^{1}H Nuclear Magnetic Resonance Study on the Binding of Cytidine Monophosphate Inhibitors to Ribonuclease A†

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ABSTRACT: An nmr chemical exchange method has been used to determine the pH dependence of the H_5, H_6, and H_1' chemical shifts of the 3′- and 5′-cytidine monophosphate·ribonuclease A complexes (CMP·RNase A). The H_6 and H_1' protons of the 5′-CMP·RNase A complex follow similar bell shaped pH–chemical shift profiles with $pK_1 \sim 3.9$ and $pK_2 \sim 4.8$. Only a single titration curve was observed for the H_5 resonance of the 5′-CMP complex with $pK = 4.9$. Evidence is presented which suggests that the lower arm of the bell profiles is associated with protonation of the dianionic phosphate while the $pK \sim 4.9$ found for all three proton–chemical shift profiles apparently results from a pH-dependent conformational change of the complex. Simple titration curves with $pK \sim 6.0$ are observed for the H_5, H_6, and H_1' resonances of the 3′-CMP·RNase A complex. The chemical shift differences between the inhibitors in solution and when bound to the enzyme suggest very different glycosidic conformations for the cytosine base in the 3′- and 5′-CMP complexes. The results are analyzed in terms of a syn conformation for the 3′-CMP complex. Implications for the mode of catalysis in RNase A are considered.

Nmr has been shown to be an extremely effective technique for probing the solution structure of the active site of an enzyme. For small proteins it is sometimes possible to directly observe individual resonances of groups on the protein and under favorable conditions assignment of these signals to specific amino acid residues can be made (Jardetzky and Wade-Jardetzky, 1971; Meadows, 1972; McDonald and Phillips, 1968; Meadows *et al.*, 1967). A second, potentially more broadly applicable nmr method is to observe the resonances of a small molecule as it rapidly exchanges between the solution and the active site of the enzyme. This dynamic chemical exchange technique has yielded both structural and kinetic information on a number of enzymes (Fischer and Jardetzky, 1965; Sykes, 1969; Gerig, 1968; Schmidt *et al.*, 1969). We have previously shown in a ^{31}P nmr chemical exchange study on the binding of 3′-cytidine monophosphate (3′-CMP) to bovine pancreatic ribonuclease A (RNase A) how this method has made possible the elucidation of the ionization states and chemical environment about the phosphate in the enzyme·inhibitor complex (Gorenstein and Wyrwicz, 1973). In this paper we wish to describe a companion ^{1}H nmr study on the binding of 3′- and 5′-CMP to RNase A. This work extends and modifies earlier results of Jardetzky and coworkers (Meadows and Jardetzky, 1968; Meadows *et al.*, 1969). By correlating these results with the known three-dimensional structure of RNase (Richards and Wyckoff, 1971), it has become possible to achieve a detailed molecular understanding of the mode of inhibitor binding. In particular, we show how it is possible to use the chemical shift information to define the conformation of the cytosine base about the glycosidic bond in the enzyme complex. The ability to determine the nucleotide base conformation in the enzyme complex is potentially significant in light of the recent realization that certain enzymes involved in polynucleotide synthesis will function with nucleotides having only an anti conformation (Kapuler *et al.*, 1970; Ikehara *et al.*, 1971).

Experimental Section

Materials. The bovine pancreatic RNase A and the CMP inhibitors used in this work were obtained from Sigma. The type XII-A enzyme was generally used without further purification although in several runs heavy-metal ion impurities were removed by passing the enzyme solution through a Chelex-100 ion-exchange resin (see Gorenstein and Wyrwicz, 1973 for a discussion of these impurities and the possible problems they can pose). The 3′- and 5′-CMP were routinely purified by the resin treatment. Solutions were generally lyophilized in H_2O although D_2O was employed for several runs. All nmr solutions were prepared with 10^{-3} M or greater EDTA and 0.2 M NaCl in 99.9% D_2O. The "pH" (uncorrected pH meter reading) was adjusted with 1 M NaOD or DCl and measured on a Radiometer PHM 26 pH meter fitted with a Type G2222C glass semimicroelectrode and Type K4112 calomel electrode. Smaller aliquots were measured on a Radiometer G2221C/K1301 micro electrode assembly. All solution pH's were measured before and after the nmr run and both agreed within 0.02 pH unit.

Nmr Methods. The ^{1}H nmr spectra were recorded either on a Bruker HFX-90 high resolution nmr spectrometer operated in the continuous wave (cw) mode at 90 MHz or on a Bruker B-KR 322S pulsed spectrometer/HFX-90 spectrometer. Either a specially constructed, Pivan Engineering, data acquisition system (4K data points) or a Nicolet 1080 Fourier transform data system was used for signal averaging in the cw or pulsed mode operation of the respective spectrometers. In the pulsed, Fourier transform experiment 8K data points were acquired with a sweep width of 1000 Hz and resolution therefore of 0.4 Hz. A fluorine (C_6F_6) external lock was used with frequencies measured on either Hewlett-Packard 5612A 12.5-MHz of 5248M 100-MHz counters. In order to selectively reduce the intensity of the HDO peak a partially relaxed Fourier transform (PRFT) (Freeman and Hill, 1970; Vold *et al.*, 1968) pulse sequence with an additional "homo spoiler" pulse was employed. All spectra were taken at 24 ± 1°.

Computer Analysis of Data. A generalized non-linear least-squares program (Dye and Nicely, 1971) which was modified for use on a chemistry department PDP 11/45 computer (32K

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words) was used to obtain "best fits" for the nmr spectra and the chemical shift and titration curves. The recorded spectra or titration curves were digitized onto punch tape (100–200 data points) by manually retracing the curves on a modified Varian G-2500 recorder with a full scale off-setting potentiometer. The analog output of the recorder was fed into an analog to digital converter ($4^1/_2$ digits). The digital output as well as a digital time signal were then sent to an ASR-33 teletype and a permanent punch tape, digitized record of the original curve was created. The doublet region for the H_6 proton and the doublet of doublets for H_5 and H_1' were separately digitized and the data were fit to either the sum of two or four Lorentzian line shapes for the respective spectral regions. In order to keep to a minimum the number of adjustable parameters in the Lorentzian line-shape equation, the coupling constants, $J_{1',2'}$ = 2.8–3.4 Hz and $J_{5,6}$ = 7.2–7.6 Hz, were generally assumed to be constant for all spectra in a given run. These values were derived from spectra taken at low enzyme to inhibitor ratios where the peaks were well resolved. In addition the individual Lorentzian lines were constrained to equal integrated areas so that only the overall integrated area of the nmr curve was adjusted along with the chemical shifts and line widths for each doublet. Although the equal area constraint for both resonances of the doublet is not strictly justified for an AB coupling pattern (Pople *et al.*, 1959), in our case $\Delta_{AB} \gg J_{AB}$ and correction for the proper spectral intensities would make little difference in the computed spectra.

