products (**3** and **7**) were formed in only trace amounts, **7** slightly greater than **3**.

(2) The esters **1** (53.7 mg, 0.195 mmol), **5** (52.4 mg, 0.190 mmol), **8** (53.0 mg, 0.192 mmol), and **9** (50.1 mg, 0.182 mmol), each in 15 ml of benzene in Pyrex tubes, were degassed and irradiated simultaneously in a merry-go-round apparatus with 15 RPR-3000-Å lamps. After 5.5 hr of irradiation **1** disappeared very slowly (4%) and its main product **3** was formed (1%). The ester **5** disappeared much faster (20%) and the main product **7** was formed in 10% yield while the other two products **6** and **4** appeared in about 1% yield. After 31.1 hr 5% of **1** and about 60% of **5** had disappeared. After 43.7 hr no significant changes of esters **8** and **9** could be observed. The products **3** and **7** were formed only in trace amounts (much smaller yields than in the irradiations at 2537 Å).

Fluorescence Measurements for Esters 1, 5, 8, and 9. Fluorescence emission spectra were determined with an Aminco-Bowman spectrofluorimeter using the IP 28 photomultiplier with an excitation wavelength of 313 nm. The uv spectra of the esters in dioxane and cyclohexane were identical in shape and absorbance, thus indicating a lack of solvent effect for the four esters' uv absorptions and permitting direct comparison of the fluorescence and absorption results for the two solvent systems.

Fluorescence Quantum Yields (Φ_f). The measured fluorescence intensities (I_f) at 336 nm were used to calculate the relative quantum yields (Φ_f) using ester **5** as the standard. The absorbance values (A) were obtained at 313 nm using more concentrated solutions ($\times 10$). The quantum yields were then calculated according to the following expression[32]

$$\frac{\Phi_f}{\Phi_f'} = \frac{I_f(1 - 10^{-A'})}{I_f'(1 - 10^{-A})}$$

Natural Lifetimes (τ_0). The natural radiative singlet lifetimes (τ_0) were determined for the four esters by integration of the uv absorption band and using the following expression[19]

$$\tau_0 = \frac{3.5 \times 10^8}{\bar{\nu}_m^2 \epsilon_m \overline{\Delta\nu}_{1/2}}$$

where $\bar{\nu}$ is the mean wavelength in reciprocal centimeters, ϵ_m is the extinction coefficient at λ max, and $\bar{\nu}_{1/2}$ is the half-width of the band in reciprocal centimeters.

Fluorescence Decay Time (τ_s). The fluorescence decay times were calculated from the Stern–Volmer relationship for oxygen quenching of the fluorescence emission in cyclohexane[20] according to the following expression

$$L_0/L = 1 + \tau_s k_q[Q']$$

where L and L_0 are the fluorescence intensities with and without air, τ_s is the mean decay time of the deaerated solution, k_q is the quenching rate constant for oxygen, and [Q'] is the concentration of dissolved oxygen.

The value of $k_q[Q']$ was taken as 6×10^7 sec^{-1}.[20] The fluorescence intensity measurements were first obtained with samples saturated with air and then repeated after argon or nitrogen degassing to give the L and L_0 values.

Phosphorescence Measurements of Esters 1, 5, and 8–10. Phosphorescence emission spectra were determined in methylcyclohexane–isopentane (1:3) glass at 77°K using 280-nm excitation wavelength. All esters (10^{-4} M) showed the characteristic three-band emission; the observed 0,0 bands were at 470 (**1**), 475 (**5**), 475 (**8**), 485 (**9**), and 475 nm (**10**). Essentially the same spectra were obtained when the glass was a 5:2:2 mixture of ether–isopentane–ethanol.

Acknowledgments. The financial support of this project by the Research Corporation and the University of Kansas Research Fund and the aid of Margaret Wallace (naphthalene calculations) are gratefully acknowledged.

