Conformational Study of Cyclic Nucleotides. Lanthanide Ion Assisted Analysis of the Hydrogen-1 Nuclear Magnetic Resonance Spectra[1]

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Abstract: ^{1}H NMR spectra of five 3′,5′-cyclic ribonucleotides with different bases [adenine (I, 3′,5′-cAMP), urasyl (II, 3′,5′-cUMP), cytosine (III, 3′,5′-cCMP), guanine (IV, 3′,5′-cGMP), hypoxanthine (V, 3′,5′-cIMP)] 3′,5′-cyclic deoxyribosylthymidine monophosphate (VI, 3′,5′-cTMP), and 2′,5′-cyclic arabinosylcytidine monophosphate (VII, 2′,5′-ara-cCMP) were analyzed using lanthanide ions as shift reagents. The preferred conformation of each these molecules was determined from the vicinal proton–proton and proton–phosphorus coupling constants. Thus the conformation of the sugar moieties was found to be 3′-endo for I–V and the conformation of the ribose phosphate ring of these 3′,5′-cyclic nucleotides was essentially the same regardless of the type of base. An unusual long-range phosphorus–proton coupling (0.7–0.9 Hz) across five single bonds was observed for the C_1′H of all of I–V. The structural implication of these couplings is given. The conformation of 3′,5′-cTMP (VI) was confirmed to be 4′-exo as reported previously. The conformational difference between the ribo- (I–V) and the deoxyribo- (VI) nucleotides is discussed in terms of the stereoelectronic effect of the C(2′) hydroxyl group. The complete analysis of the ^{1}H NMR spectra of VII led us to propose a preferred conformation in which the arabinose moiety adopted a 2′-endo conformation. An unexpectedly large vicinal phosphorus–proton coupling (31 Hz) between the phosphorus and one of the C(5′) protons was observed. This questions the reliability of the Karplus-type relation of the vicinal phosphorus–proton coupling constants vs. the dihedral angles.

An increasing interest in elucidating the biological roles of various cyclic nucleotides[2-4] has led to comparative conformational studies of these molecules whose chemical structures differ. The crystal structures of some cyclic nucleotides have been determined;[5-8] however, the structures in solutions, specifically considering the influence of the base on the solution conformation, are not yet clear.

An extensive ^{13}C NMR study of 3′,5′-cyclic nucleotides was reported by Lapper et al.[9] However, the conformational information available through ^{13}C NMR, that is, the chemical shifts and the three-bond ^{13}C–^{31}P coupling constants (J_{POCC}), has been shown to be less reliable than that available from ^{1}H NMR data.[9] Attempts to analyze the ^{1}H NMR spectra of these compounds were severely hampered by the extensive overlap of lines, and so far only the ^{1}H NMR spectra of 3′,5′-cAMP (I) and 3′,5′-cTMP (VI) have been successfully analyzed.[10] In doing so, Blackburn et al. concluded that the six-membered phosphate containing rings were in the chair conformation, and the ribose rings were described as the 3′-endo-4′-exo for I and as the 4′-exo for VI.[10] Barry and coworkers also studied the conformation of I using lanthanide ion induced chemical shifts and relaxation times.[11] They concluded, in agreement with previous results, that the probable conformation was a 3′-endo type. The evidence from which the probable conformations were derived was, however, not very convincing, especially in view of the rather large scattering in the shift ratios obtained for I (ref 11; Table I), which form the basis of the calculations. We feel that, in the present time, the understanding of the mechanism whereby the shifts are induced by the lanthanide reagent is not good enough to allow extensive analysis of conformation of complex molecules without a series of controversial assumptions.[12] Because of this a straightforward analysis is probably somewhat more reliable. In this paper, we analyze the ^{1}H NMR spectra of five different 3′,5-cyclic ribonucleotides, as well as 3′,5′-cTMP, and emphasize that shift reagents were employed only as a means of increasing the chemical shift separation so that each proton could be analyzed in detail. Still, the questions of whether the conformations or the coupling constants are changed in the presence of shift reagents remain to be considered, as does the effect of the very low pH needed to keep the components in solution. These questions will be discussed presently.

