culate the ^{31}P chemical shielding tensors of organophosphates have not been satisfactory,[18] yet the collection of empirical data from molecules of the structures $ROPO_2OH$, $ROPO(OH)_2$, and $(RO)_2PO_2$ seems to indicate that as a first-order approximation the shielding tensors of the organophosphates are aligned with the planes containing the P-O bonds. The deviations from this alignment and the variations of tensor orientation seen from molecule to molecule indicate that the ^{31}P shielding tensors are sensitive to more subtle environmental effects as well as to the P-O bond distribution, and in fact similar sensitivities have been reported for ^{13}C[19-21] and ^{19}F[7,22] chemical shielding tensors.

Acknowledgments. The authors would like to thank Helena Ruben for help in determining the crystal orientations. This research was supported in part by the U.S. Energy Research and Development Administration and in part by the National Cancer Institute (Grant No. CA 14828). S.J.K. was a Postdoctoral Fellow of the National Cancer Institute of the National Institutes of Health (Grant No. 1 F22 CA02169-01).

References and Notes

(1) J. S. Waugh, L. M. Huber, and U. Haeberlen, *Phys. Rev. Lett.*, **22**, 180-181 (1968).
(2) A. Pines, M. G. Gibby, and J. S. Waugh, *J. Chem. Phys.*, **59**, 569-590 (1973).
(3) U. Haeberlen, "High Resolution NMR in Solids: Selective Averaging", Academic Press, New York, N.Y., 1976, p 162.
(4) M. Mehring, "High Resolution NMR Spectroscopy in Solids", Springer Verlag, New York, N.Y., 1976, Chapter 5.
(5) S. J. Kohler and M. P. Klein, *Biochemistry*, **16**, 519-526 (1977).
(6) M. Mehring, R. G. Griffin, and J. S. Waugh, *J. Chem. Phys.*, **55**, 746-755 (1971).
(7) R. G. Griffin, J. D. Ellett, Jr., M. Mehring, J. G. Bullitt, and J. S. Waugh, *J. Chem. Phys.*, **57**, 2147-2155 (1972).
(8) A. Pines, M. G. Gibby, and J. S. Waugh, *Chem. Phys. Lett.*, **15**, 373-376 (1972).
(9) H. W. Spiess, *Chem. Phys.*, **6**, 217-225 (1974).
(10) H. W. Spiess, R. Grosescu, and U. Haeberlen, *Chem. Phys.*, **6**, 226-234 (1974).
(11) J. Seelig and H-U. Gally, *Biochemistry*, **15**, 5199-5208 (1976).
(12) S. J. Kohler and M. P. Klein, *Biochemistry*, **15**, 967-971 (1976).
(13) S. J. Kohler, J. D. Ellett, Jr., and M. P. Klein, *J. Chem. Phys.*, **64**, 4451-4458 (1976).
(14) M. Sundaralingam and E. F. Putkey, *Acta Crystallogr., Sect. B*, **26**, 790-800 (1970).
(15) E. Alver and S. Furberg, *Acta Chem. Scand.*, **13**, 910-924 (1959).
(16) J. Kraut, *Acta Crystallogr.*, **14**, 1146-1152 (1961).
(17) R. G. Griffin, manuscript in preparation.
(18) S. J. Kohler, unpublished results.
(19) R. G. Griffin, A. Pines, S. Pausak, and J. S. Waugh, *J. Chem. Phys.*, **63**, 1267-1271 (1975).
(20) R. G. Griffin and D. J. Ruben, *J. Chem. Phys.*, **63**, 1272-1275 (1975).
(21) A. Pines, J. J. Chang, and R. G. Griffin, *J. Chem. Phys.*, **61**, 1021-1030 (1974).
(22) R. G. Griffin, H-N. Yeung, M. D. LaPrade, and J. S. Waugh, *J. Chem. Phys.*, **59**, 777-783 (1973).

A Proton and Phosphorus Nuclear Magnetic Resonance Study of Ternary Complexes of Cyclic Adenosine 3′:5′-Monophosphate, Adenosine 5′-Triphosphate, and Mn^{2+}

