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References and Notes

(1) (a) University of California San Diego; (b) College of Staten Island, C.U.N.Y.
(2) Extensive recent reviews are available: (a) F. Naider and M. Goodman, *Biorg. Chem.*, **3**, 177 (1977); (b) R. T. Ingwall and M. Goodman, *Org. Chem. Ser. Two, 1976*, **6**, 153–182 (1976); (c) V. Hruby in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins", B. Weinstein, Ed., Marcel Dekker, New York, N.Y., 1974, pp 1–189; (d) M. Goodman, C. Toniolo, and F. Naider in "Peptides, Polypeptides and Proteins", E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, Ed., Wiley-Interscience, New York, N.Y., 1974, pp 308–319.
(3) The following abbreviations are used: ^{1}H NMR (proton magnetic resonance), CD (circular dichroism), IR (infrared), Boc (*tert*-butyloxycarbonyl), Met (methionine), Gly (glycine), OMe (methoxy), NH (amide), α-methine (α-CH), Fourier transform (FT).
(4) E. S. Pysh and C. Toniolo, *J. Am. Chem. Soc.*, **99**, 6211 (1977).
(5) (a) V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov, and Yu. A. Ovchinikov, *Tetrahedron*, **25**, 493 (1969); (b) J. L. Dimicoli and M. Ptak, *Tetrahedron Lett.*, **No. 23**, 2013 (1970); (c) M. Goodman, N. Ueyama, and F. Naider, *Biopolymers*, **14**, 904 (1975); (d) M. Goodman, N. Ueyama, and F. Naider, *ibid.*, **14**, 916 (1975).
(6) (a) M. Goodman, A. S. Verdini, C. Toniolo, W. D. Phillips, and F. A. Bovey, *Proc. Natl. Acad. Sci. U.S.A.*, **64**, 444 (1969); (b) P. A. Temussi and M. Goodman, *Ibid.*, **68**, 1767 (1971).
(7) Assignments for water-soluble, nonprotected homooligopeptides have been reported by (a) A. Nakamura and O. Jardetzky (*Biochemistry*, 7, 1226 (1968)) and (b) M. Sheinblatt (*J. Am. Chem. Soc.*, **88**, 2845 (1966)), who pioneered the determination of the amino acid sequences of water-soluble homodi- and tripeptides by pH titrations. M. Anteunis and J. Gelan (*ibid.*, **95**, 6502 (1973)) report that pH titrations at high fields (300 MHz) combined with lanthanide shift reagents allow sequential assignments for tetraalanine and pentaglycine. These methods do not appear usable for protected oligopeptides.
(8) The chemical syntheses of complete series of Boc-Met$_n$-OMe oligopeptides has been reported: (a) the mixed anhydride method to the nonamer by F. Naider and J. M. Becker (*Biopolymers*, **13**, 1011 (1974)) and (b) the acyl-azide method to the heptamer by F. M. Bonora and C. Toniolo (*ibid.*, **13**, 2179 (1974)). Toniolo and co-workers report that their Boc-Met$_n$-OMe oligopeptides are insoluble in $CHCl_3$ at the hexapeptide level.[14] We find limited solubility for the hexamer and heptamer in this solvent, and increased solubility with addition of Me_2SO-d_6.
(9) J. Champi, A. S. Steinfeld, J. M. Becker, and F. Naider, *Biopolymers*, in press.
(10) Note that the glycyl methylene protons are equivalent in Boc-Gly-Met$_5$-OMe, Boc-Met-Gly-Met$_4$-OMe, and Boc-Met$_4$-Gly-Met-OMe. Chemically distinct glycyl methylene protons are found in Boc-Met$_2$-Gly-Met$_3$-OMe, Boc-Met$_3$-Gly-Met$_2$-OMe, and Boc-Met$_5$-Gly-OMe.
(11) Our assignment of the lowest field α-CH resonance (4.61 ppm) to the C-terminal residue of the Boc-Met$_n$-OMe homooligopeptides (n = 2–7) agrees favorably with similar assignments made by Pysh and Toniolo for *dilute* norvaline dimers, trimers, and tetramers in $CDCl_3$.[4] Pysh and Toniolo make their assignment by noting an unusual downfield shift for the lowest field α-CH with temperature.[4]
(12) A. A. Ribeiro, M. Goodman, and F. Naider, unpublished work.
(13) A. Bundi, C. Grathwohl, H. [illegible]ckmann, R. M. Keller, G. Wagner, and K. Wüthrich, *J. Magn. Reson.* [illegible], 191 (1975).
(14) M. Palumbo, S. Da Rin, G. [illegible]onora, and C. Toniolo, *Makromol. Chem.*, **177**, 1477 (1976).
(15) A. A. Ribeiro, M. Goodman, and F. Naider, unpublished work.
(16) NOTE ADDED IN PROOF. Since this work was completed, we have synthesized the deuterated tripeptide, Boc-(L-Met)$_2$-L-α-deuterio-Met-OMe, and hexapeptide, Boc-(L-Met)$_5$-L-α-deuterio-Met-OMe. The NMR spectra of the trimer at 4.8×10^{-3} M and the hexamer at 2.2×10^{-3} M in $CDCl_3$ agree with the assignments obtained by the "guest–host" procedure.