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Conformation of Cyclic β-Adenosine 3′,5′-Phosphate in Solution Using the Lanthanide Shift Technique[1a]

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Abstract: The preferred conformation of cyclic β-adenosine 3′,5′-phosphate in aqueous solution has been determined from shifts of proton magnetic resonance signals caused by lanthanide ions. The conformation of the ribose and phosphate groups is consistent with the structure in the crystalline state. At pD 5.3, the purine base is in the syn conformation, with the best agreement (R = 0.048) of calculated and observed shifts at a glycosyl torsion angle of 86°. Reasonable error limits for the torsion angle determination are ±22°. Experiments were performed over a pD range of 2.2–5.3 with a nucleotide concentration range of 0.02–0.072 M, and a lanthanide nucleotide ratio ranging from 0 to 15, without significant changes in the association constant of the lanthanide–nucleotide complexes. K_{eq} at pD 5.3, 13.0 ± 1.7, and at pD 2.2, K_{eq} = 14.2 ± 2.9.

A very versatile hormonal messenger is cyclic β-adenosine 3′,5′-phosphate (3′,5′-AMP).[2] The crystal structure of 3′,5′-AMP shows two molecules in the asymmetric unit which have very different orientations of the purine about the glycosyl bond.[3] In one molecule, the torsion angle is "anti" (−50°), while in the other it is "syn" (102°). The activation energy for rotation about the glycosyl bond may be sufficiently low that the orientation in the crystal may be determined by crystal packing forces and hydrogen bonding considerations rather than the intrinsic lowest energy orientation of the base as is more probable in solution. The structure of the molecule in solution is of interest in determining the intimate mechanism of action of the molecule and in comparing solution structures of the cyclic nucleotides with those of the noncyclic nucleo-

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tides to determine possible conformational requirements for cyclase action. Hence, a method of determining the conformation in solution is necessary.

The conformation of the base of the nucleotides and nucleosides has been investigated by several means including studies of nmr chemical shifts and coupling constants,[4,5] use of the Overhauser effect,[6-8] and optical studies;[9-17] but all of these methods provide limited structural information. The use of the lanthanide ion to produce shifts in nuclear magnetic resonance spectra has been discussed recently by several authors.[18-26] In essence, the method consists of addition of a solution of a particular lanthanide ion to a solution of substrate capable of complexing with the lanthanide. Perturbations of the nmr spectrum are then interpreted in terms of contract or pseudocontact interactions. The pseudocontact interactions are governed by the McConnell–Robertson equation (eq 1),[27] where $\Delta H/H_0$ is the isotropic shift caused by the lanthanide ion, D consists of parameters relating to the magnetic anisotropy of the metal ion, ϕ is the angle between the magnetic anisotropy axis (generally lying along the resultant vector of vectors aligned along the bonds between the lanthanide ion and the substrate) and the vector connecting the lanthanide ion and the observed nucleus, and r is the distance from the lanthanide to the observed nucleus. In the case of shifts due to pseudocontact interaction, the positions of observed nuclei may be deduced from the magnitude of the observed shift and from a determination of the association constant for the lanthanide–substrate complex.

$$\Delta H/H_0 = D(3\cos^2\phi - 1)/r^3 \qquad (1)$$

This procedure has been used to determine the conformation in aqueous solution of the important hormonal messenger, 3′,5′-AMP. Pr^{3+} was used to provide accurate assignments of shifted peaks and Ho^{3+} to give broadening information. 8-D-3′,5′-AMP was synthesized to verify the assignment of the H(2) and H(8) resonances. The conformation was determined for solutions with a range of pD values from 2.2 to 5.3 at low nucleotide concentration (0.02–0.72 M) to avoid base-stacking complications.

Experimental Section

Reagents. Pr_2O_3 or Ho_2O_3 (MacKay Chemical Co., 99.9%) was dissolved in $HClO_4$ and evaporated to dryness using a flash rotary evaporator. Several dissolution–evaporation cycles were performed with D_2O (99.8%). On final dissolution, the pD was about 2.0 (pD = pH meter reading + 0.40).[28] The rare earth solutions were titrated using the "pendulum method."[29] Excess standardized EDTA[30] solution was added to the rare earth solution and back titrated with $CuSO_4$ solution using 1-(2-pyridylazo)-2-naphthol solution as the end point indicator. 3′,5′-AMP (Sigma Chemical Co.) was used without further purification. The pD of the solutions of lanthanide and nucleotide was adjusted with $DClO_4$ and NaOD using a Beckman Research pH meter and a Sargent S-30070-20 combination electrode.

