL PSFT-100 Fourier transform instrument at 28 °C. A spectrum of cyclic CMP without or with ethidium bromide gave rise to virtually identical ^{1}H chemical shifts in the aromatic region. The lowest field triplet of ethidium bromide was found to undergo cyclic CMP induced chemical shift changes, however. It was the center peak of this lowest field triplet that

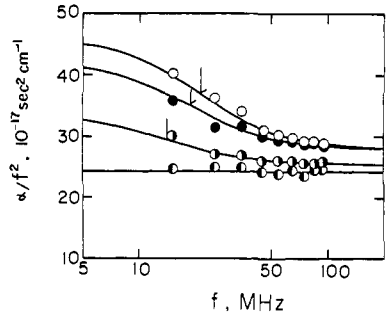

Figure 2. Ultrasonic absorption of 0.2 M cyclic CMP (◐) and in the presence of 0.04 (◑), 0.08 (●), and 0.1 M (○) ethidium bromide at 25 °C as determined on the instrument at Rutgers.

Table I. Results of Ultrasonic Absorption Measurements at 25 °C[a]

cyclic CMP (M)	ethidium bromide (M)	δ	$10^3\mu_{max}$	$10^{17}B$ (s^2 cm^{-1})	f_r (MHz)
0.20		0		24.0	
0.20	0.02	0.095	0.030	25.0	10
0.20	0.04	0.188	0.084	25.5	14
0.20	0.06	0.281	0.13	27.5	17
0.20	0.08	0.371	0.20	28.0	19
0.20	0.10	0.458	0.28	28.0	21
0.15	0.07	0.446	0.18	27.0	20

[a] δ is from eq 3 and μ_{max} is from eq 2.

was employed in a determination of the $K_{heterostack}$ between ethidium bromide and cyclic CMP. Very precise chemical shift measuerments were performed on this peak with respect to 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt (DSS) with ethidium bromide at 2.4 × 10^{-3} M and the cyclic CMP concentration ranging from 5 × 10^{-3} to 2 × 10^{-2} M. A plot of $1/\Delta\delta$ vs. 1/[cyclic CMP] (essentially a double-reciprocal plot) led to a $K_{heterostack}$ of ca. 100 ± 30 M^{-1}. The large error is due to the very small bound chemical shift (difference in the chemical shift of ethidium bromide in the absence and presence of cyclic CMP) of 5–6 Hz. The $K_{heterostack}$ was determined at the probe temperature of the NMR instrument, 28 °C.

Results and Discussion

Figure 2 illustrates some of the ultrasonic absorption spectra obtained in aqueous solution of cyclic CMP per se (no excess absorption observed) and in the presence of varying ethidium bromide concentrations. All of the excess absorptions are characteristic of a single relaxation process and can be analyzed according to the equations

$$\alpha/f^2 = A/[1 + (f/f_r)^2] + B \quad (1)$$

or

$$\mu = (\alpha/f^2 - B)fc = \frac{2\mu_{max}(f/f_r)}{1 + (f/f_r)^2} \quad (2)$$

where α is the sound absorption coefficient, f_r is the relaxation frequency, A and B are constants, μ is the excess absorption per wavelength, μ_{max} is the maximum excess absorption per wavelength, and c is the velocity of sound.

In view of the small amplitude of the effects observed and not being able to extend the measurements to lower frequencies, the errors in f_r are larger than normal for an ultrasonic study. It is estimated that the f_r is good only to ±10%.