Role of the Pyrimidine Base in Ribonuclease A Hydrolysis of RNA. Determination of the Conformation of Cyclic β-Cytidine 2',3'-Phosphate and Cyclic β-Uridine 2',3'-Phosphate in Solution

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Abstract: Ribonuclease A (RNase) cleaves ribonucleic acid at the 3'-pyrimidine nucleotide sites, producing 2',3' cyclic nucleotides which subsequently react with RNase to produce 3' nucleotides. The relative orientation of the pyrimidine base relative to the ribose moiety is an important aspect of the mechanism of cleavage and subsequent hydrolysis of the cyclic intermediates. Using NMR chemical-shift changes induced by the complexation of Pr^{3+} and Dy^{3+} to the phosphate group of cyclic β-cytidine 2',3'-phosphate (2',3'-cCMP) and cyclic β-uridine 2',3'-phosphate (2',3'-cUMP), the glycosyl angle, as defined by Donahue and Trueblood, is 120 ± 16° for 2',3'-cCMP and 116 ± 16° for 2', 3'-cUMP, both syn conformations. The broadening of ^{1}H resonances caused by Gd^{3+} is consistent with the angles deduced from shift data. Circular dichroism (CD) spectra and ^{1}H–^{1}H spin–spin coupling constants are the same for uncomplexed and complexed cyclic nucleotides, showing that complexation does not change their conformation. The constancy of the CD spectra at elevated temperatures (to 85 °C) shows a minimum rotation barrier for base rotation of 25 kcal/mol. Previously proposed mechanisms for the RNase-catalyzed hydrolysis of RNA have invoked the interaction of the C(2) carbonyl oxygen atom with the enzyme as a possible feature of the selection of 3'-pyrimidine nucleotides as cleavage sites as well as interaction of this atom with the phosphate group in the second step of the hydrolysis. The syn conformation of the pyrimidine bases and the high rotation barrier, however, strongly suggest that the role of the C(2) oxygen atom is not a decisive factor in the mechanism of RNA hydrolysis by RNase.

In the hydrolysis of RNA catalyzed by bovine pancreatic ribonuclease A (RNase), the cyclic pyrimidine nucleotides, cyclic β-cytidine 2',3'-phosphate (2',3'-cCMP) and cyclic β-uridine 2',3'-phosphate (2',3'-cUMP), are formed. Subsequently, RNase catalyzes the hydrolysis of these cyclic nucleotides specifically to the 3'-monophosphates.[1,2] Acidic or basic hydrolysis in the absence of enzyme leads to mixtures of the 2' and 3' isomers. The enzyme RNase has been particularly well studied,[3,4] including full sequencing[5] and structural determination via x-ray diffraction.[6] In view of the known structural detail of the enzyme, several proposals for the intimate mechanism of hydrolysis of the cyclic intermediates to the 3'-monophosphates have been suggested.[7-15] In these mechanisms, consideration is given to the role of the pyrimidine

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ring as a possible source of hydrogen-bonding interactions and as a binding site for enzyme interaction. Of importance in drawing such conclusions is the conformation of the cyclic nucleotide intermediates and the relative difficulty of altering the equilibrium conformation. The structures of 2',3'-cCMP and the closest analogue to 2',3'-cUMP which has been structurally characterized, uridine 2',3'-*O,O*-cyclophosphorothioate,[17] provide an excellent basis for the determination of the conformation of 2',3'-cCMP and 2',3'-cUMP in aqueous solution. We have analyzed these conformations using lanthanide-induced proton nuclear magnetic resonance chemical-shift and isotropic broadening techniques.

Circular dichroism (CD) spectra taken at various temperatures (5–85 °C) were used to investigate the barrier to rotation about the glycosyl bond. CD experiments were also done to ensure the equivalence of the conformations with and without bound lanthanide ions, and to verify that structures of the naturally derived molecules are the same as the structures found using synthetically cyclized nucleotides.

Experimental Section

Materials. The following compounds were purchased from the indicated companies: Sigma Chemical Co., 2',3'-cCMP, 2',3'-cUMP (Na salts), RNA from Torula yeast and ribonuclease A from bovine pancreas; Aldrich Chemical Co., gadolinium oxide (99.9%), praseodymium oxide (99.9%), lutetium oxide (99.9%) and D_2O (99.8%); Merck and Co., sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), an internal reference standard, and acetic-d_4 acid (99.5%).

The cyclic nucleotides were used at concentrations of 0.10 M in D_2O without further purification. Solutions of the rare earth ions were made by rotary evaporation from DCl solution and concentrations determined by titration with EDTA.[19] The pH of the cyclic nucleotide solutions containing rare earth ions was 3.0 ± 0.2, as determined using a Sargent pH meter, Model DR.

The cyclic nucleotides were also obtained from natural sources by the hydrolysis of RNA with ribonuclease A at 25 °C for 1.0 h. The cyclic nucleotides were isolated by dialysis with VWR-260 dialysis tubing followed by paper chromatography. The chromatography system used was Whatman No. 3 paper as the stationary phase and 2-propanol/water (70:30) as the eluent.[2]

Instrumentation. Circular dichroism spectra were obtained using a Cary Model 61 spectropolarimeter. ^1H NMR spectra were obtained on a JEOL 100-MHz spectrometer and on a Varian EM 360A spectrometer.

Lanthanide Shift Experiments. NMR spectra of the cyclic nucleotides were taken with ratios of lanthanide to cyclic nucleotide varying from 0 to 12. Chemical shifts were calculated relative to DSS for all protons.