Ionization Constants. Titrations of the nucleotides in H_2O and D_2O were performed on a Radiometer SBR2c/Titrator 11 automatic titrator at 24.0°. The nucleotides in 0.2 M NaCl were titrated with standardized NaOH (NaOD) solutions. The titration curve was digitized and converted into a punch tape record. A titration curve of a blank solution containing only 0.2 M NaCl was subtracted from the titration data of the nucleotides and the two ionization constants for the nucleotides in the pH region studied were obtained from the corrected curve by a non-linear least-squares fit to eq 1. This equation describes the

$$\psi_{obsd} = \frac{1}{f_{H_2A}}\psi_{H_2A} + \frac{1}{f_{HA}}\psi_{HA} + \frac{1}{f_A}\psi_A = \frac{\alpha\beta\psi_{H_2A} + \beta\psi_{HA} + \psi_A}{1 + \beta + \alpha\beta} \quad (1)$$

general behavior of any observable parameter, ψ, which is dependent upon the ionization state of a dibasic acid where the f's are the Michaelis pH functions (Dixon and Webb, 1964) for the diprotonated, H_2A, the monoprotonated, HA, or the unprotonated species, A, and $\alpha = (H^+)/K_1$, $\beta = (H^+)/K_2$. K_1 and K_2 are the first and second ionization constants of the dibasic acid. Ionization constants from the chemical shift *vs.* pH data were evaluated as well by an unweighted least-squares fit to this equation.

Results and Discussion

The chemical shift study described in this paper has utilized the nmr chemical exchange method for obtaining information on the enzyme·inhibitor (E·I) complex. It is assumed that the small molecule inhibitor exchanges between two sites, the free solution and the enzyme active site. If the chemical shift of the E·I complex, δ_{EI}, is different from that of the inhibitor in solution, δ_I, and if the inhibitor exchanges between the two sites sufficiently rapidly (Sykes, 1969)

$$E + I \rightleftharpoons E\cdot I$$

then the observed chemical shift, δ_{obsd}, will represent a weighted average of chemical shifts for the two different environments

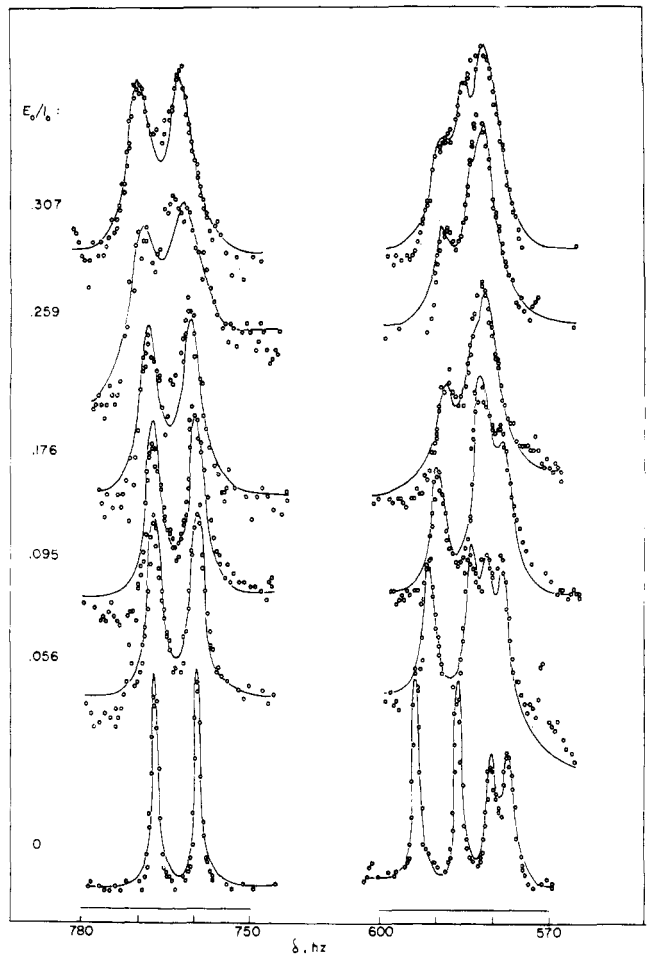


FIGURE 1: Digitized ^{1}H nmr spectra (O) for the H_6, H_5, and H_1' protons of 5′-CMP at various E_0/I_0 ratios, pH 6.5. Solid curves represent computer-calculated spectra assuming Lorentzian line shapes.

$$\delta_{obsd} = \frac{(E\cdot I)}{I_0}\delta_{EI} + \frac{(I)}{I_0}\delta_I \quad (2)$$

Rearranging, and since under most of our conditions the enzyme is saturated with the inhibitor, $(E\cdot I) \sim E_0$, and

$$\delta_{obsd} - \delta_I = \Delta\cdot E_0/I_0 \quad (3)$$

where $\Delta = \delta_{EI} - \delta_I$. Thus the chemical shift of the E·I complex may be obtained from the slopes of plots of δ_{obsd} *vs.* E_0/I_0.

In these chemical exchange studies we have monitored the cystosine base H_6 and H_5 protons and the ribose ring H_1' proton of 3′- and 5′-CMP. Other resonances of the inhibitors are obscured by the enzyme signals. The six-line spectra (resulting from coupling between the H_5 and H_6 protons and between the H_1' and H_2' protons) were computer simulated by assuming simple Lorentzian line shapes. The chemical shifts and line widths at half-height[1] were either found by an iterative nonlinear least-squares computer fit (see Experimental Section) or alternatively the shifts and line widths were varied until a visual best fit of the computed and original spectrum was obtained. This more laborious "human iteration method" was especially necessary for those spectra where the H_5 and H_1' peaks were so

[1]This paper describes only the chemical shift effects. The line width changes are presented in Gorenstein and Wyrwicz (1974).