The excess absorption amplitude, A, and the relaxation frequency, f_r, increased with the concentration of ethidium bromide. On the other hand, the relaxation frequency is independent of cyclic CMP concentration when the ratio of concentrations of cyclic CMP to ethidium bromide is maintained constant (Table I). This result suggests a unimolecular process. The model proposed previously for the behavior of 2′-deoxyadenosine in the presence of ethidium bromide[9] is applicable to the present problem. The mechanism can be written as in Scheme I, where A and S are the anti and syn conformers and A′ and S′ are the same when

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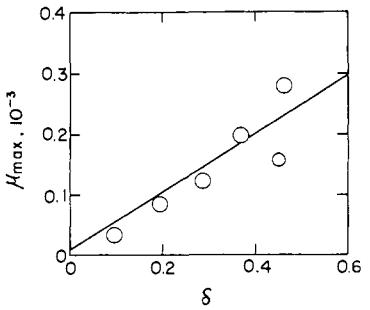
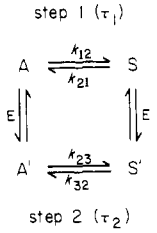


Figure 3. Plot of μ_{max} vs. fraction of ethidium bromide bound cyclic CMP, δ, for a total concentration of 0.2 M nucleotide according to eq 3 and 4.

Scheme I



bound to ethidium bromide (E), respectively. The maximum excess sound absorption per wavelength, μ_{max}, can be determined from the ultrasonic absorption measurements. This quantity is related to the volume and enthalpy changes of the reaction.[12] This quantity can be given as a linear function of the fraction of bound nucleotide, δ, as follows[9] (see eq 4′ in ref 12):

$$\mu_{max} = p\delta + q \tag{3}$$

p and q are approximately constant when the total concentration of nucleotide is maintained constant. The quantity δ can be estimated by

$$\delta = \frac{1}{2KC_N}[K(C_N + C_E) + 1 - ([K(C_N - C_E) + 1]^2 + 4KC_E)^{1/2}] \tag{4}$$

It is assumed that self-stacking of cyclic CMP is negligible.[13] The quantity K is the heterostacking constant ($K = (A')/[(E)(A)] = (S')/([E][S])$) between cyclic CMP and ethidium bromide; C_N and C_E are their analytical concentrations, respectively. In Figure 3 we have a plot of μ_{max} vs. δ at a total nucleotide concentration of 0.20 M. Within experimental error this is a linear relationship.

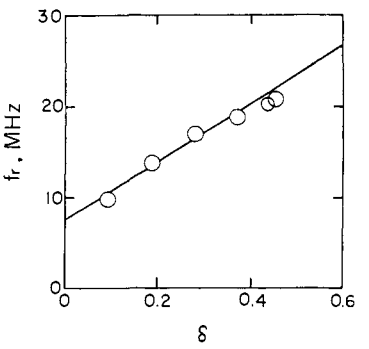


Figure 4. Empirical relationship between the relaxation frequency f_r and the fraction of ethidium-bound nucleotide.

The intercept, q, is very close to zero. This means that the amplitude of the excess absorption for step 1 in Scheme I is too small to be observed regardless of the relaxation frequency. If the amplitude of the effect without bound ethidium bromide were exactly zero, however, we would expect that upon addition of ethidium bromide to cyclic CMP a relaxation would appear with a constant relaxation frequency. The data in Table I show that at low values of δ the relaxation frequency seems to shift to lower values. We have interpreted that in the same manner as in our previous study[9] in which we are observing an amplitude weighted average relaxation frequency. That is, we are always fitting the data to a single relaxation when in fact there may be a small effect due to the unbound form. There would then be two relaxations and the fitting would eventually be shifted toward the values characteristic of the unbound form. One can write[9]

$$\langle\tau^{-1}\rangle = \frac{\mu_{max_1}\tau_1^{-1}(1-\delta)}{\mu_{max_1} + (\mu_{max_2} - \mu_{max_1})\delta} + \frac{\mu_{max_2}\tau_2^{-1}\delta}{\mu_{max_1} + (\mu_{max_2} - \mu_{max_1})\delta} \tag{5}$$