The differences in chemical shifts ($\Delta\nu$) between the complexed and uncomplexed nucleotides were calculated and used to form ratios of the form $\Delta H(i)/\Delta H(1')$. These experimental shift ratios were compared to shift ratios calculated for possible cyclic nucleotide conformations. A computer program, BURLESK,[19] was used to compute possible conformations and to compare the calculated and experimental ratios. An agreement factor, R, was calculated using eq 1:

$$R = \left[\frac{\sum_i[(\Delta H/H)_{o,i} - (\Delta H/H)_{c,i}]^2}{\sum_i(\Delta H/H)_{o,i}{}^2}\right] \quad (1)$$

where $(\Delta H/H)_{o,i}$ are the experimentally observed shift ratios and $(\Delta H/H)_{c,i}$ are the calculated shift ratios.[20]

Results and Discussion

Requirements for Use of the Lanthanide Shift Techniques. The conformations of cyclic β-cytidine 2',3'-phosphate and cyclic β-uridine 2',3'-phosphate were determined in aqueous solution from lanthanide ion induced proton chemical-shift and broadening experiments. The addition of paramagnetic lanthanide ions to solutions of molecules containing protons causes shifts in the proton nuclear magnetic resonance frequencies. The magnitude of the shift experienced by protons in an axially symmetric complex due solely to pseudocontact interaction was expressed by Bleaney:[22]

$$\frac{\Delta H}{H_0} = g^2\beta^2\frac{J(J+1)(2J-1)(2J)}{60(kT)^2}(D_z)\left(\frac{3\cos^2\theta - 1}{r^3}\right) \quad (2)$$

where $D_z = \langle r_f{}^2\rangle\langle J|\alpha|J\rangle A_2°$ in which r_f is the electronic radius of the 4f electrons, $A_2°$ is an energy coefficient, θ is the angle between the magnetic anisotropy vector of the lanthanide ion and the nucleus whose shift is measured, r is the distance between the lanthanide ion and this nucleus, and the other symbols have their usual meanings. If there are several observable nuclei in the substrate which exhibit shifts due to the lanthanide ion, a ratio of chemical shifts using the above expression will give a simpler form containing only conformational parameters:

$$\frac{\Delta H(i)}{\Delta H(j)} = \frac{(3\cos^2\theta_i - 1)(r_j{}^3)}{(3\cos^2\theta_j - 1)(r_i{}^3)} \quad (3)$$

At a particular lanthanide ion concentration, the change in the proton resonance frequency relative to the resonance frequency of an internal standard (DSS) for each observable proton divided by the chemical-shift change for a normalizing proton (H(1') was chosen for this study) gives the set of experimental shift ratios. Equation 3 can be used in conjunction with atomic coordinates of the lanthanide-substrate complex to obtain sets of calculated shift ratios for various possible conformations. These sets of calculated shift ratios are compared to the set of experimental shift ratios. The solution conformation is defined when a good agreement factor, R, is obtained for a single set of calculated shift ratios.

Several aspects of the lanthanide-substrate interaction must be investigated before the use of eq 2 is justified. It is necessary to show: (1) there is a single predominant binding site of the lanthanide to the substrate, (2) the interaction between the lanthanide and the substrate is purely pseudocontact, and (3) the magnetic anisotropy axis is axially symmetric. Furthermore, the value of the determinations using the equation depends on whether: (1) the complexation of the lanthanide ion affects the conformation of the molecule of interest and (2) the experimental conditions necessary for the lanthanide shift experiment change the conformation of the molecule.

A hard acid such as lanthanide ion would be expected to bind to the phosphate group much more strongly than to any other group on the cyclic nucleotides, and various aspects of the experimental data support this intuitive argument. Chemical-shift data collected at pH 3.0 ± 0.2 in acetate-d_4 buffer, with praseodymium(III) and dysprosium(III), were analyzed according to the equation:[23]

$$K_{eq} = \delta\Delta/([Ln^{3+}]_{tot}\Delta^2 - [Nuc]_{tot}\delta\Delta - [Ln^{3+}]_{tot}\delta\Delta + [Nuc]_{tot}\delta^2) \quad (4)$$

where K_{eq} is the formation constant of the lanthanide–nucleotide complex, δ is the measured shift at a particular total lanthanide ion concentration ($[Ln^{3+}]_{tot}$) and a particular total nucleotide concentration ($[Nuc]_{tot}$), and Δ is the extrapolated shift for the fully complexed nucleotide. The best fit K_{eq} value for the complex at 2',3'-cCMP with Pr^{3+} determined by this analysis is 2.5 ± 1.4. Figure 1, drawn for $K_{eq} = 2.5$, illustrates that a single interaction predominates in the formation of this complex. The corresponding value for Pr^{3+} and 2',3'-cUMP is $K_{eq} = 6.7 \pm 1.7$. The same pattern of shifts is evident in each case where Dy^{3+} is used in place of Pr^{3+}, with Dy^{3+} causing larger shifts. These equilibrium constants are in very reasonable agreement with binding constants found for other lanthanide–nucleotide complexes. These values include: 6 ± 2 for binding of Ho^{3+} to 5'-AMP,[24] 10 ± 2 for Eu^{3+} and 5'-AMP,[24] 5.3 ± 1.4 for the binding of Pr^{3+} to 3',5'-cAMP,[19] 13.0 ± 1.7 for Ho^{3+} to 3',5'-cAMP or 3',5'-cGMP,[19] and 8.0 ± 0.6 for Pr^{3+} and 3',5'-cGMP.[19] Another indication of a single pre-

Table I. Shift Ratios of 2',3'-cCMP and 2',3'-cUMP Protons in the Presence of Pr^{3+} and Dy^{3+}

Nucleotide	Ln^{3+}	H(6)	H(5)	H(2' + 3')	H(4')	H(5')
2',3'-cCMP	Pr^{3+}	0.283 ± 0.003	0.106 ± 0.002	1.229 ± 0.005	1.148 ± 0.005	0.328 ± 0.002
	Dy^{3+}	0.282 ± 0.003	0.096 ± 0.002	1.300 ± 0.005	1.118 ± 0.005	0.360 ± 0.002
Calcd (120°)		0.282	0.092	1.246	1.163	0.403
2',3'-cUMP	Pr^{3+}	0.271 ± 0.003	0.078 ± 0.002	1.266 ± 0.005	1.189 ± 0.005	0.387 ± 0.002
	Dy^{3+}	0.272 ± 0.003	0.060 ± 0.002	1.300 ± 0.005	1.124 ± 0.005	
Calcd (116°)		0.274	0.091	1.246	1.163	0.403