Experimental Section

3′,5′-Cyclic nucleotides were obtained from Boehringer Mannheim Biochemicals and from Sigma Chemical Co. 2′,5′-Ara-cCMP was kindly supplied by Dr. W. J. Wechter of the Upjohn Co. Europium and praseodimium nitrate were obtained from Aldrich Co. All were used as received. The nucleotides were dissolved in D_2O at a concentration of 0.2 *M* with a small amount of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. The pD (uncorrected meter reading) was adjusted at first to 1.0, so as to avoid a precipitate forming on the addition of lanthanide nitrate. Successive portions of 0.1 *M* lanthanide salt were then added directly to the solutions in the NMR tube, until we got a good separation of every peak, and after that the pD was readjusted to 1.0 and the spectra were recorded.

^{1}H NMR spectra were run on Varian XL-100-FT and HR-220 spectrometers. Spectrum simulations were done on a Varian 620-L (16K) computer using the program supplied by Varian. An iterative analysis of the 3′,5′-cTMP spectrum was performed by the LAOCN-3 program as an eight-spin system.

Results and Discussion

Conformation of 3′,5′-Cyclic Mononucleotides. The well-separated ribose portion of the ^{1}H NMR spectra of I–V is illustrated by the series of spectra in Figure 1. These spectra were obtained by successive addition of small portions of lanthanide salt until well-resolved lines were observed. First-order analysis patterns are given on the basis of ^{1}H-{^{31}P} and ^{1}H-{^{1}H} experiments and confirmed by computer simulation of the spectra. The spin–spin coupling constants obtained are listed in Table I. These coupling constants did not depend on the lanthanide ion concentrations, which imply that the conformation of the ring systems and the coupling constants are insensitive to the complex formation.[13] The coupling constants for I are generally in good agreement with those measured by Blackburn et al. without shift reagent.[10] This supports our suggestion that the ribose ring conformation does not change upon complexing with the lanthanide ion. As their spectra were obtained at neu-