Nuclear Magnetic Resonance Spectra. Nmr spectra were recorded on a Varian HA 60 spectrometer operating at 29 ± 1° equipped with a Varian C1024 time averaging computer. The low concentration of nucleotide used required 20–150 scans for good peak definition. The sweep width settings of the C1024 unit were calibrated with TMS in $CHCl_3$. External lock and field sweep mode were used. All shift values are given relative to DSS (Merck) as internal standard.

Sample Preparation Procedure. Lanthanide ion and nucleotide solutions were adjusted to the sample pD value within ±0.10 unit. Nucleotide solution (500λ) was added to an nmr sample tube and weighed. An aliquot of lanthanide ion solution (5–50λ) was then weighed into the sample tube. After thorough mixing, an nmr spectrum was run; 10–15 such additions were performed for each nucleotide sample. To test for possible decomposition, the spectrum of the stock solution of nucleotide was rerun at the end of the series. For some samples, the lanthanide ion was removed by ion-exchange resin (Dowex 50W-X8, Li^+ form) and the initial nucleotide spectrum was obtained on observation of the effluent solution.

Preparation of 8-D-3′,5′-AMP. 8-Br-3′,5′-AMP was synthesized by direct bromination of 3′,5′-AMP[31] using 3% Br_2 in H_2O and adjusting the pH to about 5 to prevent precipitation of 3′,5′-AMP in the protonated form. The H_2O was removed by flash evaporation and the 8-Br-3′,5′-AMP suspended in THF (Fisher—dried over Linde 4A sieves for several days). The suspension was contained in a three-necked flask equipped with mechanical stirrer, dry N_2 inlet and thermocouple, and a pressure equalizing addition funnel with N_2 inlet. The 8-Br-3′,5′-AMP suspension and a suspension of a 20-fold excess of $LiAlD_4$ (Alfa Inorganics) in the addition funnel were deaerated. The $LiAlD_4$ was added slowly to the rapidly stirred nucleotide suspension. N_2 flow and stirring were continued overnight. The temperature was about 15° due to THF evaporation. The reaction volume was maintained at about 200 ml by periodic additions of deaerated THF.

The bromination of 3′,5′-AMP was followed by observation of

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(30) Abbreviations used: 3′,5′-AMP, cyclic β-adenosine 3′,5′-phosphate; EDTA, ethylenediaminetetraacetic acid; TMS, tetramethylsilane; DSS, trimethylsilylpropanesulfonic acid; THF, tetrahydrofuran.
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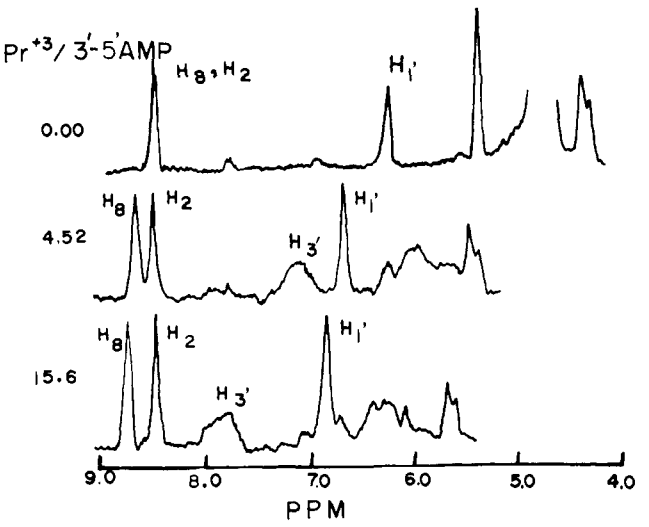

Figure 1. Typical nmr spectra of cyclic β-adenosine 3′,5′-phosphate alone (upper) and with added $Pr(ClO_4)_3$ (lower spectra). Chemical shifts are relative to internal DSS.