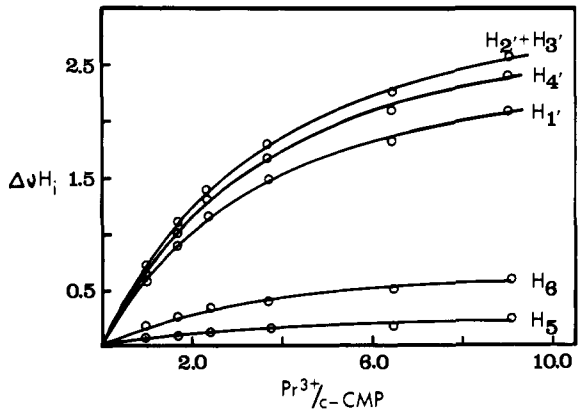

Figure 1. A plot of the shifts induced by the addition of Pr^{3+} solution as a function of the ratio of Pr^{3+} to 2',3'-cCMP.

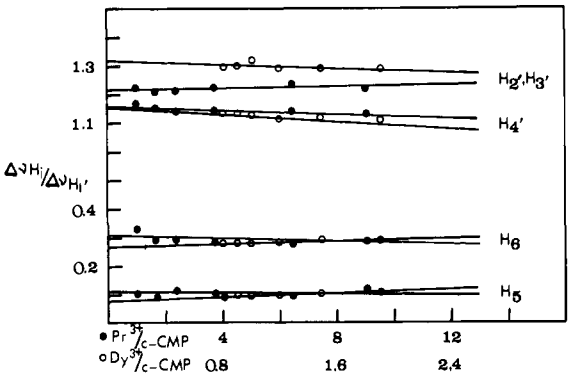

Figure 2. A plot of the ratios of the shifts induced by Pr^{3+} and Dy^{3+} for the observable protons relative to the shifts induced for H(1').

dominant binding site is the constancy of the shift ratios as the lanthanide ion concentration is increased. Table I gives the ratios for each proton of 2',3'-cCMP in the presence of Pr^{3+} and Dy^{3+}, and a plot of these data, Figure 2, illustrates the constancy of the shift ratios.

The agreement between the shift ratios shown in Figure 2 can also be used to indicate the fulfillment of requirement 2. The amount of contact contribution to the chemical shift can be investigated by using two lanthanide ions, whose contact interactions are of opposite sign. In this case Pr^{3+} and Dy^{3+} were used.[25] The fact that the shift ratios for these two ions were the same (the absolute magnitudes differ due to the J and D_z terms of eq 2, with Dy^{3+} giving much larger shifts) indicates that the contact interactions must not be very large. The absence of significant contact contribution to the shift is predicted since, in the case of protons, four bonds between a lanthanide ion and a proton are generally sufficient to eliminate most contact interaction, and the 2',3' cyclic nucleotides have a minimum of five such bonds. A good fit of the calculated ratios for a set of protons whose relationships are well defined is an indication of compliance with the third requirement. The good agreement found for the chemical-shift ratios of the five ribose protons to the predicted ribose structure (calculated agree with experimental within ±6%) supports an axially symmetric magnetic anisotropy axis. Although crystal structures of lanthanide complexes of molecules show they are generally not axially symmetric in the solid state, it is apparent from the excellent agreement of the axially symmetric model in many studies of relatively rigid molecules that rearrangement in solution is capable of producing time-averaged axial symmetry.[26-30] This feature is again verified by our data.

Ensuring that the binding of the lanthanide or the experimental conditions used (eg., pH and temperature) do not change the conformation from that of the molecule under physiological conditions is less straightforward. Discussion of the application of NMR and CD techniques in this regard is delayed until these results have been presented.

Conformation of Synthetic Cyclic Nucleotides. The solution conformation of the lanthanide-cyclic nucleotide complex is defined by: the direction and degree of puckering of the ribose ring, the orientation of the phosphodiester ring, the relative populations of the conformers existing for rotation about the C(4')-C(5') bond, and the orientation of the pyrimidine base with respect to the ribose ring.

The crystal structure of 2',3'-cCMP,[16] the crystal structure of a close analogue of 2',3'-cUMP, 2',3'-*O,O*-cyclophosphorothioate,[17] and the assumed binding of the lanthanide ion to O(6) and O(7) of the phosphodiester ring serve as a basis for obtaining calculated shift ratios, and therefore the solution conformation. The range of fluctuations possible for the ribose and phosphodiester rings is indicated by the crystal structures, and by molecular models. Due to the rigidity imposed on the ribose ring by the phosphodiester ring, O(1') is the only atom found to pucker in the solid state.[16,17] In one form of the cCMP anion (anion A),[16] O(1') is displaced 0.48 Å endo; in the other form (anion B), O(1') is 0.03 Å exo; in the analogue of cUMP,[17] O(1') is 0.29 Å exo. This displacement is an important aspect of the conformation since the degree and direction O(1') is displaced affect the distance between the pyrimidine ring and the C(5') carbon. The orientation of the phosphodiester group can be described by the angle between the plane defined by C(2')-C(3')-O(3')-O(2') and the plane O(2')-P-O(3'). This angle in the solid state varies from 11.6° exo in the 2',3'-*O,O*-cyclophosphorothioate to 26.1° endo in anion B of 2',3'-cCMP, where exo refers to the P atom being displaced away from the ribose ring (the phosphorus atom is on the same side of the C(2')-C(3')-O(3')-O(2') plane as H(2') and H(3')).