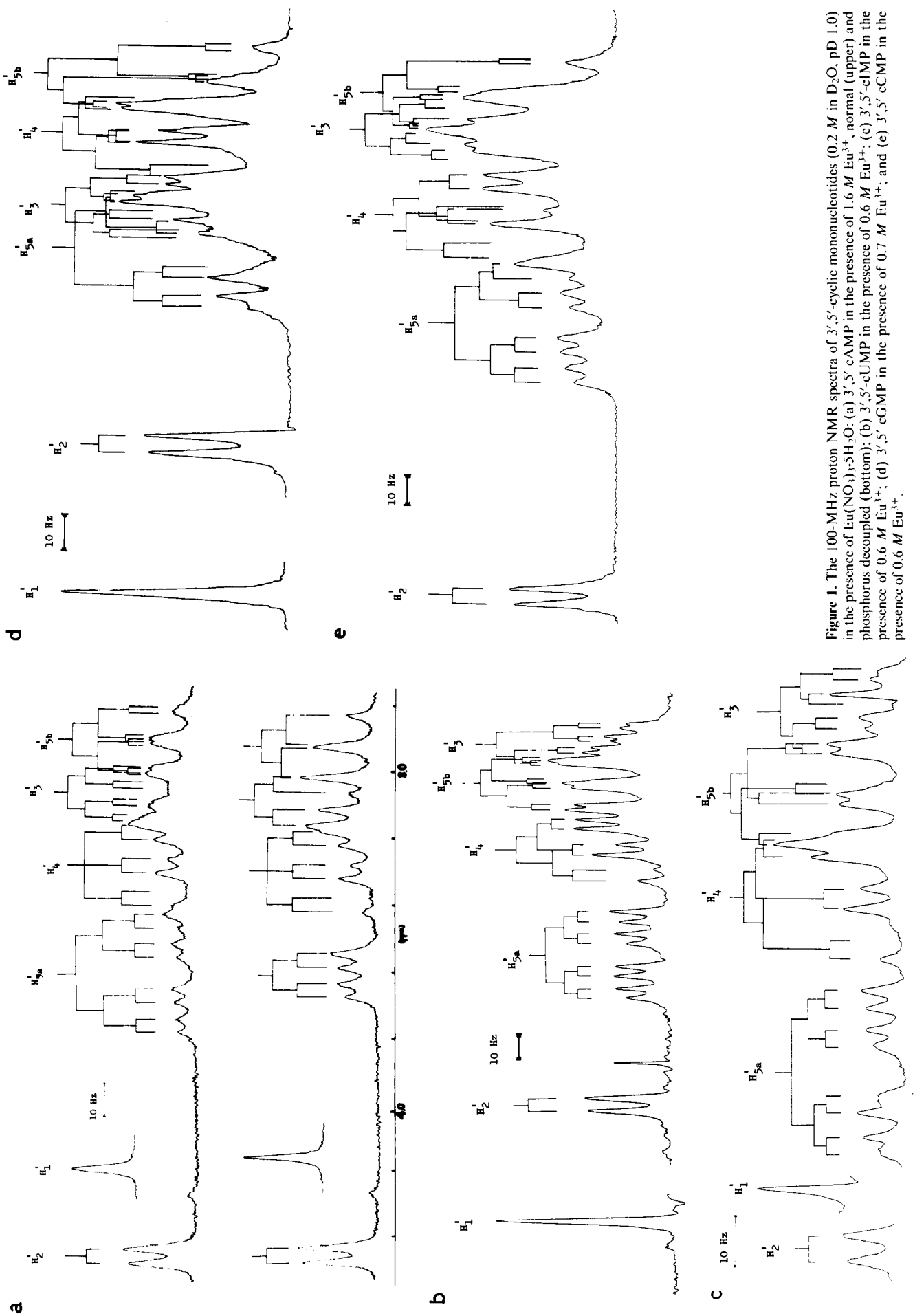

Figure 1. The 100-MHz proton NMR spectra of 3′,5′-cyclic mononucleotides (0.2 *M* in D_2O, pD 1.0) in the presence of $Eu(NO_3)_3 \cdot 5H_2O$: (a) 3′,5′-cAMP in the presence of 1.6 *M* Eu^{3+}, normal (upper) and phosphorus decoupled (bottom); (b) 3′,5′-cUMP in the presence of 0.6 *M* Eu^{3+}; (c) 3′,5′-cIMP in the presence of 0.6 *M* Eu^{3+}; (d) 3′,5′-cGMP in the presence of 0.7 *M* Eu^{3+}; and (e) 3′,5′-cCMP in the presence of 0.6 *M* Eu^{3+}.

Table I. Spin–Spin Coupling Constants for Various Cyclic Nucleotides Obtained from Lanthanide Shifted Spectra

Nuclei (*i*, *j*)	$J_{i,j}$ ($\phi_{i,j}$)[a]	cAMP I	cUMP II	cCMP III	cGMP IV	cIMP V	$J_{i,j}$ ($\phi_{i,j}$)[b]	cTMP VI	$J_{i,j}$ ($\phi_{i,j}$)[c]	ara-cCMP VII
1′, 2′[d]	0 (100)	0.4[f]	0.1[f]	0.1[f]	0.3[f]	0.1[f]	2.3 (120)	2.4 [2.7][h]		
1′, 2″[d]							9.0 (0)	8.9 [8.6]	3.6 (50)	3.2[i]
2′, 2″								−13.0 [−12.7]		
2′, 3′	5.2 (40)	4.7	5.0	4.5	4.7	5.0	6.7 (30)	8.0 [8.3]		
2″, 3′							7.5 (150)	9.9 [9.4]	0 (80)	0
3′, 4′	9.8 (170)	9.9	9.8	10.0	9.1	9.8	9.8 (170)	9.0 [9.0]	−0.3 (90)	0
4′, 5′a[e]		4.7	3.5	3.8	3.0	3.4		4.8 [4.7]		3.1
4′, 5′b[e]		10.5	9.8	9.5	9.0	9.8		9.7 [9.8]		0
5′a, 5′b		−9.6	−8.4	−8.5	−8.0	−7.8		−9.8 [−9.7]		−13.0
1′, P		0.8[g]	0.8[g]	0.7[g]	0.7[g]	0.9[g]		0 [0]		0
2″, P		0	0	0	0	0		0 [0]		22.2
3′, P		1.6	1.8	1.8	2.0	2.0		1.5 [1.5]		0
5′a, P		20.6	20.8	21.6	20.2	20.2		20.0 [20.0]		31.0
5′b, P		1.6	1.8	1.5	1.8	2.0		2.0 [2.2]		4.1