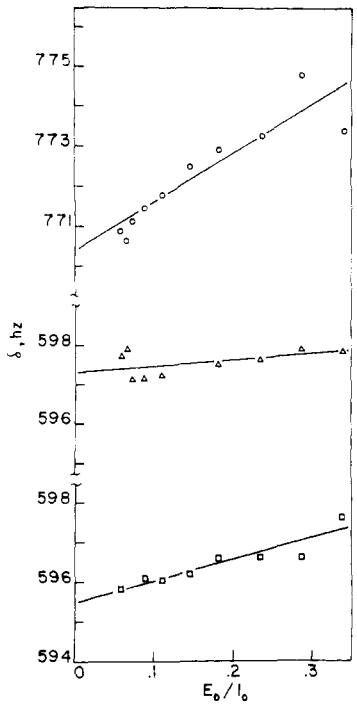


FIGURE 2: Observed chemical shift, δ_{obsd}, *vs.* E_0/I_0 ratio for the H_6(O), H_5(Δ), and H_1'(□) protons of 5′-CMP at pH 4.0 in 0.2 M $NaCl/D_2O$.

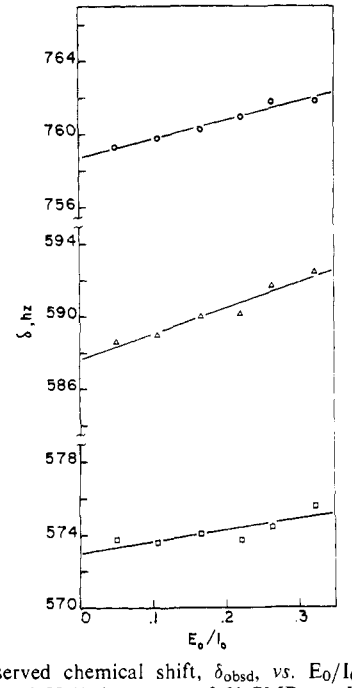


FIGURE 3: Observed chemical shift, δ_{obsd}, *vs.* E_0/I_0 ratio for the H_6(O), H_5(Δ), and H_1'(□) protons of 3′-CMP at pH 4.5 in 0.2 M $NaCl/D_2O$.

broad and overlapping that a unique computer fit became impossible. A typical example of the observed and computer simulated spectra at various E_0/I_0 ratios is shown in Figure 1. Generally the enzyme concentration was kept constant at *ca.* 0.009 M, although in several runs both enzyme and inhibitor concentrations were varied (only small errors were introduced by this alternative method; see Gorenstein and Wyrwicz, 1973). Several of the δ_{obsd} *vs.* E_0/I_0 plots are shown in Figures 2 and 3. The chemical shifts were fitted to eq 2 with a least-squares computer program and the δ_I and δ_{EI} obtained from

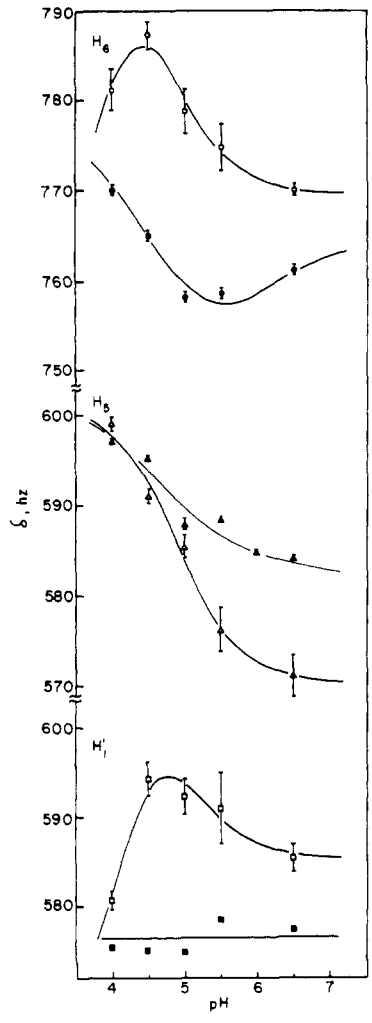


FIGURE 4: Chemical shift, δ, *vs.* pH (uncorrected pH meter reading) for the H_6(O), H_5(Δ), and H_1'(□) protons of 5′-CMP in 0.2 M $NaCl/D_2O$. Filled symbols represent δ_I, the chemical shift of the inhibitor, free in solution. Unfilled symbols represent δ_{EI}, the chemical shift of the enzyme·inhibitor complex. Curves were generated from eq 1 and the "best fit" parameters given in Tables I and III.

these plots at different pH's are presented in Figures 4 and 5.[2]

It should be noted that δ_I obtained from eq 2 may be different from δ_I obtained in the absence of protein. The difference arises from the change in the bulk susceptibility of the solution. However, the pH-dependent shifts of δ_I in the two solutions are quite comparable.

It has previously been noted that the nucleotide proton chemical shifts (Jardetzky and Wade-Jardetzky, 1960) are influenced by such factors as ring-current and keto group magnetic anisotropy (Schweizer *et al.*, 1965, 1968; Chan *et al.*, 1964; Prestegard and Chan, 1969), proximate ionizable groups (Danyluk and Hruska, 1968), and conformations of the rings (Fujiwara and Vetsuki, 1968; Smith and Jardetzky, 1968). In the CMP nucleotides both the N-3 and phosphate groups have pK's in the pH range here studied. Schweizer *et al.* (1968) have previously noted that protonation of the phosphate group has no effect on the H_5 and H_6 protons of 3′-CMP but has a substantial shielding effect on the H_6 proton (shifted upfield by

[2]Unfortunately, the error bars (representing standard deviations) are quite substantial in these plots, particularly in Figure 5. Because only a limited range of E_0/I_0 ratios was studied, the standard deviations in the slopes of plots such as shown in Figures 2 and 3 were in certain cases quite large, reflecting the large error brackets in Figure 5.

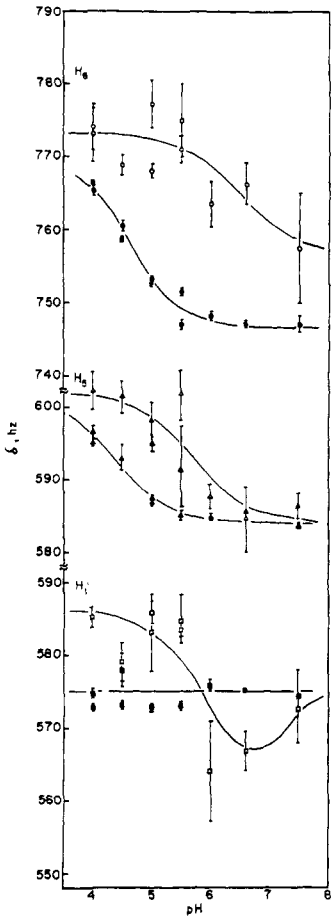


FIGURE 5: Chemical shift, δ, *vs.* pH (uncorrected pH meter reading) for the H_6(○), H_5(△), and H_1'(□) protons of 3′-CMP in 0.2 M NaCl/D_2O. Filled symbols represent δ_I, the chemical shift of the inhibitor, free in solution. Unfilled symbols represent δ_{EI}, the chemical shift of the enzyme·inhibitor complex. Curves were generated from eq 1 and the "best fit" parameters given in Tables I and III.