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Abstract: Nuclear magnetic resonance was used to investigate the self-association of cAMP and ATP, the association of cAMP with ATP, and the interactions of the nucleotide dimers with Mn^{2+} in Tris-DCl (pH 7.6), (uncorrected meter reading) at 25 °C. The concentration dependences of the H_8, H_2, and $H_{1'}$ proton chemical shifts of the nucleotides were used to determine association constants of 4.2 M^{-1}, 0.45 M^{-1}, and 3.0 M^{-1} for the formation of $(cAMP)_2^{2-}$, $(ATP)_2^{8-}$, and $cAMP \cdot ATP^{5-}$, respectively, from the monomeric species. The association constants for formation of $MncAMP^{+}$, $Mn(cAMP)_2$, and $MncAMP \cdot ATP^{-3}$ from Mn^{2+} and the nucleotides were obtained from measurements of the transverse nuclear relaxation times of the $H_{1'}$ and ^{31}P nuclei of cAMP. The values obtained for the constants were 14.1 M^{-1}, 53.0 M^{-2}, and 41 700 M^{-2}, respectively. Interatomic distances between the Mn^{2+} and the H_8, H_2, $H_{1'}$, and ^{31}P nuclei of cAMP in the metal complexes were calculated from longitudinal nuclear relaxation times. In the $Mn(cAMP)_2$ complex the metal is coordinated to the phosphate of one cAMP molecule and to the adenine ring of the other. Base stacking between the bases also occurs. A similar structure is found for the $MncAMP \cdot ATP^{3-}$ complex: the Mn^{2+} is coordinated to the triphosphate chain of the ATP and to the adenine ring of the cAMP.

Nuclear magnetic resonance has been widely used in the study of the interactions of adenine nucleotides with metals.[1] The paramagnetic ion, Mn^{2+}, binds simultaneously to the three phosphates and to the adenine ring of ATP.[2-7] The adenine ring is separated from Mn^{2+} by a water molecule which is coordinated to the metal ion and is hydrogen bonded to N_7 of the adenine ring.[8,9] Prior to the study reported here the interaction of cAMP with Mn^{2+} had not been studied in detail, but the interaction of cAMP with lanthanide ions has been investigated.[10] (cAMP is used as an abbreviation for cyclic adenosine 3′;5′-monophosphate.)

Adenine nucleotides associate in aqueous solution through base stacking,[11-16] and the association processes involved have been investigated using several techniques including NMR[5,17,18] and vapor pressure osmometry.[19] The formation of AMP dimers,[5] AMP · ATP dimers,[12] and ATP dimers[12,13] has been demonstrated. Higher aggregates are also very likely formed.[11,13,14,19,20]

Evidence has been provided for the formation of ternary complexes consisting of adenine nucleotide dimers liganded to metal ions.[12,16] These ternary complexes are formed by the base stacking of the two adenine rings and the metal ion binding to the phosphate(s) of one nucleotide only.[12,16] The metal ion is then further coordinated to one or both of the adenine rings.[12,16] For example, in the metal-$(ATP)_2$ complex the metal ion binds directly (inner-sphere coordination) to the three phosphates of one of the ATP molecules and to three water molecules. The adenine rings are both outer-sphere coordinated via hydrogen bonds to two of the coordinated water molecules.[16]

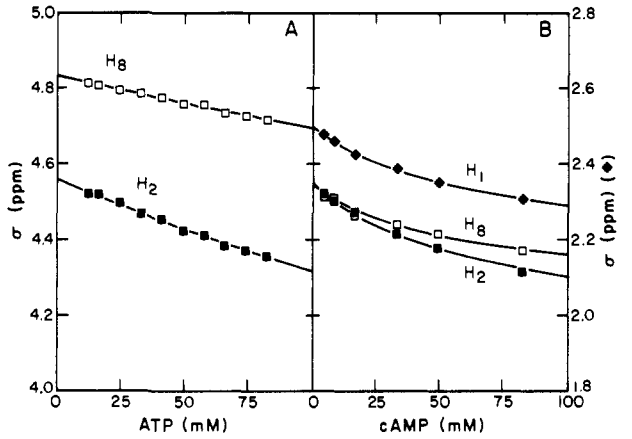

Figure 1. Plots of the concentration dependences of the chemical shifts, σ, of (A) the H_8 (□) and H_2 (■) protons of ATP and (B) the H_8 (□), H_2 (■), and $H_{1'}$ (♦) protons of cAMP at 25 °C. For ATP the Na^+ concentration was 0.4 M, and for cAMP it was 0.1 M. The lines were obtained by a nonlinear least-squares fit of the data to eq 1.

In this work, nuclear magnetic resonance has been used to obtain evidence for the formation of $(cAMP)_2^{2-}$, cAMP · ATP^{5-}, and $(ATP)_2^{8-}$ dimers and for the formation of $MncAMP^+$, $Mn(cAMP)_2$, and MncAMP · ATP^{3-} in aqueous solution. The association constants for formation of the complexes were determined and also the distances between the H_8, H_2, $H_{1'}$, and ^{31}P nuclei of cAMP and the Mn^{2+} in the three metal complexes. Structures of the complexes consistent with the measured distances are discussed.