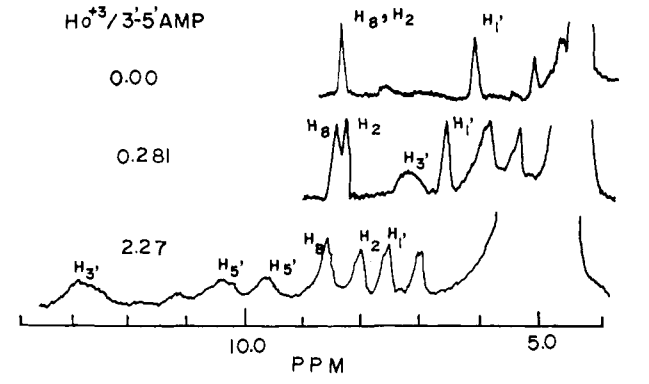

Figure 2. Typical nmr spectra of cyclic β-adenosine 3′,5′-phosphate alone (upper) and with added $Ho(ClO_4)_3$ (lower spectra). Chemical shifts are relative to DSS.

the disappearance of the H(8) resonance for aliquots taken from the reaction solution. The formation of 8-D-3′,5′-AMP was determined by the magnitude of the D resonance using a Varian wide line spectrometer and comparing the area under the peak with weighed sample of D_2O in H_2O. The peak magnitude was not affected by repeated evaporation with H_2O.

Computation. A CDC 6600 computer was employed. The programs MSEARCH and BURLESK[32] were used to assign the metal position and to compute reasonable conformations, respectively. The program PDIGM[23] was also used, giving very similar results. The *R* values quoted within are from the latter program.

Results and Discussion

At present, the interpretation of nmr shifts due to complexation with a lanthanide ion will only give accurate structural information if the interaction is predominantly pseudocontact. For protons, unlike ^{13}C, several studies of the chelated lanthanide shift reagents in nonaqueous media indicate that only pseudocontact interactions are significant.[18,26,33] As a test for this particular system, the aquated lanthanide ions, a comparison of the relative shifts induced by Pr^{3+} and Ho^{3+} was used to ascertain the importance of contact interaction. For ^{17}O shifts,[34] as well as ^{15}N shifts, in nitrilotriacetate complexes,[35] the signs of the predominantly contact shifts for Pr^{3+} and Ho^{3+} are opposite and the magnitudes of the shifts are relatively large. Hence, one might anticipate that these two ions would have a total shift interaction at each proton as a result of variable portions of contact and pseudocontact interactions. It the contact shift is a significant fraction of the total shift for protons in 3′,5′-AMP, different ratios of shifts of corresponding protons caused by Pr^{3+} and Ho^{3+} should be observed. The ratios of the shifts of corresponding protons caused by these two ions, however, are essentially the same (±2%), suggesting the absence of significant contact interaction. The interpretation of the shifts, therefore, assumes only pseudocontact interaction.

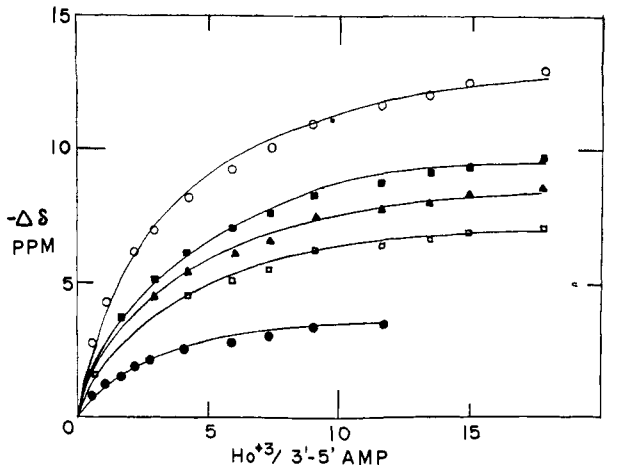

Figure 3. A plot of the shifts induced by the addition of $Ho(ClO_4)_3$ solution as a function of the ratio of Ho^{3+} to 3′,5′-AMP. From top to bottom, the curves represent the shifts of H(3′), H(5′A), H(5′B), H(4′), and H(2′).