[a] Estimated values of spin–spin coupling constants and dihedral angles (in degrees) for the 3′-endo conformation. [b] As in footnote *a* but for the 4′-exo conformation. [c] As in footnote *a* but for the 2′-endo conformation. [d] 2′ and 2″ denote the C(2′) protons which are trans and cis relative to the C(1′) proton, respectively. [e] 5′a denotes pro-*R*, 5′b denotes pro-*S* hydrogen attached to the C(5′) (cf. Figures 3 and 6). [f] Estimated to within ±0.2 Hz by the reduction of the H(1′) line width during irradiation of H(2′) (Figure 2). [g] These values were obtained at neutral pH and without shift reagent. The signs were not determined. [h] Values in the brackets were obtained from the 220-MHz spectra without adding shift reagent. [i] These values are the average of those obtained with and without adding shift reagents. Both values agreed within 0.2 Hz.

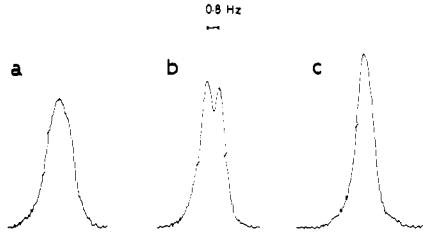

Figure 2. The 100-MHz proton NMR spectra of the $H_{1'}$ signal of 3′,5′-cAMP: (a) normal; (b) $H_{2'}$ decoupled; and (c) phosphorus decoupled.

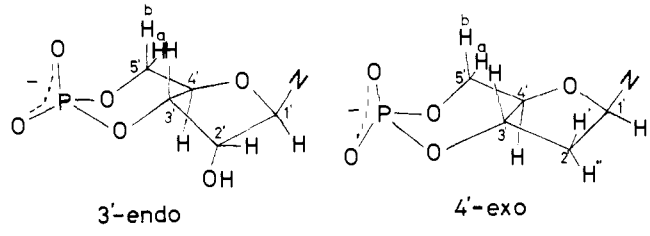

Figure 3. Preferred conformation of 3′,5′-cyclic nucleotides. In the 3′-endo conformation, atoms 4′-O-1′-2′ are all coplanar, and 3′ is above this plane. In 4′-exo, atoms O-1′-2′-3′ are coplanar with 4′ below the plane.

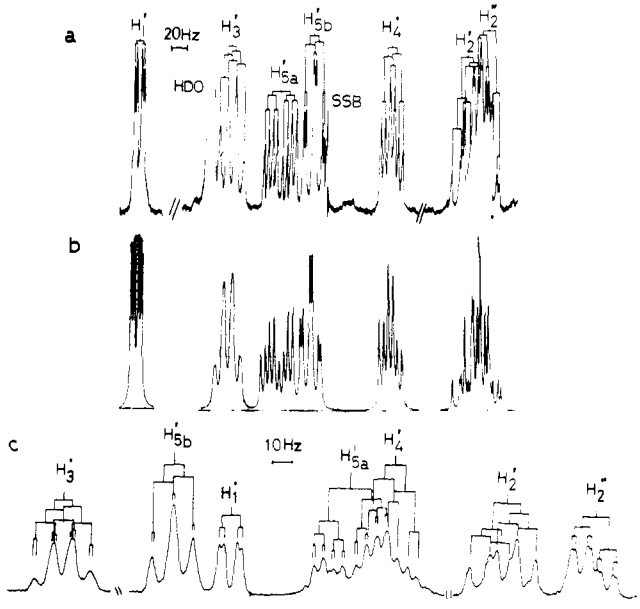

Figure 4. (a) The 220-MHz proton NMR spectrum of 3′,5′-cTMP (0.2 *M* in D_2O, pD 1.0 at 34°); (b) the simulated spectrum; (c) the 100-MHz proton NMR spectrum in the presence of 0.8 *M* Pr^{3+}.

tral pH, the similarity of the coupling constants also implies a lack of dependence of this ring conformation on the pH.