Experimental Section

Chemicals. cAMP in its acid form and disodium ATP (grade I) were purchased from Sigma Chemical Co. D_2O (99.8%) was obtained from Aldrich Chemical Co. and all other chemicals used were the best available commercial grades. Heavy metal contaminants were removed from all solutions by passage through a 4-5-mL column of chelating resin (Chelex 100, 100-200 mesh, sodium form, Bio-Rad Laboratories).

Nuclear Magnetic Resonance Measurements. The NMR measurements were carried out in D_2O solutions of 20 mM Tris-DCl (pH 7.6, uncorrected pH meter reading) at 25 °C. The Na^+ concentration was held constant in a given series of measurements by appropriate addition of NaCl. The NMR measurements were made on a Brucker X-90 spectrometer with a fast Fourier transform accessory operating at 90 MHz for ^1H and 36.43 MHz for ^{31}P nuclei. The probe temperatures were determined from a thermocouple placed in the probe prior to any measurements. The uncertainty in the temperature is about ±1 °C. Chemical-shift measurements were made using the methyl proton peak of the Tris buffer in the solutions as the reference peak and all chemical shifts are quoted in parts per million from this peak. Longitudinal relaxation time (T_1) measurements were made using the inversion recovery technique [(d-180 °C-τ-90 °C) pulse sequence, where d is a delay time greater than $5T_1$]. Measurements of the transverse relaxation times (T_2) were made from the spectral line width at one-half of the resonance peak height, $\Delta\nu_{1/2}$, using the relationship:

$$T_2 = 1/\pi\Delta\nu_{1/2}$$

The proton T_2 measurements were made using the spectrometer in the continuous wave mode, whereas the lower sensitivity of the instrument to ^{31}P nuclei necessitated the use of the Fourier transform accessory for the determination of the phosphorus T_2 values. The T_2 values reported are the averages of three-five determinations.

Data Analysis. The data were fit to the theoretical equations by the nonlinear regression procedure of Cornish-Bowden and Koshland (1970),[21] modified as described by Wharton et al.[22] The combinations of the various ionic species present in the solutions of cAMP, ATP, and $MnCl_2$ were calculated using the iterative procedure described by Storer and Cornish-Bowden.[23] A combination of this method and the nonlinear regression procedure was used in obtaining the association constants from the data.

Table I. Association Constants Determined from NMR Measurements[a]

Reaction	K_a
$2cAMP^- \rightleftharpoons (cAMP)_2^{2-}$	4.2 M^{-1}
	4.4 M^{-1} [b]
$2ATP^{4-} \rightleftharpoons (ATP)_2^{8-}$	0.45 M^{-1}
	0.53 M^{-1} [b]
$cAMP^- + ATP^{4-} \rightleftharpoons cAMP{\cdot}ATP^{5-}$	3.0 M^{-1} [b]
$Mn^{2+} + cAMP^- \rightleftharpoons MncAMP^+$	14.1 M^{-1}
$Mn^{2+} + 2cAMP^- \rightleftharpoons Mn(cAMP)_2$	53.0 M^{-2}
$Mn^{2+} + cAMP^- + ATP^{4-} \rightleftharpoons MncAMP{\cdot}ATP^{3-}$	41 700 M^{-2}
$Mn^{2+} + ATP^{4-} \rightleftharpoons MnATP^{2-}$	56 250 M^{-1} [c]

[a] 25 °C and pH 7.6 (uncorrected meter reading). [b] Determined with mixture of cAMP and ATP. [c] Reference 24.

Results

Measurement of cAMP and ATP Association Constants. The concentration dependences of the H_8, H_2, and $H_{1'}$ proton chemical shifts of the nucleotides were used to measure the association constants for the formation of cAMP dimers, ATP dimers, and mixed dimers of cAMP and ATP. The data can be quantitatively analyzed without considering the formation of larger aggregates. The resonance peaks of all three protons were shifted upfield with increasing concentrations of the nucleotides (Figure 1).

For solutions of cAMP, the H_8, H_2, and $H_{1'}$ chemical shifts were measured as a function of the cAMP concentration (0.01-0.1 M). Since a single sharp resonance is observed for each proton, it is assumed that the chemical exchange between the different environments is in the fast exchange region. Therefore, the data obtained for each individual proton can be fit to the following equation to obtain values for the association constant characterizing dimer formation from monomeric cAMP:

$$\sigma_{obsd} = f_D\sigma_D + (1 - f_D)\sigma_M \quad (1)$$

Here σ_{obsd} is the observed chemical shift, σ_M and σ_D are the chemical shifts of the protons in the monomer and dimer, respectively (σ_D actually is the average of the chemical shifts σ_{D1} and σ_{D2} of the two protons in the dimer, $(\sigma_{D1} + \sigma_{D2})/2)$), and f_D is the fraction of the total cAMP in the form of the dimer. The fraction f_D can be related to the concentrations by the association constant:

$$K_a = (D)/(M)^2 \quad (2)$$

and the mass conservation relationship:

$$(C_T) = (M) + 2(D) \quad (3)$$

where (C_T) is the total cAMP concentration, (M) and (D) are the concentrations of monomer and dimer, respectively, and $f_D = 2(D)/(C_T)$. The value of K_a obtained by fitting the data to eq 1 is given in Table I, and the lines in Figure 1B have been calculated with this constant according to eq 1.