The atomic coordinates from each crystal structure were used to calculate coordinates for the lanthanide ion, allowing the phosphate of the phosphodiester to vary from 26° exo to 26° endo, and allowing the Ln-P distance to vary from 2.4 to 2.9 Å. The best fit of experimental shift ratios with calculated shift ratios is observed using the atomic coordinates of anion A and an Ln-P distance of 2.5 Å. The protons H(1') and H(4') of the ribose are the most sensitive probes to the degree of O(1') displacement, and the agreement is good for these protons with O(1') endo 0.48 Å (within 5% except H(5')), and poor using the planar ribose coordinates (>10%), or coordinates placing O(1') exo (>15%). An endo displacement at 0.48 Å places the phosphodiester ring 22° endo, as found for anion A.[16]

Table II. Calculated Shift Ratios of Various Possible Glycosyl Angles for H(5) and H(6) Relative to H(1′)

Glycosyl angle, deg	H(5)	H(6)
24	0.148	0.312
84	0.100	0.245
100	0.093	0.250
108	0.092	0.259
112	0.091	0.266
116	0.095	0.274
132	0.107	0.320
148	0.130	0.388
168	0.178	0.492
200	0.214	0.628
228	0.231	0.659
264	0.322	0.599
296	0.222	0.512
344	0.186	0.391
360	0.171	0.357

In the case of the ribose pucker, solution studies of these molecules employing ^1H–^1H coupling constant measurements indicate a slight shift of C(3′) to an endo conformation.[31] Due to the closeness of the H(2′) and H(3′) resonance frequencies to one another, and to the sensitivity of their calculated ratios to the lanthanide–cyclic phosphate bond distance, resolution of this displacement is not possible using our shift data. However, a larger chemical-shift change for H(3′) than for H(2′) at high Dy^{3+} concentrations indicates a shift of C(3′) to an exo conformation.

Relative populations of the various conformations available to the exocyclic C(5′) H_2OH group have been previously determined using H^1–H^1 coupling constants.[32] The similarity of the coupling pattern for H(4′) and the two H(5′) protons with and without lanthanide ion indicates that these populations are not significantly altered by lanthanide complexation.

Using the anion A crystal-structure coordinates and the best fit lanthanide ion position, shift ratios were calculated for the protons on the pyrimidine base as the base was rotated by 4° increments about the C(1′)–N(1) glycosyl bond. The calculated shift ratios for various possible glycosyl angles are shown in Table II. Comparison with experimental shift ratios indicated that the best fit glycosyl angle for 2′,3′-cCMP is ϕ_{CN} = 120 ± 16°. The R value for ϕ_{CN} = 120° is 0.06, and the agreement between the calculated and experimental shift ratios for H(6) is within 1% at 112° and greater than 10% outside the stated limits. The best fit glycosyl angle of 2′,3′-cUMP has an R value of 0.05, ϕ_{CN} = 116 ± 16°. The calculated and experimental shifts for H(6) again are in agreement within 1% at the best fit angle and differ by greater than 10% outside the stated limits. The lower limit for the glycosyl angle is also required by the presence of van der Waals' contacts between the C(2) carbonyl oxygen and the H(2′) proton. It is of interest to compare these values to the glycosyl angles found for the molecules in the solid state; 2′,3′-cCMP has ϕ_{CN} = 112° in the syn region and 2′,3′-*O*,*O*-cyclophosphorothioate has ϕ_{CN} = 12.9° in the anti conformation.

In addition to the use of lanthanide-induced shifts, information about the conformation can also be obtained by the addition of the isotropic line broadening lanthanide ion, gadolinium(III). The effect of the addition is an increase in the relaxation rate of protons depending on the inverse sixth power of the protons' distance from the gadolinium ion.[24c] The width of proton signals at half-height can be used as a measure of the relaxation rate and thus of lanthanide–proton distances. Ratios of the amount of broadening of the cyclic nucleotide resonances in the presence of the isotropic lanthanide ion, Gd^{3+}, are shown in Table III, along with values calculated for possible particular conformations of the pyrimidine base about the glycosyl bond.

Table III. Experimentally Observed Broadening Ratios and Broadening Ratios Calculated for Various Possible Glycosyl Angles for H(5) and H(6) Relative to H(1′)

Exptl broadening ratios	Glycosyl angle, deg	Calcd broadening ratios	
		H(5)	H(6)
2′,3′-cUMP	24	0.02	0.08
H(5) 0.06 ± 0.01	84	0.03	0.16
H(6) 0.20 ± 0.03	100	0.03	0.20
	108	0.03	0.22
2′,3′-cCMP	112	0.03	0.24
H(6) 0.32 ± 0.02	116	0.04	0.25
	132	0.04	0.29
	148	0.04	0.32
	168	0.04	0.34
	200	0.04	0.28
	228	0.04	0.20
	264	0.03	0.12
	296	0.02	0.09
	344	0.02	0.07
	360	0.02	0.07

The conformations deduced from broadening experiments are in agreement with shift experiment conformations. The anomalous broadening of the H(5) on the cytidine ring reported by Chan et al.[33] was interpreted in terms of some binding of a paramagnetic ion to the amine group. Since the r^{-6} dependence of the broadening makes this parameter more sensitive than the shift ratios to the presence of small amounts of Gd^{3+} binding to a second site on the molecule, a similar situation may apply here.