Upon irradiation of H(2′), doublets resulted for the C(1′) protons of all of I–V in neutral deuterium oxide. This splitting, which was almost constant for all the compounds (0.7–0.9 Hz), was assigned as the coupling between the phosphorus and H(1′) by double irradiation of the phosphorus (Figure 2).

Using Table I in conjunction with the estimated vicinal proton–proton coupling constants for various ribose conformations proposed by Smith and Jardetzky,[14] we could easily select the 3′-endo structure for the ribose of I–V. We compare in Table I the vicinal coupling constants for the 3′-endo conformation with those estimated using the more recent empirical relations of Blackburn et al.[10] The observed and the calculated values are obviously in good agreement. There are small differences in the ribose conformation of I–V between this (3′-endo) and the previous report (3′-endo-4′-exo), but these are probably not significant or important. The phosphate ring is seen to be a chair form, in agreement with earlier results since the proton–proton as well as the proton–phosphorus vicinal coupling constants are the same in both cases (Figure 3).

A most interesting observation is that there is virtually no change in the ribose conformation with the different bases. This is in stark contrast to the observations for acyclic 5′-ribonucleotides in which the $J_{1',2'}$ of the purine nucleotides are much larger than those of the pyrimidine nucleotides.[15] It was not clear whether these differences in $J_{1',2'}$ were due to an effect of substitution of bases directly on the coupling or to a change in conformation of the ribose ring upon substitution. With the lack of base dependence of $J_{1',2'}$ and $J_{1',P}$ in the 3′,5′-cyclic nucleotides, we might conclude that the effect of substitution directly on the coupling is minor and that there in fact are different ribose ring conformations in acyclic 5′-pyrimidine ribonucleosides and acyclic 5′-purine ribonucleosides. The conformational similarity of these cyclic nucleotides suggests that the different biological activities found among these compounds are not governed by the ribose or phosphate ring conformations.

We analyzed the 100- and 220-MHz spectra of 3′,5′-cTMP (VI) with and without lanthanide ions (Figure 4).

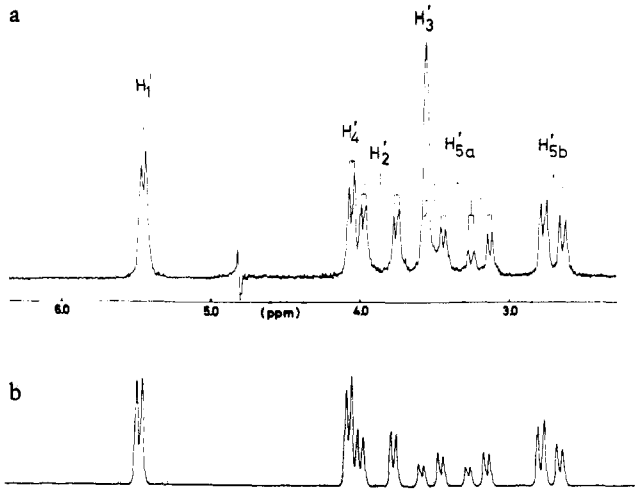

Figure 5. (a) The 100-MHz proton NMR spectrum of 2',5'-ara-cCMP (0.2 M in D_2O) in the presence of 0.8 M Eu^{3+}; (b) simulated spectrum without H(3').

Table II. Comparison of the Observed Coupling Constants of the Phosphate Ring Moiety of VII with the Predicted Values for the Four Structures Shown in Figure 6

Nuclei[a] (i, j)	Obsd $J_{i,j}$[b]	Predicted $J_{i,j}$ $(\phi_{i,j})$[c] VIIa	VIIb	VIIc	VIId
4', 5'a	3.2	5.2 (40)	0 (90)	2 (60)	6.7 (30)
4', 5'b	0	0 (80)	6.7 (30)	2 (60)	7.5 (150)
2', P	22.2	22 (180)	12 (150)	4 (120)	0 (90)
5'a, P	31.0	22 (180)	0 (90)	2 (60)	17 (170)
5'b, P	4.1	2 (60)	12 (150)	22 (180)	4 (50)

[a] For notations of nuclei see Figure 6. [b] The averaged values (Hz) from the spectra analyzed with and without shift reagent. [c] $J_{i,j}$ are represented in Hz and $\phi_{i,j}$ are in degrees. All angles are crude estimates from Dreiding models and could easily be changed by ±10°.