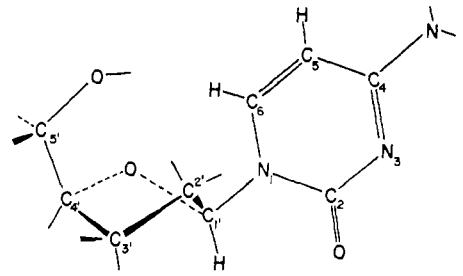


FIGURE 6: Structure of the 2′-endo-, anti conformation for the cytidine nucleotide.

0.080 ppm) and a much smaller effect (0.012 ppm upfield shift) on the H_5 proton of 5′-CMP. This has been interpreted to indicate that the dianionic phosphate deshields only the proximate H_6 proton of 5′-CMP in the predominantly anti[3] conformation of the base (see Figure 6). Any change in the conformation of the base about the glycosidic bond in the E·I complex would be expected to alter the deshielding magnetic anisotropy of the phosphate group. In addition N-3 ring protonation in the pH region 4–5 is known to shift (Lee *et al.*, 1972) downfield the H_5 and H_6 resonances of both 5′- and 3′-CMP by 0.2–0.3 ppm. Our own results (see Figures 4 and 5) for the pH dependence of the H_5, H_6, and H_1' resonances of the free nucleotides are in general agreement with the earlier work. A computer fit of the chemical shift titration data for the free inhibitor H_5 and H_6 protons to eq 1 gives pK's which agree within the experimental error to the ionization constants derived from the potentiometric titrations (Table I). The agreement confirms the general utility of these chemical shift effects as sensitive probes of local magnetic environments.

CMP·RNase A Chemical Shifts. As shown by Figures 4 and 5 the chemical shift titration curves for the H_5, H_6, and H_1' resonances of the CMP·RNase A complexes are quite different from the curves for the free-solution inhibitors. Before discussing these E·I complex titration curves, it should be noted that our results differ from those previously reported by Meadows and Jardetzky (1968) at a single pH. A comparison of our own chemical shift differences, Δ, and those of the earlier workers is shown in Table II.

While there is general agreement between the 3′-CMP results of Meadows and Jardetzky and the present study, there is a dramatic discrepancy between the 5′-CMP results. We find a large *upfield* shift for the H_5 resonance of 5′-CMP while Meadows *et al.* (1969) report only a small *downfield* change for the same signal. In addition we observe a much larger downfield shift for the H_1' signal of 5′-CMP. Jardetzky reported that each of the three resonances of the 5′-, 3′-, and 2′-CMP complexes gave similar chemical shift differences. On this basis, they concluded that the cytosine ring in all three complexes was similarly bound. This conclusion is no longer tenable if, in fact, their Δ values for 5′-CMP are incorrect. The source of the discrepancy is made apparent by studying the original spectra. At concentrations of 5′-CMP ~ 0.02–0.03 M and RNase ~ 0.009 M, the H_5 and H_1' signals are broad and overlapping so that at even lower 5′-CMP concentrations (effectively, higher E_0/I_0 ratios) it is quite understandable that the peak assignments were reversed in the crossover. This would explain why, as noted by Jardetzky, their H_5 plot (Figure 7, p 499, Meadows *et al.*, 1969) shows first a downfield shift, then with increasing inhibitor concentration, an upfield shift. If correction is made for this reversed assignment, then similar values for the chemical shift differences for the H_5 and H_1' signals of 5′-CMP are obtained.

5′-CMP·RNase A. The H_6 and H_1' resonances of the complex follow bell-shaped chemical shift–pH profiles (Figure 4), while only a single titration curve is observed for the H_5 resonance. Assuming that only one ionizable group of the complex is responsible for each of the arms of the profiles a least-squares fit of the data of these plots to eq 1 yields the apparent pK's and chemical shifts given in Table III.

The 5′-CMP·RNase A chemical shifts and the pH dependence of the shifts are shown to be quite different from those of the inhibitor free in solution. Thus, the H_6 resonance of the free inhibitor first shifts upfield then downfield with increasing pH while the H_6 resonance of the complex shifts exactly the opposite. However, there *are* important similarities and they provide clues as to the interpretation of these effects. The H_5 resonance does not show an upfield shift with increasing acidity in the pH region where the H_6 peak is markedly shifted. This is the behavior expected for 5′-CMP protons which are perturbed by protonation of the anisotropic phosphate. The magnitude and direction of the shift for the low pH arm of the H_6 titration curve would be explained then by ionization of a proximate phosphate group with pK ~ 3.9. It is significant that we have observed in a ^{31}P nmr study a pK ~ 4.4 for ionization of the

[3]Donohue and Trueblood (1960) first noted that the purine or pyrimidine bases could exist in either of two preferred conformations, anti or syn. Pyrimidine bases normally are in the anti conformation with H_6 over the ribose ring.

TABLE I: Apparent pK's and Chemical Shifts of 5′- and 3′-CMP in 0.2 M NaCl/D_2O Solution, 24°.

Inhibitor	(Proton)	Ionization Constants[a] pK_1	Ionization Constants[a] pK_2	Chemical Shifts[a,b] (Hz) δ_{H_2A}	δ_{HA}	δ_A
5′-CMP	(H_6)	4.42 ± 0.2	6.17 ± 0.2	777 ± 1	755 ± 2	764 ± 5
	(H_5)	4.52 ± 0.1	6.30 ± 0.2	601 ± 1	586 ± 1	582 ± 10
	(H_1')			576 ± 1	576 ± 1	576 ± 1
	(Potentiometric Titration)	4.46 ± 0.02	6.17 ± 0.04			
		4.32 ± 0.01[c]	6.06 ± 0.01[c]			
3′-CMP	(H_6)	4.45 ± 0.15		772 ± 3	747 ± 1	
	(H_5)	4.44 ± 0.15		600 ± 2	584 ± 0.5	
	(H_1')			575 ± 1	575 ± 1	
	(Potentiometric Titration)	4.15 ± 0.04	5.65 ± 0.03			
		4.06 ± 0.01[d]	5.69 ± 0.01[d]			

[a] Errors represent either linear estimates of the standard deviation derived from the computer analysis (see Dye and Nicely (1971), references) or simply best estimates when not enough data points were available to achieve convergence to realistic values for the chemical shifts. [b] Chemical shifts in Hz from Me_4Si at 90 MHz for the different protonic species. Chemical shifts, δ_I, derived from intercepts of δ_{obsd} *vs.* E_0/I_0 plots. (RNase A) ≈ 0.009 M. [c] In H_2O. [d] 30.0°.