In order to use this equation, an estimate of τ_1^{-1} is needed. This can be obtained from the empirical relationship discovered between f_r [$=\tau^{-1}/(2\pi)$] and δ (Figure 4). This gives $\tau_1^{-1} \simeq 8$ MHZ. Using this value one has $\mu_{max_1}/\mu_{max_2} < 0.3$. Thus, it was found, as in the case of deoxyadenosine, that the binding of ethidium bromide produces increased amplitudes and frequencies. A unimolecular process gives a relaxation time $\tau^{-1} = k_f + k_r$. If one assumes that the equilibrium constant for the syn–anti process is fairly large, one can write $\tau^{-1} \cong k_f$. Comparing the kinetic results for unbound cyclic CMP to 2′-deoxyadenosine one has

$$\frac{\tau^{-1}_{CMP}}{\tau^{-1}_{ADO}} \cong \frac{k_{CMP}}{k_{ADO}} = \frac{\exp[-\Delta G^{\ddagger}_{CMP}/(RT)]}{\exp[-\Delta G^{\ddagger}_{ADO}/(RT)]} \tag{6}$$

$$\frac{8\text{ MHz}}{35\text{ MHz}} = 0.229 = \exp(\Delta G^{\ddagger}_{ADO} - \Delta G^{\ddagger}_{CMP}) \tag{7}$$

Since $\Delta G^{\ddagger}_{ADO} \cong 4$ kJ/mol was estimated before[9]

$$\Delta G^{\ddagger}_{CMP} = 7.6\text{ kJ/mol} \tag{8}$$

To our knowledge this is the first estimate of the free energy barrier[14] to syn–anti interconversion in a pyrimidine nucleotide.

To check the numerical values one can consider the following results: for the solution employing 0.15 M cyclic CMP and 0.075 M ethidium bromide, μ_{max} and f_r should be 0.22×10^{-3} and 20 MHz, respectively (compare with the values in Table I).

To rule out heterostacking interaction as a cause of the excess absorption, consider the following:

$$N + E \underset{k_r}{\overset{k_f}{\rightleftharpoons}} NE \tag{9}$$

(12) For the nucleotide one can write

$$\mu_{max} = [\pi/(2\beta RT)](\Delta V_s)^2\Gamma^{-1} \tag{1′}$$

with

$$\Delta V_s = \Delta V - (\Theta/\rho C_p)\Delta H \text{ and } \Gamma^{-1} = K_u C_T/1 + K_u \tag{2′}$$

where ΔV is the standard volume change, ΔH is the enthalpy change, β is the adiabatic compressibility, Θ is the thermal expansion coefficient, ρ is the density, C_p is the specific heat at constant pressure, C_T is the total concentration which participates in the reaction, and K_u is the syn–anti equilibrium constant for free (uncomplexed) nucleotide. In the presence of ethidium bromide

$$\mu_{max} = \frac{\pi}{2\beta RT}[(\Delta V_{sa})^2\Gamma_a^{-1} + (\Delta V_{su})^2\Gamma_u^{-1}] \tag{3′}$$

$$= \frac{\pi}{2\beta RT}\left[(\Delta V_{sa})^2\frac{K_a}{(1+K_a)^2}\delta C_T + (\Delta V_{su})^2\frac{K_u}{(1+K_u)^2}(1-\delta)C_T\right] \tag{4′}$$

where the subscripts a and u refer to ethidium-associated and uncomplexed nucleotides, respectively (i.e., steps 2 and 1 in Scheme I, respectively), and δ is the fraction of bound nucleotide.

(13) P. O. P. Ts'o "Basic Principles in Nucleic Acid Chemistry", Vol. 1, Academic Press, New York, 1974, p 453.

(14) In view of the assumptions employed in calculating $\Delta G^{\ddagger}_{\text{cyclic CMP}}$ (which is a free energy barrier) and the experimental errors, it is difficult to say anything about $\Delta H^{\ddagger}$. However, it was shown before that, when corrected for stacking, $\Delta S^{\ddagger}$ for the syn–anti conformational isomerization is small (see ref 7). Therefore, there should be a rough equality between $\Delta G^{\ddagger}$ and $\Delta H^{\ddagger}$.