All cases gave practically identical results. Also in this case the pH of the solution and the shift reagent, similar to I-V, did not affect the ribose ring conformation and the coupling constants.

Since we got the same coupling constants as Blackburn et al.[10] did, the conformation of VI can be described as the 4'-exo form for the ribose ring and the chair form for the phosphate ring. As we have shown above, 3',5'-ribocyclic nucleotides are in the 3'-endo conformation, so the small conformational difference between these two classes must be accounted for by the effect of the presence or absence of a C(2') hydroxy group on the sugar conformation. Using Dreiding models it is clear that a 3'-endo conformation can easily be converted to a 4'-exo form by pushing the O(2') down and away from the base. By doing this, the C(2')-O bond is nearly trans to the C(1')-N bond so electrostatic repulsion between the O(2') and the base is minimized (Figure 3). This consideration is an apparent contradiction to the frequently claimed concept of hydrogen bonding of the 2'-hydroxyl group to the N(3) of purines.[5,6] The lack of any conformational differences between the pyrimidine and the purine cyclic nucleotide, however, strongly suggests that this type of hydrogen bonding does not exist for the cyclic nucleotides (at least not in aqueous solutions).

The 3'-endo conformation for I-V might also account for the unusual ^{31}P-$^{1}H(1')$ coupling constant. That is, the long-range coupling through many single bonds has been known to be observed (or to show a maximum value) when all the nuclei involved in the coupling path are arranged in a single plane.[18] For I-V, the repulsive interaction between the O(2') and the base is probably the reason for forcing the H(1')-C(1')-C(2')-C(3')-O(3')-P nuclei into a nearly co-

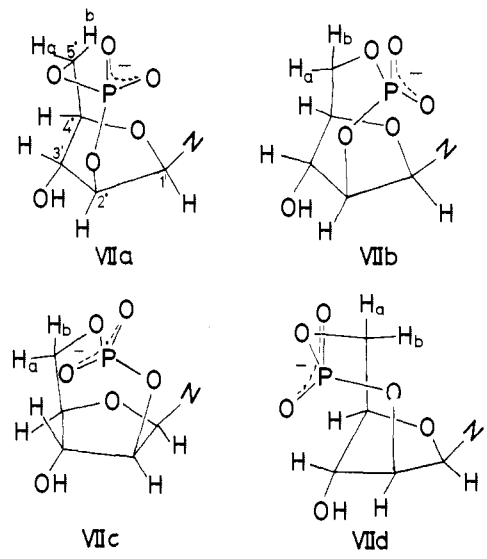

Figure 6. Possible conformations of VII.

planar zigzag array. The fact that the H(1') of 3',5'-cTMP did not show a resolvable coupling to the phosphorus nucleus supports this argument. There might be alternative explanations to the difference in the $J_{1',P}$ between ribose and deoxyribose nucleotides, such as a substitution effect of the 2'-hydroxyl group on the long-range coupling. We cannot a priori eliminate this ambiguity; however, the lack of $J_{1',P}$ in 3'-AMP and 3'-CMP indicates that this type of proton-phosphorus coupling would only be observable for special spatial situations. As reported previously the proton-phosphorus coupling across four bonds (J_{POCCH}) showed a similar stereospecificity.[19-21] The long-range couplings are not as fully understood in theoretical terms as vicinal couplings.[18] The present observations, however, suggest the usefulness of these couplings in structural considerations.

Conformation of 2',5'-Ara-cCMP (VII). A partial analysis of the 60-MHz ^{1}H NMR spectrum of VII was reported by Wechter.[22] Although the spin-spin coupling constants and chemical shifts reported by him turn out to be correct, the failure to analyze the lines from the H(4') and H(5') is found to lead to an unlikely conformation (structure c in Figure 6) by which the large downfield shift (1 ppm) of the H(3') with respect to that of ara-cytidine itself was explained. We analyzed the 100- and 220-MHz ^{1}H NMR spectra of VII with and without shift reagents and by computer simulations. At 100 MHz the resonances were also studied by homonuclear decoupling experiments and ^{31}P double irradiation. Exactly the same conclusions were obtained from the 100- and 220-MHz data (Table II). The 100-MHz spectrum of VII in the presence of Eu^{3+} ion is shown in Figure 5. This indicates again the conformational insensitivity of cyclic nucleotide toward metal complexation.