Pr^{3+} causes relatively little line broadening (Figure 1). Since the peaks remain essentially unbroadened, their positions are easily determined as different amounts of lanthanide ions cause them to shift. Ho^{3+} causes measurable broadening (which is proportional to $1/r^6$) and allows a determination of the proximity of the protons. The magnitude of the shifts is much greater for Ho^{3+} than for Pr^{3+} (Figure 2), allowing more accurate relative shift values to be obtained. The shifts were used to determine the association constant for the lanthanide–nucleotide complex, since the equation for the association constant, eq 2, may be expressed in the

$$K_{eq} = [\text{complex}]/[\text{lanthanide}][\text{nucleotide}] \quad (2)$$

form of eq 3 for a 1:1 complex, where δ is the observed

$$K_{eq} = \delta\Delta/([La^{3+}]_{TOT}\Delta^2 - \delta\Delta - [La^{3+}]_{TOT}\delta\Delta + \delta^2) \quad (3)$$

shift for a given proton, Δ is the shift for that proton extrapolated to complete complexation, and $[La^{3+}]_{TOT}$ is the total lanthanide ion concentration. Los Alamos Non-Linear Least-Squares Program[36] was used to fit Δ and K_{eq} with δ and $[La^{3+}]_{TOT}$ values. The fit of the values to experimental points (Figure 3) is supporting evidence for a single 1:1 complex.

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The association constant at 29 ± 1° shows little variation with pD (for Ho^{3+} and 3′,5′-AMP at pD 5.3, K_{eq} = 13.0 ± 1.7, and at pD 2.2, K_{eq} = 14.2 ± 2.9). The values for the association constant are calculated from the shifts of the six protons which have the largest shift values. The value for Pr^{3+} is at pD 2.1, K_{eq} = 5.3 ± 1.4. These values may be compared with the values found for 5′-AMP,[25] using Ho^{3+}, K_{eq} = 6 ± 2 at 28° and pH 2; and using Eu^{3+}, K_{eq} = 10 ± 2 at 28° and pH 2.

Before discussing the conformation of the lanthanide–nucleotide complex, the effect of complexation on the conformation of the free nucleotide should be considered. The lanthanide ion may affect a substrate in several ways when it complexes; the most important of these being the attraction of electron-rich groups toward the positively charged lanthanide ion and the restriction of conformational changes (*e.g.*, ring puckering, phosphate group rotation in noncyclic nucleotides, etc.) near the lanthanide complexation site due to the large mass of the solvated lanthanide ion. These effects are of minimal importance in 3′,5′-AMP since there are no nucleophilic sites on the adenine ring which approach the lanthanide ion closely and the cyclic phosphate group to which the lanthanide ion is attached is in the stable chair form which has been shown to be rather rigid by Smith, *et al.*[37]

The conformational structure was deduced by comparing the shift ratios caused by the lanthanide ion for calculated trial structures with observed shifts. Shift ratios formed by dividing the induced shift found for each proton by that of an arbitrary normalizing proton were used to eliminate the factor, D (eq 1). Some deviation from a constant value for the shift ratios is apparent at low lanthanide ion concentrations (Figure 4), presumably due to ionic strength effects.[25] This assumption appears to be valid because the addition of La^{3+} or Lu^{3+}, which cause very little shift by themselves, to a dilute solution of Ho^{3+} and 3′,5′-AMP causes the resonances to shift toward the values at high ionic strength. The value used as the observed shift is the value found by extrapolation of the induced shift *vs.* lanthanide/nucleotide ratio curve to a ratio value of zero (*i.e.*, zero ionic strength) (Figure 2).

H(2) was assigned from the shift of H(2) in 8-D-3′,5′-AMP. It is also consistent with experiments performed with very narrow sweep widths (37 Hz) in which the H(2) and H(8) resonances are resolved. In these experiments, small aliquots of a dilute solution of Pr^{3+} were added, causing correspondingly small separations in these peaks. The peaks separated in a normal manner (as those in Figure 1) and evidently did not "cross." Therefore, H(2) is the resonance which shifts upfield, while H(8) shifts downfield. No solutions for a conformation were found when the assignments for H(2) H(8) were interchanged.

To determine the preferred conformation, the metal ion position was first determined by requiring agreement of trial structure computer shift ratios with observed shift ratios for the ribose unit. In computing trial structure-observed shift agreement, the observed shifts were allowed free assignment. That is, the calculation was done assuming that each shifted peak could