TABLE II: Inhibitor Chemical Shift Differences, Δ, for the Binding of Inhibitors to RNase A (pH 5.5, 0.2 M NaCl in D_2O).

Inhibitor	Δ(Hz)[a] H_5	H_6	H_1'
3′-CMP[b,d]	−6	−22	−3
3′-CMP[c,e]	−11.5	−24	−11
5′-CMP[b,d]	−5	−17	−3
5′-CMP[c,e]	+17	−12	−13

[a] Negative Δ's refer to downfield shifts of the resonances of the inhibitor complex relative to the inhibitor alone. [b] 100 MHz, 32°. [c] 90 MHz, 24°. [d] Meadows *et al.* (1969). [e] This work.

secondary phosphate in the 3′-CMP·RNase A complex (Gorenstein and Wyrwicz, 1973).[4] Further support for this hypothesis is derived from the absence of any chemical shift titration effect in this pH region for the H_6 resonance of 3′-CMP·RNase A (see Figure 5). As discussed earlier for free 3′-CMP, the phosphate is not near the cytosine ring in any conformation, and no perturbation of the H_5 and H_6 resonances is therefore to be expected.

The specific perturbation of the H_6 resonance by the titrable 5′-phosphate indicates that the cytosine ring is in the anti conformation in *both* the complex and free solution. The lowering of the pK for the secondary phosphate by 2.5 pK units is not unreasonable considering the highly positive local environment of the enzyme (Richards and Wyckoff, 1971).

[4] Rüterjans *et al.* (1971) have reported a pK ~ 5.6 for the 5′-CMP·RNase complex. However, they obtained this value by titrating a 1:1 mixture of 5′-CMP and RNase A and monitoring the ^{31}P chemical shift of this solution. This method cannot be used to obtain accurate ^{31}P chemical shifts of the complex since the enzyme will not be completely saturated throughout the pH range of the titration. The chemical exchange method circumvents this difficulty and a ^{31}P nmr study of 5′-CMP in progress should provide an accurate pK for the secondary phosphate of the complex.

Both the H_6 and H_5 resonances are perturbed by an ionizable group with a pK ~ 4.8. The sign of the chemical shift difference is the same as that produced by protonation of N-3 on the cytosine ring in the free 5′-CMP, and the pK is in good agreement for assignment to this group, again considering the positive environment of the enzyme. In addition, Anderson *et al.* (1968) have concluded on the basis of inhibitor binding curves and uv spectral difference curves that the N-3 protonated CMP inhibitors are bound equally as well as the unprotonated species.

Our results would indicate that N-protonation could be a reasonable explanation for the chemical shift changes, but considering the normally highly specific binding behavior of enzymes, it would certainly seem remarkable. Protonation of N-3 would disrupt any binding of the cytosine ring at N-3 to the enzyme, and, in fact, Richards and Wyckoff (1971) on the basis of their X-ray diffraction study have indicated that an enzyme–ring hydrogen bond exists at this site. In addition, the difference between the H_5 and H_6 chemical shifts of the N-3 protonated and unprotonated forms in the free solution 5′-CMP is 15–20 Hz. Yet the chemical shift difference for these resonances in the 5′-CMP·RNase A complex for titration of the pK 4.8 group is larger, 30–35 Hz. Finally, this hypothesis fails to explain the 13-Hz *upfield* shift of the H_5 resonance of the complex at pH 6.5. Since both the complex and free inhibitor N-3 would be unprotonated at this pH this upfield shift must be attributed to some other proximate group or groups at the active site. For these reasons we feel that protonation of N-3 is probably not responsible for the titration curve of H-5 and the high pH arm of the bell profile for H-6.

Instead, we propose that these upfield shifts of the H_5 and H_6 resonances with increasing pH are due to the magnetic anisotropy of the Phe_{120} residue. The X-ray studies of RNase A and RNase S complexes (Kartha *et al.*, 1967; Richards and Wyckoff, 1971) show that the H_5 and H_6 protons of pyrimidine inhibitors are in close proximity to the aromatic ring of Phe_{120}. As noted by Jardetzky (Meadows *et al.*, 1969) the ring current magnetic anisotropy of this group should shift the H_5 and H_6 pyrimidine ring resonances of the complex upfield from the resonances of the inhibitor in solution. This hypothesis would require that some titrable group with pK ~ 4.8 is re-

TABLE III: Apparent pK's and Chemical Shifts for the Inhibitor·RNase A Complexes in 0.2 M NaCl/D_2O Solutions, 24°.

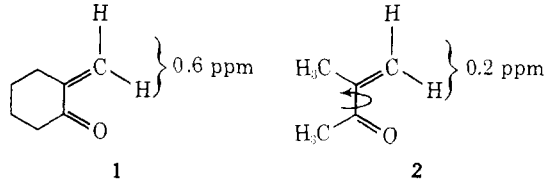
Inhibitor·Complex	(Proton)	Ionization Constants[a]		Chemical Shifts (Hz)[a,b]		
		pK_1	pK_2	δ_{H_2A}	δ_{HA}	δ_A
5′-CMP·RNase A	(H_6)	$\leq 3.9 \pm 0.3$[c,d]	4.7 ± 0.2	754 ± 20[c,d]	805 ± 10	770 ± 1
	(H_5)		4.9 ± 0.2		602 ± 3	570 ± 2
	(H_1')	$\leq 3.95 \pm 0.3$[c,d]	4.9 ± 0.1	552 ± 20[c,d]	608 ± 3	585 ± 0.5
3′-CMP·RNase A	(H_6)	6.5 ± 0.4			773 ± 2	757 ± 5
	(H_5)	5.7 ± 0.3			602 ± 3	584 ± 0.4
	(H_1')	6.0 ± 0.3	(7.1)[d]		586 ± 4	556 ± 3
						(580 ± 5)[c,d]