Effect of Lanthanide Binding on Conformation. Conformations determined by the complexation of lanthanide ions have biological significance only if the conformation of the molecule can be shown to be the same in the complexed and uncomplexed biological form. The equivalence of these two conformations for the cyclic nucleotides can be tested by separate techniques for two portions of the molecule. The absence of conformational change in the ribose upon complexation can be deduced from proton-proton coupling constants. Although it is not always possible to calculate dihedral angles directly from coupling constants, there is general agreement with the premise that conformational changes altering dihedral angles between protons will also cause changes in the coupling constants. The proton-proton coupling constants for the ribose protons are not changed by the addition of the nonshifting, nonbroadening lutetium(III) ion under conditions in which the complexation is calculated (using the equilibrium constant values for Pr^{3+}) to be greater than 30% complete. Hence, the ribose unit conformation is unchanged by lanthanide ion binding. Two other interesting features arise from the constancy of the coupling pattern. First, the ^{31}P–^1H coupling constants are also unchanged, showing that the relationship between the phosphate group and the ribose is unchanged by lanthanide complexation. Secondly, the coupling constants between H(4′) and the two H(5′)'s are determined by the amounts of the gauche–gauche, gauche–trans, and trans–gauche rotamers of the C(5′) H_2OH group. Since they are also unchanged, the rotamer distribution is not affected by lanthanide ion complexation.

A different experiment is necessary to demonstrate that complexation does not alter the position of the pyrimidine base about the glycosyl bond, since there are no coupling constants observed between the pyrimidine ring protons and the ribose protons. The pyrimidine nucleotides exhibit circular dichroism spectra which arise from the dissymetric environment of the pyrimidine base chromophore and which should be very sensitive to the disposition of the base relative to the ribose ring. The fact that the CD spectra of 2′,3′-cCMP and 2′,3′-cUMP

are not markedly changed by complexation with Dy^{3+} (Figure 3) substantiates the assumption that lanthanide-induced shift and broadening results can be used to determine the conformations of these nucleotides when they are not complexed by lanthanide ions.

Size of Barrier of Base Rotation. To apply the solution conformation of 2',3'-cCMP and 2',3'-cUMP to the deduction of the RNase hydrolysis mechanism requires knowledge of the magnitude of the barrier to rotation about the glycosyl bond, i.e., on how easy it is to convert from the syn conformation to the anti. In the case of 3',5'-cAMP, for example, there are two types of molecules in the solid state; one has the anti conformation, and the other is syn.[34] However, only the syn form is found in solution at pD = 5.3 and 29 °C.[19] Since the syn form can convert to the anti form on crystallization, the glycosyl rotation barrier is low enough that either form could be proposed as a substrate for an enzyme. This deduction is based on the observation that enzyme–substrate interactions are often similar in nature to crystal-packing interactions, so that conformational changes brought about by crystallization (such as the syn–anti rotation of 3',5'-cAMP) may also be brought about by the binding of a substrate to an enzyme.

We were interested in determining if the interconversion for the 2',3' cyclic pyrimidines would be more difficult than for 3',5'-cAMP, as the relative rigidity of two fused five-membered rings and the proximity of H(2') and the C(2) carbonyl oxygen atom in the solid state indicate.[16] The sensitivity of CD to conformational change was again used. The absence of change in the CD spectra of 2',3'-cUMP and 2',3'-cCMP after 15 min at a series of temperatures up to 85 °C allows a minimum barrier to rotation of 25 kcal/mol to be calculated. Furthermore, neglecting conformations which require contact at less than the van der Waals radii, the shift ratios for H(5) and H(6) are minimum values for the best fit glcosyl angle, thus fortuitously removing the possibility of the experimentally determined glycosyl angle being an average of two or more conformations.

The sensitivity of CD to conformational changes was also used to determine the possible effect of the low pH, which was employed due to insolubility of the lanthanide complexes at high pH. The absence of change in the CD spectrum of 2',3'-cUMP as a function of pH is strong evidence that the conformation determined at pH 3.0 also exists at biological pH. The fact that the molar ellipticity change for 2',3'-cCMP has the form of a titration curve indicates a change in the electronic wave functions involved in the transitions due to protonation. To support this conclusion, nuclear Overhauser experiments were performed on 2',3',-CMP at pH 3.0 and 7.0. Irradiation of H(6) causes an enhancement of 30% for H(1') at pH 3.0 and an enhancement of 27% at pH 7.0, indicating that the changes in the CD spectra are not due to a change in the conformation of the base about the glycosyl bond, but are rather due to protonation of the pyrimidine ring (pK_a = 4.3).

Correlations of CD Spectra and Conformation. The determination of the conformation of these nucleotides in solution by NMR techniques allows comment on conclusions drawn from CD spectra by earlier workers. Interest in the conformation of nucleosides and nucleotides has led investigators to attempt correlating the sign and magnitude of transitions in the CD and ORD spectra with the relative orientation of the pyrimidine ring and the ribose ring, even though CD and ORD are unable to distinguish between weighted average positions and single conformers.

CD data on a large variety of cytidine and uridine derivatives reported by Miles and his colleagues[35] were used to construct a diagram giving the molecular ellipticity of the long wavelength (B_{2u}) transition as a function of the torsion angle, ϕ_{CN}. The diagrams constructed assumed the preferred conformation of the β anomers of cytidine and isopropylidenecytidine to be

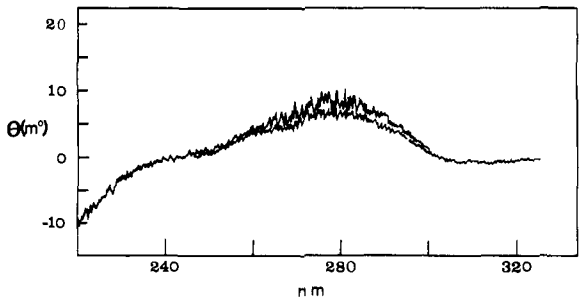

Figure 3. Circular dichroism spectra: upper spectrum, [2',3'-cCMP] = 1.6 mM; lower spectrum, [2',3'-cCMP] = 1.6 mM; $[Dy^{3+}]$ = 0.5 M.