The coupling constant between H(1') and H(2') (3.1 Hz) in the arabinose ring indicate that the dihedral angle between these two protons, $\phi_{1',2'}$ is about 50°. Somewhat surprisingly both $J_{2',3'}$ and $J_{3',4'}$ were too small to observe, which however suggests that both $\phi_{2',3'}$ and $\phi_{3',4'}$ are around 90°. The dihedral angle of approximately 50° between H(1') and H(2') is the maximum possible value without creating any deformations. Any other conformations are then expected to exhibit a smaller $\phi_{1',2'}$ and, therefore, a larger $J_{1',2'}$ than observed. A further study of the molecular model indicates that keeping $\phi_{1',2'}$ maximum might be essential to minimize the steric interaction between the phos-

phate group and the base. In the proposed arabinose conformation the C(1′), C(3′), C(4′), and O(1′) nearly form a plane, and the arabinose ring is best described by a fixed 2′-endo structure.

The seven-membered phosphate ring should be in one (or several) of the four conformations, which can be predicted by model building (Figure 6). All of these conformers could exist with the same arabinose conformation (2′-endo). In Table II we list the dihedral angles in question, as estimated roughly by Dreiding models along with the observed coupling constants.

We can easily eliminate a possibility of conformer VIId because in this conformer $\phi_{2',P}$ predicts $J_{2',P} = 0$ Hz rather than the observed 22.2 Hz.[23-26] An interesting feature of this conformer is that it, as the only one, contains a "boat" form for the seven-membered ring. The preferred conformation of seven-membered phosphate rings has not been studied in full detail, but in the case of cycloheptane the "chair" form is known to be more stable than the "boat" form.[27] Conformation VIIc which was implied previously as the preferred conformation is unlikely since it does not completely satisfy the observed spin couplings; for example, the expected $J_{2',P}$ should be approximately 4 Hz (corresponding to $\phi_{2',P} = 120°$) instead of the observed 22.2 Hz (see Note Added in Proof). This structure also has large steric interactions between the H(3′) and the phosphate oxygen, as has been pointed out previously.[22] Conformer VIIb also is unlikely; the proton at C(5′) (tentatively designated as 5′a), which is expected to show approximately 0-Hz coupling with the H(4′), must also have a very small coupling with ^{31}P. The observed coupling was 30.5 Hz!

The remaining conformer (VIIa) may be in agreement with all coupling constants; it accounts for the $J_{4',5'a}$ and $J_{4',5'b}$ values better than the other conformers and also explains the very large $J_{2',P}$ and $J_{5',P}$. The seven-membered phosphate ring of VIIa is in a "chair" form and is without any serious steric hindrance. In this conformation the two protons attached to C(5′) are assigned as illustrated in Figure 6. Apparently the H(3′) is far apart from the phosphate group in the VIIa conformer, and then the chemical shift difference of this proton with respect to that of ara-cytidine may be due primarily to the conformation difference of the arabinose ring.

The most significant result of the analysis is undoubtedly the observation of an extremely large (30.5 Hz) vicinal phosphorus coupling constant. This value is approximately 50% larger than usual trans coupling.[23-26] As this molecule is seemingly almost strain-free, we are not certain about the reason for this abnormality. At any rate, the quantitative use of the empirical relation for ϕ-$^3J_{PH}$[23-26] and extended later to the ϕ-$^3J_{PC}$ relation[9] must be done with care.

Note Added in Proof

After this paper had been accepted, we analyzed the crystal structure of VII. The results showed the solid state conformation of VII is very similar to that of VIIc, which has been ruled out from the solution conformation by the vicinal proton-phosphorus coupling constants (J_{POCH}). It was revealed that the bond angles, ∠P-O-C, are about 10° larger than those in the six-membered phosphates or acyclic phosphates, which might be the reason for the "extremely" large J_{POCH} in this molecule. A full account of the results which includes a revised NMR assignment will be published shortly.