[a,b] See footnotes to Table I. [c] Since only 1 data point was used to fit this arm of the profile, the chemical shifts and pK are only poorly defined. [d] The existence of this arm of the profile is questionable.

sponsible for juxtaposing the aromatic ring of the Phe_{120} and the pyrimidine ring. This group need *not* be at the active site. Instead, it may merely be responsible for causing a conformational change of the complex which moves these two groups into closer proximity. Hammes has previously noted that a group with a pK of 5.8 is responsible for a pH-dependent conformational change of the 3′-CMP·RNase A complex (Hammes and Walz, 1969). This group is thought to be His_{48} which is affected by inhibitor binding in this pH region (Meadows and Jardetzky, 1968; Meadows *et al.*, 1969). This residue is believed to be responsible for a conformational change of the complex which serves to "close the cleft" (Hammes, 1968; Medows *et al.*, 1969) on the substrate or inhibitor and bring into play key catalytic and binding groups at the active site.

Interpretation of the H_1' chemical shift titration curve is made difficult by the multiplicity of factors which might be associated with such shifts (Prestegard and Chan, 1969). Whatever factors are responsible, they must be much more efficacious in the complex than in the free solution, where only minor perturbations of the H_1' resonance are found with a change in pH. In contrast, the complex follows a well-defined bell-shaped profile with pK's of 4 and 4.9 and large chemical shift differences of >20 Hz for each arm of the profile. Since the high pH arm of the H_1' profile follows the same pH dependency as the H_5 and H_6 resonances, it is probably simplest to group the phenomena together, *i.e.*, associate it with the conformational change of the complex. (In fact this pH perturbation of the H_1' chemical shift of the complex, which is not found in the free solution inhibitor, is perhaps one of the best arguments *against* the assignment of the p$K \sim 4.8$ in the H_5 and H_6 plots to the protonation of N-3.) The 20-Hz upfield chemical shift resulting from deprotonating His_{48} or some other group could be due to the juxtaposition of H_1' and a magnetically anisotropic group such as the aromatic ring of Phe_{120}, the imidazolium ring of His_{119}, the keto group of the cytosine ring, or virtually any of a number of other groups at the active site. All are possible candidates for effectors of this shift, though at present we prefer an explanation based upon the deshielding anisotropy of the 2-keto group. A proton is maximally deshielded by a carbonyl group when it lies in the plane of the carbonyl group (Jackman, 1959). Thus, in the two α,β-unsaturated ketones the vinyl hydrogens cis to the keto group are both shifted downfield relative to the trans vinyl hydrogens. Most importantly, however, the difference in chemical shift between the two vinyl hydrogens is much larger in compound **1** than in the acyclic ketone **2**, where free rotation about the 2,3 single bond is allowed.

Apparently only in the planar s-cis conformation is the keto group maximally deshielding. Schweizer *et al.* (1973) attribute



1 **2**

to the keto group anisotropy the 0.25 ppm downfield shift of the H_1' proton in 5-methylcytidine relative to 6-methylcytidine. They argue that for steric reasons, the 5-methylcytidine must exist in the anti conformation while the 6-methylcytidine must exist in the syn conformation (2-keto group over the ribose ring). Again, the 2-keto group will cause maximum deshielding of the H_1' proton only when the base is in the anti conformation. There must still be considerable rotational freedom about the glycosidic bond in the free nucleotide (Sundaralingam, 1969; Haschemeyer and Rich, 1967). In fact, Lavalle and Coulter (1973) argue that 3′- and 5′-CMP exist as syn-anti mixtures with the anti conformer predominating. The H_1' nucleus will therefore only experience a fraction of the maximal possible deshielding field of the keto group. It is relevant then that at all pH's studied, *the chemical shift of the H_1' proton of the E·I complex is shifted downfield relative to the inhibitor in solution.* This would indicate that the cytosine base is more restricted to the anti conformation in the E·I complex. The foregoing examples indicate that constraining a proton to the plane of a carbonyl group rather than allowing it to freely rotate through the plane will lead to an additional 0.4 ppm upfield shift for this proton. The 0.3 ppm difference between the chemical shifts of the H_1' resonance in the free 5′-CMP and the monoprotonated state of the complex (the "acid stable" HA, isomer) provides some support then for this keto group anisotropy explanation. In addition if we assume that the keto group anisotropy is responsible for the pH dependent chemical shift changes of the H_1' proton as well, then it would follow that the conformational change of the complex induces a change in the cytosine base conformation. The upfield shift with increasing pH could result from rotation of the glycosidic bond so that the base moves into less of an anti conformation.

3′-CMP·RNase A Chemical Shifts. Meadows *et al.* (1969) have argued on the basis of the similarity of the chemical shift differences for the H_5, H_6, and H_1' protons in 5′-CMP and 3′-CMP (as well as 2′-CMP) that the cytosine ring of the nucleotides binds in all cases in essentially the same fashion. We have already noted that at the single pH of Jardetzky's study this is not evident from the corrected data (Table II). In addition, as shown by Figures 4 and 5, there are several important differ-

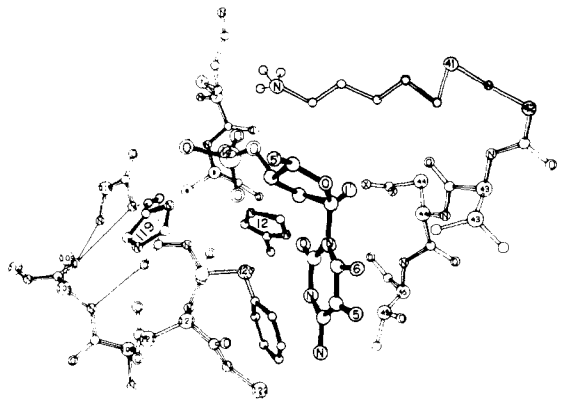


FIGURE 7: Model for the "base stable" conformational isomer of the 3′-CMP·RNase A complex showing some of the nearest neighbor amino acid residues of the protein and a syn conformation for the cytosine base. In contrast to Richards and Wyckoff's (1971) X-ray structure from which this model was derived, the Lys_{41} ϵ-amino group is assumed to be juxtaposed to the dianionic phosphate.