in the anti range ($\phi_{CN} = -30 \pm 30°$) in H_2O, while isopropylidenecytidine in dichloroethane and 6-methyluridine and 6-methylcytidine were assumed to be in the syn conformation. The diagram predicts an anti conformation for the β anomers of cytidine and uridine derivatives exhibiting a positive ellipticity for the B_{2u} transition and a syn conformation for molecules exhibiting a negative ellipticity for the long-wavelength transition. The CD spectrum of 2',3'-*O*-isopropylidene-O^2-5'-cyclouridine exhibits a positive peak corresponding to the B_{2u} transition whereas the base is in a syn conformation. These data were discussed by the authors and their usefulness questioned due to the considerable torsional strain encountered for molecules of this type.

Hart and Davis[36] reported nuclear Overhauser effect (NOE) measurements of isopropylidenecytidine and isopropylideneuridine indicating a predominance of the syn conformer in H_2O and in Me_2SO, although both of these molecules exhibit positive peaks for the long-wavelength CD transition. They suggested that syn rotamers are characterized by a smaller ellipticity for this transition than the anti rotamers.

The positive ellipticity of the B_{2u} transitions found for 2',3'-cCMP and 2',3'-cUMP in conjunction with the syn conformation and high barrier to rotation for these molecules makes it necessary to reconsider the validity of the assumptions used in deriving Miles's diagram. The magnitude of the B_{2u} transition in comparison to that found for the isopropylidene derivatives is consistent with the hypothesis of Hart and Davis.

The Mechanism of Enzymatic Hydrolysis. The hydrolysis of ribonucleic acid by pancreatic ribonuclease normally occurs only if the 3' nucleotide has a pyrimidine base. In the hydrolysis of other polymeric 3',5' diesters of nucleosides, those containing pyrimidine bases in the 3' position are hydrolyzed at much faster rates than other 3',5' nucleoside diesters. Furthermore, of the cyclic nucleotides, only those which contain pyrimidine bases or very closely related analogues are hydrolyzed to the 3'-phosphate monoesters. The specificity of ribonuclease is attributable either to increasing the concentration of the moiety containing pyrimidine at the active site by a specific binding, or to a specific catalysis by the base. Our finding that 2',3'-cCMP and 2',3'-cUMP are in the syn conformation relates to the controversy concerning the enzyme's specificity. Witzel[13] proposed catalysis involving the pyrimidine ring via a hydrogen-bonding interaction between the C(2) carbonyl oxygen of the base and the oxygen bonded to C(2') of the ribofuranose ring. This proposal has been questioned by various studies, and the arguments for and against the involvement of O(2) have been discussed by Richards and Wyckoff.[4] Since our studies show both cyclic nucleotides in the syn conformation with a large barrier to rotation about the glycosyl bond, the C(2) carbonyl oxygen is not positioned for the proposed hydrogen-bonding interaction, eliminating this feature of the mechanism of hydrolysis. Since our data show that the specificity of the enzyme reaction cannot be related to the nucleophilicity of

O(2), the explanation for the difference in rates of hydrolysis between the various substrates must be based on the variation of the substrate position and orientation. Since the K_m values for 2′,3′-cCMP and 2′,3′-cUMP hydrolysis by RNase A (3.4 and 5.0 mM, respectively[13]) and their hydrolysis rates, k_2 (5.55 and 2.25 s^{-1} at 27 °C and pH 7.0, respectively[13]), are so similar, it seems reasonable these differences can arise from the different C(4) substituents and pK_a values of N(3) for 2′,3′-cCMP and 2′,3′-cUMP. While the NH_2 and C=O groups may be hydrogen bonded to the same group in RNase, and N(3) of each cyclic nucleotide may interact with the same residue, the strength of these interactions would not be expected to be the same. It would be interesting to obtain activation parameters for the enzymatic hydrolysis so that more light could be shed on the intimate mechanism.