Table II. Ultrasonic Relaxation Results on Aqueous Solution of 0.2 M Cytosine in the Presence of 0.1 M Ethidium Bromide, pH 6.6, 25 °C

f (MHz)	α/f^2 ($\times 10^{-17}$ s^2 cm^{-1})[a]
15	26.0, 28.0
25	23.0, 24.7
35	26.6, 26.7
45	25.7, 25.9
55	25.3, 25.4
65	25.9, 25.9
75	25.0, 24.1
85	25.0, 24.8
95	24.6, 24.3

[a] Duplicate determinations performed.

The relationship between the relaxation time and the concentration is

$$\tau^{-1}_{\text{hetero}} = k_f([N_{eq}] + [E_{eq}]) + k_r \quad (10)$$

$$\tau^{-1}_{\text{hetero}} = k_r([1 - K'(C_E - C_N)]^2 + 4K'C_N)^{1/2} \quad (11)$$

where N_{eq} and E_{eq} are equilibrium concentrations, C_E and C_N are the stoichiometric concentration (total) of ethidium and nucleotide, respectively, and $K' = [NE_{eq}]/([N_{eq}][E_{eq}])$. The solubility of the ethidium bromide increases upon addition of nucleoside or nucleotide. This strongly implies complexation and a K' much larger than unity (according to both ultrasonic absorption and ^{1}H NMR, K' is near 100). Therefore, eq 11 can be approximated as

$$\tau^{-1}_{\text{hetero}} \cong k_r K'(C_N - C_E) \quad (12)$$

If heterostacking were responsible for the excess absorption, the relaxation frequency should decrease with increasing ethidium bromide concentration. Since the opposite is observed experimentally, the heterostacking mechanism can be ruled out.

Table II presents data on the α/f^2 vs. f for 0.2 M cytosine in the presence of 0.1 M ethidium bromide at pH 6.6. There is no relaxation observed, again ruling out heterostacking as the source of the relaxation observed for cyclic CMP.

Table III. Relaxation Times for Two Steps in Scheme I and Heterostacking Constant for 2'-Deoxyadenosine and Cyclic CMP

	τ_1^{-1} (s^{-1})	τ_2^{-1} (s^{-1})	K (M^{-1})
2'-deoxyadenosine	1.2×10^8	2.4×10^8	400
cyclic CMP	4.8×10^7	2.3×10^8	100

The unimolecular relaxation therefore can be assigned to the syn–anti glycosyl isomerization.

Conclusions

It was demonstrated that binding to a receptor can have a profound effect on the barrier to glycosyl syn–anti isomerization in nucleosides and nucleotides. A previous study employed lanthanide-induced chemical shift and line-width changes to establish the glycosyl conformation of cyclic CMP.[15] The conclusion of that study was that the syn conformer predominates in solution. However, a very large barrier to conformational interconversion was also suggested. In the absence of ethidium bromide, the result reported here (the lack of an observed relaxation due to very small amplitude) is consistent with the presence of one predominant conformer. However, the barrier to glycosyl interconversion clearly cannot be very different in the absence than in the presence of ethidium bromide. We have now demonstrated (Table III) that the early theoretical work suggesting higher glycosyl barriers for pyrimidine than for purine nucleosides was essentially correct qualitatively.[16] NMR, either high resolution *or* nuclear Overhauser enhancement studies, cannot detect a small, minor conformer population. The technique of shifting the relaxation to observable ranges by addition of stacking agents gives useful information about the unbound conformers as well.

Registry No. Cytidine cyclic 2',3'-monophosphate, 633-90-9; ethidium bromide, 1239-45-8.

(15) D. K. Lavallee and R. B. Myers, *J. Am. Chem. Soc.*, **100**, 3907 (1978).

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