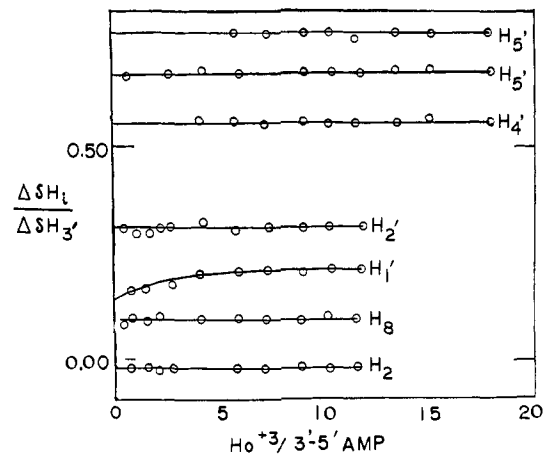

Figure 4. A plot of the ratios of the shift induced by Ho^{3+} for each proton relative to the shift induced for H_3'.

have arisen from any of the original peaks. Broadening was used as an agreement criterion, allowing rapid elimination of many trial structures. The assignments of the H(2′), H(3′), H(4′), and H(5′) resonances were decided in this manner. The assignments for H(1′), H(2), and H(8) were obvious from the observed spectra. Structures with only one oxygen complexed to the lanthanide ion were attempted without any reasonable results. For the model with both free phosphate oxygen atoms complexing the lanthanide ion, the metal position was varied from 2.0 to 3.5 Å from the complexing atoms. The symmetry axis angles were varied by 60°. Reasonable agreement was obtained with a metal ion–oxygen distance of 2.7–3.0 Å (R = 0.040 for 2.9 Å) and with the metal ion on a vector which lies in the O–P–O plane passes through the phosphorus atom, bisecting the O–P–O angle (Figure 6). Using this structure for phosphate-ribose conformation, the purine base was then rotated about the glycosyl bond through 360° in 4° steps. Shift ratios for the purine base at each glycosyl bond angle were computed and agreement with observed ratios was required.

Slight improvement of the R value (to 0.036) could be obtained by movement of the lanthanide symmetry axis 10–15° from the perpendicular bisector for various positions of the purine ring. The angular deviation giving the lowest R value fluctuates about the perpendicular bisector as the base is rotated. The fluctuation argues that the slight degree of improvement is fortuitous. Hence, the criteria for accepting a solution as reasonable were only applied to calculations with the metal ion along the perpendicular bisector. Recent careful studies of the calculation of the symmetry axis have demonstrated that it is directed along the metal–oxygen bond for all cases studied.[21,38] For complexes in which the lanthanide ion bind to more than one oxygen atom, the symmetry axis lies along the resultant of the metal–oxygen bonds,[25c] as found for 3′,5′-AMP.

The following criteria for the choice of reasonable glycosyl angle gave the indicated results: (1) a difference between the observed and calculated shift ratios no greater than 2% for either H_2 or H_8 (*i.e.*, H_2/H_3 = −0.020 ± 0.020, H_8/H_2 = 0.080 ± 0.020) gives 90 ± 14°; (2) a difference between the observed and cal-

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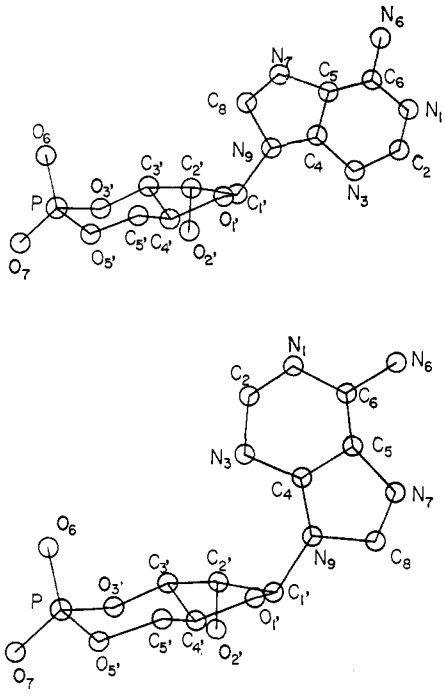

Figure 5. The structures found in crystalline 3′,5′-AMP: (upper) the anti conformation, $\phi_{CN} = -50°$; (lower) the syn conformation, $\phi_{CN} = 102°$.

culated difference in shift ratios between H_2 and H_8 no greater than 4% (*i.e.*, $H_8/H_3 - H_2/H_3 = 0.100 \pm 0.040$) gives 86 ± 22°; and (3) minimum $R \pm 1\%$ gives 82 ± 20°.[39] For all cases, any rotation angle which would require internuclear distances closer than van der Waals radii were rejected. It should be noted that the agreement of the calculated shift ratios for the "best" model structure and the observed shift ratios was within 1.0% except for H(1′), which shows a difference of 6.2%. This resonance shows the greatest ionic strength dependence and is not well fit by any reasonable position for H(1′).