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References and Notes

(1) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).
(2) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).
(3) C. B. Anfinsen and F. H. White, Jr. *Enzymes, 2nd Ed.*, **5**, (1960).
(4) F. M. Richards and H. W. Wyckoff, *Enzymes, 3rd Ed.*, **4**, (1971).
(5) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 227 (1963).
(6) G. Kartha, J. Bello, and D. Harker, *Nature (London)*, **213**, 862 (1967).
(7) D. Findlay, D. G. Herries, A. P. Matthias, B. R. Rabin, and C. A. Ross, *Nature (London)*, **190**, 781 (1961).
(8) D. Findlay, D. G. Herries, A. P. Matthias, B. R. Rabin, and C. A. Ross, *Biochem. J.*, **85**, 152 (1962).
(9) A. Deavin, A. P. Matthias, and B. R. Rabin, *Biochem. J.*, **101**, 14c (1966).
(10) D. A. Usher, *Proc. Natl. Acad. Sci. U.S.A.*, **62**, 661 (1969).
(11) D. A. Usher, D. I. Richardson, Jr., and F. Eckstein, *Nature (London)*, **228**, 663 (1970).
(12) J. H. Wang, *Science*, **161**, 328 (1968).
(13) H. Witzel, *Prog. Nucl. Acid Res.*, **2**, 221 (1963).
(14) G. C. K. Roberts, E. A. Dennis, D. H. Meadows, J. S. Cohen, and O. Jardetsky, *Proc. Natl. Acad. Sci. U.S.A.*, **62**, 1151 (1969).
(15) G. G. Hammes, *Acc. Chem. Res.*, **1**, 321 (1968).
(16) C. L. Coulter, *J. Am. Chem. Soc.*, **95**, 570 (1973).
(17) W. Saenger and F. Eckstein, *J. Am. Chem. Soc.*, **92**, 4712 (1970).
(18) The disparity of the glycosyl angle found in 2′,3′-cCMP (syn, ref 16) and in the analogue of 2′,3′-cUMP, which is 2′,3′-*O*,*O*-cyclophosphorothioate (anti, ref 17), which are cyclized by different methods, suggested the possibility that the conformation would be dependent on the synthetic method of cyclization. Since all NMR experiments were carried out using synthetically cyclized nucleotides, we determined if molecules formed by the hydrolysis of RNA by pancreatic RNase have the same conformation as the synthetic materials. The exact correspondence of the CD spectra of synthetic and natural 2′,3′-CMP and of synthetic and natural 2′,3′-UMP verifies glycosyl conformational similarity.
(19) D. K. Lavallee and A. H. Zeltman, *J. Am. Chem. Soc.*, **96**, 4712 (1974).
(20) H. E. Bosshard, C. D. Barry, J. M. Fritsch, R. A. Ellis, and E. R. Marshall, *Proc. Summer Comput. Simulation Sci. Conf.*, 581 (1972). Program kindly provided by L. O. Ford, University of Oxford.
(21) M. R. Willcott, R. E. Lenkinski, and R. E. Davis, *J. Am. Chem. Soc.*, **94**, 1742 (1972).
(22) B. Bleaney, *J. Magn. Reson.*, **8**, 91 (1972).
(23) R. H. Moore and R. K. Ziegler, Los Alamos Scientific Laboratory Report LA 2367, Los Alamos, N. Mex., 1959.
(24) (a) C. D. Barry, J. A. Glasel, A. C. T. North, R. J. P. Williams, and A. V. Xavier, *Biochim. Biophys. Acta*, **262**, 101 (1972); (b) *Biochem. Biophys. Res. Commun.*, **47**, 166 (1972); (c) *Nature (London)*, **232**, 236 (1971).
(25) W. B. Lewis, J. A. Jackson, J. F. Lemons, and H. Taube, *J. Chem. Phys.*, **36**, 694 (1962).
(26) J. Briggs, F. A. Hart, and G. P. Moss, *Chem. Commun.*, 1506 (1970).
(27) R. Caple, D. K. Harriss, and S. K. Kuo, *J. Org. Chem.*, **38**, 381 (1973).
(28) R. M. Wing, J. J. Uebel, and K. K. Andersen, *J. Am. Chem. Soc.*, **95**, 6046 (1973).
(29) S. Farid, A. Ateya, and M. Maggio, *Chem. Commun.*, 1285 (1971).
(30) G. E. Hawkes, D.Leibfritz, D. W. Roberts, and J. D. Roberts, *J. Am. Chem. Soc.*, **95**, 1659 (1973).
(31) R. D. Lapper and I. C. P. Smith, *J. Am. Chem. Soc.*, **95**, 2880 (1973).
(32) D. K. Lavallee and C. L. Coulter, *J. Am. Chem. Soc.*, **95**, 576 (1973).
(33) M. Pieber, P. A. Droon, J. H. Prestegard, and S. I. Chan, *J. Am. Chem. Soc.*, **95**, 3408 (1973).
(34) K. Watenpaugh, J. Dow, L. H. Jensen, and S. Furberg, *Science*, **159**, 206 (1968).
(35) B. W. Miles, M. J. Robins, R. K. Robins, M. W. Windley, and H. Eyring, *J. Am. Chem. Soc.*, **91**, 831 (1969).
(36) P. A. Hart and J. P. Davis, *J. Am. Chem. Soc.*, **93**, 753 (1971).

^{13}C NMR Conformational Studies of Oxytocin Analogues with a Prolyl Residue in Position 3

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Abstract: Carbon-13 NMR chemical shifts and spin-lattice relaxation times (T_1) of [Pro³,Gly⁴]-oxytocin and oxytocin are compared. Assignments were also made for [Pro³]-oxytocin and [Leu²,Pro³,[α,α-²H₂]Gly⁴]-oxytocin. These studies have shown that introduction of a prolyl residue in position 3 of the cyclic portion of oxytocin causes conformational heterogeneity in the peptide backbone of the 20-membered covalent ring moiety as a consequence of cis–trans isomerism about the Tyr-Pro peptide bond. The amount of cis isomer is larger in the more sterically crowded [Pro³]-oxytocin (45%) when compared with [Pro³,Gly⁴]-oxytocin (33%). Introduction of the Pro-Gly sequence in positions 3 and 4 of oxytocin, which is thought to favor formation of a β turn, results in shorter T_1 values for the α carbons in [Pro³,Gly⁴]-oxytocin than for the same carbons in oxytocin under analogous experimental conditions, but it does not eliminate the intrinsic flexibility of the α carbon of the glycyl-4 residue. The rotational correlation times for the linear portion of the peptide backbone (residues 7 through 9) of [Pro³,Gly⁴]-oxytocin and oxytocin are similar, which may be due to dominance of the T_1 behavior by segmental motion of the tripeptide.

In continuing studies dedicated to understanding the physico-chemical properties of peptides and peptide hormones, we have been using carbon-13 nuclear magnetic resonance (^{13}C NMR) to elucidate structural and conformational characteristics of these compounds.[2] The chemical shifts of carbon-13 are sensitive to the time-averaged conformation of peptides. If conformers are interconverting slowly on the NMR time scale, separate resonances for each conformer can be observed. Carbon-13 spin–lattice relaxation times (T_1) can provide information on the relative conformational flexibility of back-

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