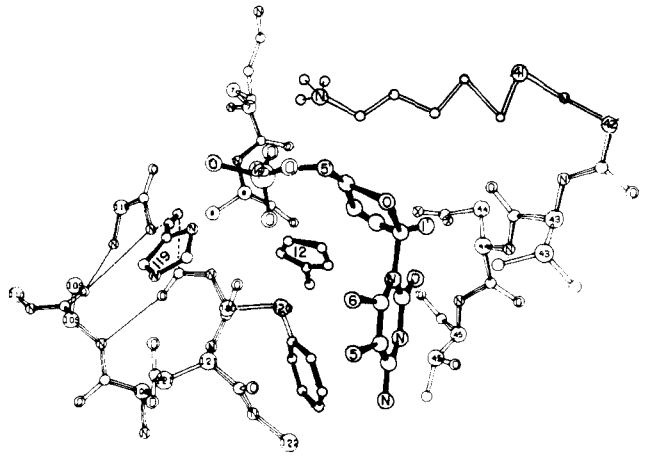


FIGURE 8: Model for the "base stable" conformational isomer of the 5′-CMP·RNase A complex showing some of the nearest neighbor amino acid residues of the protein and an anti conformation for the cytosine base.

ences between the pH–chemical shift curves for the nucleotide·enzyme complexes. A major difference between the sets of curves is the number of ionizable groups responsible for the pH dependence of the chemical shifts. In the 5′-CMP complex only the H_5 proton follows a simple titration curve whereas in the 3′-CMP complex all three protons follow simple titration curves (neglecting the high pH point for the H_1' chemical shift of the complex). In addition the pK derived from the H_6, H_5, and H_1' curves of the 3′-CMP complex is *ca.* 1 pK unit larger than the corresponding pK of the 5′-CMP complex.

Lastly, the absolute chemical shifts of the three resonances differ in the two complexes. In this regard it is significant that the chemical shifts of these protons are nearly the same for 3′-CMP and 5′-CMP free in solution (this ignores the specific perturbation of H_6 and to some extent H_5 caused by ionization of the 5′-phosphate.) On the other hand, the whole curve for the H_5 proton in the 3′-CMP complex is shifted 12 Hz downfield relative to the 5′-CMP·RNase A curve. The H_1' curve in 3′-CMP·RNase A is shifted 10–20 Hz upfield from the curve of the 5′-CMP complex. Because of the complicating specific effects of the phosphate on the H_6 resonance in the 5′-CMP complex, it is difficult to state whether the H_6 proton curves in the two nucleotide complexes would be shifted in the absence of this effect. It appears as though the shift in the H_6 curves would be small.

These pK and chemical shift changes indicate that there must be substantial differences in the geometry of the two nucleotide complexes. However, the phosphate and ribose and cytosine rings are probably still bound in essentially the same sites for the two nucleotides since a main feature of the profiles remain unchanged: an upfield shift for all resonances in *both* complexes reflecting ionization of a group with a pK between 5 and 6. We believe then, that the same interpretations that we have applied to understanding this pH-dependent chemical shift in the 5′-CMP complex applies as well to the 3′-CMP complex. We again propose that the titration curve profiles result from a pH-dependent conformational change of the complex. Although protonation of N-3 in the complex would provide an alternative explanation for the downfield shifts of the H_5 and H_6 protons, it cannot explain the change in the H_1' resonance in the same pH region.

Structure of the Enzyme·Inhibitor Complexes. The magnitude of the chemical shift changes and the absolute values of the chemical shifts provide a basis for defining the structures of the nucleotide·enzyme complexes. It is necessary, however, to make the following postulates in order to assess the chemical shift effects: (1) the phosphate in both nucleotides is bound in essentially the same location in the active site cleft; (2) Phe_{120} is largely responsible for the perturbation of the H_5 and H_6 magnetic environment; (3) the 2-keto group is largely responsible for the perturbation of the H_1' magnetic environment; (4) deprotonation of a group with pK = 5–6 induces a conformational change of the complex which moves Phe_{120} and other residues at the active site *closer* to the inhibitor.

We have already discussed some of the experimental evidence supporting postulates 2 and 3. In addition, Jardetzky has reached similar conclusions about the phosphate binding site and the effect of Phe_{120}. Thus, it has become apparent from ^1H and ^{31}P nmr studies that the protonated His_{119} and the phosphate dianion of 3′-CMP interact strongly, possible *via* a hydrogen bond. In drawing a model (shown in Figure 7) for the 3′-CMP complex we have chosen to use Richards and Wyckoff's (1971) crystallographic structure of a dinucleotide phosphate·RNase S complex as a guide. The model is intended to represent the structure of the unprotonated, tightly bound state of the complex (the "base stable" conformational isomer).

Essentially, the 3′-CMP phosphate is shown bound to the highly positive site created by the protonated side groups of His_{119}, His_{12}, and Lys_{41}. For reasons we will present shortly, the cytosine base is drawn in the unusual syn conformation. However, we do not wish to imply that the specific syn torsional angle for the glycosidic bond shown in this model is any better than many other syn angles. The aromatic ring of Phe_{120} lies at an angle with the cytosine ring, bringing the H_5 and H_6 protons close to the shielding anisotropic field of the Phe_{120}. Protonation of His_{48} probably is responsible for a conformational change which opens the cleft and swings Lys_{41} and Phe_{120} away from the inhibitor. In this monoprotonated ("HA") "acid stable" isomer the 3′-CMP is bound only loosely. This allows the cytosine ring to rotate more freely than in the tightly bound, unprotonated "A" state of the complex, thus allowing the cytosine base to assume a more normal, anti conformation.

Figure 8 shows a model of the 5′-CMP complex in the tightly bound "base stable" state. In order to bring the 5′-phosphate into approximately the same location as the 3′-phosphate, the ribose ring must be rotated by at least 50° about an axis that runs through the center of the sugar ring and perpendicular to

it. This would force the cytosine base against Phe_{120}, which would probably result in some rotation about the glycosidic bond. It would also disrupt some of the hydrogen bonds to the cytosine base and this inaccurate fit of the 5′-nucleotide to the active site shows up in the diminished binding constant for this nucleotide. Pushing together of the cytosine base and the aromatic ring results in an upfield shift of the H_5 proton in the "base stable" "A" state of the 5′-CMP complex relative to the 3′-CMP complex. Thus in this tightly bound state δ_A = 570 Hz for the 5′-CMP complex whereas δ_A = 584 Hz for the 3′ complex. In the more loosely bound (HA) state of the complex, δ_{HA} = 602 Hz for the H_5 proton in both nucleotides. If the 30 Hz or larger downfield shift caused by the 5′-phosphate is subtracted from the chemical shift of the H_6 proton, then it can be seen that the H_6 resonance of the 5′-CMP complex is also shifted upfield from the H_6 resonance in the 3′-CMP complex (again discussing only the "base stable" isomer). Thus, δ_A <740 Hz in the 5′-CMP complex but δ_A = 757 Hz in the 3′-CMP complex. If correction for the phosphate ionization is made, δ_{HA} ~773 Hz for both nucleotide complexes in the loosely bound conformation.