Of these criteria, the most reliable is the shift ratio difference since this value is most directly observed in the nmr spectrum. It does not require the subtraction of the initial H_2 and H_8 peak positions. A slight variation of this initial position is reasonable due to medium effects caused by the added lanthanide solution other than the induced chemical shift. Since the results for a glycosyl angle of 86° give the best agreement with experimental shift values, no combination of syn and anti conformations would give better agreement. Hence, we find no evidence for a significant (10% or greater) population of another conformational state.

The structure of 3′,5′-AMP found in the crystalline state is shown in Figure 5. Molecule A shows the purine in the anti position ($\phi_{CN} = -50°$) while molecule B shows the base in the syn position ($\phi_{CN} = 102°$). ϕ_{CN} is the glycosyl torsion angle and has been defined by Donohue and Trueblood[40] as the angle formed between the trace of the plane of the base and the projection of the C(1′)–O(1′) bond when viewed along the glycosidic bond. The torsion angle is taken as zero when O(1′) of the ribose and C(2) of the base are antiplanar and the sense is positive for a clockwise rotation of the C(1′)–O(1′) projection relative to the trace of the base plane when viewed in the direction C(1′) to N(9).

(39) The assumed error in a shift ratio of 2% corresponds to a 15 Hz error in measurement of the nmr peak position at the largest Ho^{3+}/3′,5′-AMP ratios used but only 3–4 Hz at the lowest ratios. The measurements at low lanthanide ion concentration are very important since the values used for the calculation result from extrapolation to zero ionic strength. A 3-Hz error is twice the standard deviation for measurement at low lanthanide ion concentration. This error limit corresponds to a large percentage of the shift ratios of H(2) and H(8), causing the large angular uncertainty cited above.

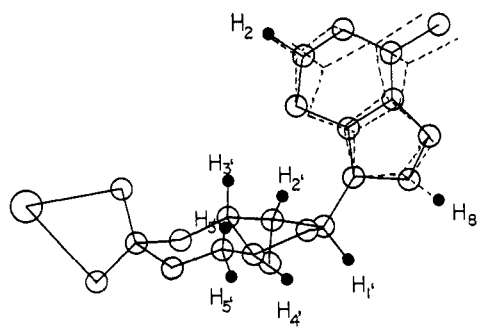

Figure 6. The structure as determined by the lanthanide ion induced shift method. The center purine ring gives the "best" solution, $\phi_{CN} = 86°$, with the other rings showing reasonable limits of the determination, 64 and 108°.

The preferred conformational structure in solution at pD 5.3 is shown in Figure 6. The goodness-of-fit factor, R, is 0.048 for a comparison of the shift ratios of this structure and the observed shift ratios. The base is in the syn conformation with best agreement at $\phi_{CN} = 86°$ and reasonable values (*vide supra*) in a range of 64–108°. In Figure 6, the centermost base is the best fit position and the dotted bases represent the reasonable limits.

Conclusion

The ribose and phosphate groups retain the structure found in the crystalline state, with the phosphate in the energetically favorable chair form. This conclusion agrees with the results of Smith, *et al.*[37] from an extensive study of nmr coupling constants. The preferred conformation of the purine results in a rather compact form of the molecule, with nearly the opposite orientation of the base from that found for 5′-AMP.[25c] It seems reasonable to assume that the purine base orientation is similar for ATP and 5′-AMP. Retention of purine base conformation may not be necessary in the formation of 3′,5′-AMP from ATP by adenyl cyclase. This method determines only the preferred conformation in solution and does not provide any measure of the rotational barrier about the glycosyl band, which may be low enough for essentially free rotation.

Work is in progress on the conformation of cyclic β-quanosine 3′,5′-phosphate to determine if conformation is an important factor in the 4 kcal/mol difference in the free energy of hydrolysis of the 3′ bond in 3′,5′-AMP and 3′,5′-GMP.[41]

(40) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960).
(41) S. A. Rudolph, E. M. Johnson, and P. Greengard, *J. Biol. Chem.*, 246, 1271 (1971).