Acknowledgment. We wish to thank Dr. William J. Wechter of the Upjohn Co. for his kind gift of 2′,5′-ara-cCMP. We also acknowledge the Administration Committee of the 220-MHz NMR system at Kyoto University for permission to use its facilities.

References and Notes

(1) A major portion of this study was presented at the 12th NMR symposium (Japan) held at Osaka University, Oct 10, 1972.
(2) E. W. Sutherland, I. Oye, and R. W. Buther, *Recent Prog. Horm. Rev.*, **2**, 623 (1965).
(3) J. F. Kuo and P. Greengarg, *Proc. Natl. Acad. Sci. U.S.A.*, **64**, 1349 (1969).
(4) G. A. Robinson and E. W. Sutherland, *Ann. N.Y. Acad. Sci.*, **185**, 5 (1971).
(5) M. Sundaralingam and J. Abola, *J. Am. Chem. Soc.*, **94**, 5070 (1972).
(6) M. Sundaralingam and J. Abola, *Nature (London)*, **235**, 245 (1972).
(7) C. L. Coulter, *Acta Crystallogr., Sect. B*, **25**, 2055 (1969).
(8) N. Yathindra and M. Sundaralingam, *Biochem. Biophys. Res. Commun.*, **56**, 119 (1974).
(9) R. D. Lapper, H. H. Mantsch, and I. C. P. Smith, *J. Am. Chem. Soc.*, **95**, 2878 (1973).
(10) B. J. Blackburn, R. D. Lapper, and I. C. P. Smith, *J. Am. Chem. Soc.*, **95**, 2873 (1973).
(11) C. D. Barry, D. R. Martin, R. J. P. Williams, and A. V. Xavier, *J. Mol. Biol.*, **84**, 491 (1974).
(12) K. Ajisaka and M. Kainosho, *J. Am. Chem. Soc.*, **97**, 330 (1975).
(13) There have been several reports claiming that such changes were observed, in cases of substrates dissolved in organic solvents, using β-diketonate lanthanides [W. G. Bantrude, H. W. Tan, and K. C. Yee, *J. Am. Chem. Soc.*, **94**, 3264 (1972); T. B. Patrick and P. H. Patrick, *ibid.*, **94**, 6230 (1972)].
(14) M. Smith and C. D. Jardetzky, *J. Mol. Spectrosc.*, **28**, 70 (1968).
(15) P. O. P. Ts'o, M. P. Schweizer, and D. P. Hollis, *Ann. N.Y. Acad. Sci.*, **158**, 256 (1969).
(16) J. Pitha, *Collect. Czech. Chem. Commun.*, **28**, 1622 (1963).
(17) A. M. Michelson, *Annu. Rev. Biochem.*, **30**, 133 (1963).
(18) M. Barfield, *J. Chem. Phys.*, **41**, 3825 (1964).
(19) M. Kainosho and Y. Kyogoku, *Biochemistry*, **11**, 741 (1972).
(20) R. H. Sarma, R. J. Myott, D. J. Wood, and F. E. Hruska, *J. Am. Chem. Soc.*, **95**, 1457 (1973).
(21) D. B. Davies and S. S. Danyluk, *Biochemistry*, **13**, 4417 (1974), and references cited therein.
(22) W. J. Wechter, *J. Org. Chem.*, **34**, 245 (1969).
(23) M. Kainosho, A. Nakamura, and M. Tsuboi, *Bull. Chem. Soc. Jpn.*, **42**, 1713 (1969).
(24) M. Kainosho and A. Nakamura, *Tetrahedron*, **25**, 4041 (1969).
(25) M. Tsuboi, S. Takahashi, Y. Kyogoku, H. Hayatsu, T. Ukita, and M. Kainosho, *Science*, **166**, 1504 (1969).
(26) D. W. White and J. G. Verkade, *J. Magn. Reson.*, **3**, 111 (1970).
(27) J. B. Hendrickson, *J. Am. Chem. Soc.*, **86**, 4854 (1964).