The H_1' chemical shifts provide a means of defining the base conformation, assuming that the 2-keto group is largely responsible for any changes in these shifts. In the "base stable" conformer of the 5′-CMP complex and the "acid stable" conformer of the 3′-CMP complex, δ = 585–586 Hz. This indicates that both nucleotides have similar anti conformations in these states of the complex. In the "base stable" isomer of the 3′-CMP complex the H_1' resonance is upfield (*ca.* 30 Hz) from the resonance of the 5′-CMP complex and most significantly, it is *upfield (ca.* 20 Hz) *from even the H_1' signals of the inhibitors in the solution.* The direction and magnitude of the chemical shift differences suggest that the cytosine base is rotated into the syn conformational range in the "base stable" isomer of the 3′-CMP complex.

Ribose Conformations and Mode of Catalysis in RNase A. X-Ray diffraction (Haschemeyer and Rich, 1967) and nmr studies (Prestegard and Chan, 1969) have shown that nearly all pyrimidine nucleotides and nucleosides exist in the "puckered" 2′-endo and/or 3′-endo ribose conformations (the 2′- or 3′- carbon atom and the base are on the same side of the plane defined by the other four atoms of the ribose ring; see Figure 6). An important exception to this general rule is 2′,3′-cyclic CMP which is shown by crystallographic (Coulter, 1973) and nmr studies (Lavalle and Coulter, 1973; Lapper and Smith, 1973) to have a much more planar ribose conformation. Especially surprising and intriguing is the unusual syn conformation found by Coulter (1973) and Lavalle and Coulter (1973) for the cytosine base of this cyclic nucleotide. As noted by Prestegard and Chan (1969), the range of torsional angles which the base can assume is limited by the nonbonded interactions between the H_6 proton (or the 2-keto group) and the ribose ring atoms. Apparently the more planar conformation of the ribose ring in the cyclic nucleotide allows the cytosine ring to adopt this unusual syn conformation. However, this interaction can probably work both ways so that the extent of ring puckering could in turn largely determine the conformational preference of the base ring. Whether the syn conformation is a consequence of or an effector of ribose ring distortion is not to be argued here. Our proposal that the cytosine base in the "base stable" state of the 3′-CMP complex is bound in the syn conformation would, however, suggest that the ribose ring is bound in a distorted planar conformation. This could be significant since constraining the ribose ring to a planar conformation could be one important catalytic function of the enzyme.

An important question yet to be fully resolved regarding the mechanism of action of RNase A is why the enzyme forms a highly strained cyclic diester intermediate in the hydrolysis of unstrained acyclic diester substrates. Model studies by Westheimer and coworkers (1968) have shown that most of the *ca.* 10^6-fold rate acceleration of strained cyclic phosphate esters results from the release of this ring strain in forming an unstrained cyclic pentacovalent intermediate. For the enzyme, however, not much would seem to be gained[5] by this pathway since it must first provide this strain energy and then ultimately release it. The strain energy involved is considerable: 8.1 kcal/mol in the hydrolysis of 2′,3′-cyclic CMP (Rudolph *et al.*, 1971). Speculations on the mode of catalysis in RNase A have largely focused on the role of the histidine and lysine residues at the active site. These groups presumably serve to lower the energy of the transition state by functioning as general acid/base catalysts. Possibly an equally important function of the enzyme is to raise the energy of the substrate, utilizing a form of strain catalysis (Jencks, 1969). An effective way for RNase A to induce strain in the acyclic diesters would be to distort the ribose ring and base into a geometry similar to that of 2′,3′-cyclic CMP. Our finding of a syn conformation for the base in the 3′-CMP complex, would support this suggestion.

Conclusions

Including Jardetzky's earlier work, all previous studies on the binding of cytidine nucleotides to RNase A have concluded that the cytosine rings in the nucleotides bind in essentially the same manner. Thus, the uv-difference spectra (Hummel *et al.*, 1961; Anderson *et al.*, 1968) and optical rotatory dispersion spectra (Deavin *et al.*, 1966) of the 3′-, 5′-, and 2′-CMP nucleotides bound to RNase A are quite similar, in spite of the fact that the uv-difference spectra of the complexes apparently result from changes in the absorption of the cytosine ring and not from any group on the enzyme (Irie and Sawada, 1967). It would therefore appear that only nmr, in contrast to other spectroscopic methods, is sensitive enough to observe the difference in binding of the nucleotides.

It is significant that the ^{1}H nmr spectra of the 3′-CMP complex is *not* perturbed in the pH region where the phosphate is protonated ($pK \sim 4.4$ derived from the ^{31}P nmr study). This indicates that the dianionic and monoanionic states of the 3′-CMP inhibitor are bound in essentially the same site with similar environments for the pyrimidine and ribose rings. Therefore, the conclusions that we have reached regarding the structure of the dianionic inhibitor complexes are likely directed as well to the monoanionic diester substrates.

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[5]Ring strain provides about 5–6 kcal/mol of the 7.5 kcal/mol difference in activation energies between the cyclic and acyclic hydrolysis. (Covitz and Westheimer, 1963. See Gerlt and Westheimer (1973) for some qualifications to this ring strain value.)

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CORRECTION

"Variability in the Tertiary Structure of α-Chymotrypsin at 2.8-Å Resolution," by Alexander Tulinsky,* Richard L. Vandlen, Carl N. Morimoto, N. Venkit Mani, and Lynn H. Wright, Volume 12, Number 21, October 9, 1973, page 4185.

"Changes in the Tertiary Structure of α-Chymotrypsin with Change in pH; 4.2–6.7," by Richard L. Vandlen and Alexander Tulinsky,* Volume 12, Number 21, October 9, 1973, page 4193.

Due to a defective pH electrode, the pH values reported in these communications are high by about 0.7 pH unit. The conclusions drawn are not thereby affected since they were made independent of absolute pH values and were generally confined to pH regions. For further details, see page 3661 of this issue (August 27, 1974).