A similar treatment was used to analyze the chemical shifts of the H_8 and H_2 protons of ATP (Figure 1A). The chemical shift of the $H_{1'}$ proton was quite insensitive to variations in the concentrations over the range 0.01-0.1 M. The value of K_a obtained for the dimerization of ATP is also given in Table I, and the lines in Figure 1A have been calculated with this constant and eq 1.

Mixtures of cAMP and ATP were used to determine the association constant for the formation of the cAMP·ATP^{5-} dimer. Because of the overlap of some of the resonance peaks of the mixture, only the chemical shifts of the H_8 proton of

ATP and the $H_{1'}$ proton of cAMP could be used in this study. The data were fit to the equation:

$$\sigma_{obsd} = f_D\sigma_D + f_D'\sigma_D' + (1 - f_D - f_D')\sigma_M \qquad (4)$$

where σ_{obsd}, σ_D, σ_M, and f_D are defined as above, σ_D' is the chemical shift of the proton in the mixed dimer, and f_D' is the fraction of the total concentration of ATP or cAMP (depending upon which proton is under consideration) in the mixed dimer. The fractions f_D and f_D' were calculated using the iterative procedure described in the Experimental Section. The values of the association constants obtained for the formation of the three types of dimers are given in Table I.

Interaction of cAMP and ATP with Mn^{2+}. The broadening of the $H_{1'}$ and ^{31}P nucleus resonance peaks of cAMP brought about by the paramagnetic ion Mn^{2+} was used to investigate the interaction of cAMP and ATP with Mn^{2+}. The value of results are shown in Figure 2. The broadening of the $H_{1'}$ resonance of cAMP by Mn^{2+} is enhanced in the presence of ATP whereas ATP narrows the Mn^{2+} broadened ^{31}P resonance of cAMP. The increase in the line width of the $H_{1'}$ resonance with increasing concentrations of cAMP in mixtures of cAMP and Mn^{2+} (Figure 2A) is unexpected since in general the line width decreases as the ratio of ligand to Mn^{2+} increases, as is the case in the presence of ATP (Figure 2B). The data were fit to the model depicted in Scheme I. Reactions 9 and 10 in this scheme

Scheme I

$$\begin{array}{ccc} Mn^{2+} + 2cAMP^- & \overset{1}{\rightleftharpoons} & MncAMP^+ + cAMP^- \\ \updownarrow 4 & & \updownarrow 2 \\ Mn^{2+} + (cAMP)_2^{2-} & \overset{3}{\rightleftharpoons} & Mn(cAMP)_2 \end{array}$$

$$\begin{array}{ccc} Mn^{2+} + cAMP^- + ATP^{4-} & \overset{5}{\rightleftharpoons} & MnATP^{2-} + cAMP^- \\ \updownarrow 8 & & \updownarrow 6 \\ Mn^{2+} + cAMP{\cdot}ATP^{5-} & \overset{7}{\rightleftharpoons} & MncAMP{\cdot}ATP^{3-} \end{array}$$

$$\begin{array}{ccc} Mn^{2+} + 2ATP^{4-} & \overset{5}{\rightleftharpoons} & MnATP^{2-} + ATP^{4-} \\ \updownarrow 11 & & \updownarrow 9 \\ Mn^{2+} + (ATP)_2^{8-} & \overset{10}{\rightleftharpoons} & Mn(ATP)_2^{6-} \end{array}$$

were omitted in fitting the data since under the conditions used their contributions to the distribution of species are small, i.e., $(Mn^{2+}) < (ATP) < (cAMP)$, and the constant characterizing ATP dimerization is a factor of six less than that characterizing the interaction of ATP with cAMP (Table I). The equation used to fit the data was:

$$\frac{1}{T_{2obsd}} - \frac{1}{T_{20}} = \frac{f_M}{T_{2M}} + \frac{f_D}{T_{2d}} + \frac{f_D'}{T_{2d'}} \qquad (5)$$

where $1/T_{2obsd}$ and $1/T_{20}$ are the overall reciprocal transverse relaxation times in the presence and absence of Mn^{2+}, respectively, and T_{2M}, T_{2D}, and $T_{2D'}$ are the transverse relaxation times for the protons in the species $MncAMP^+$, $Mn(cAMP)_2$, and $MncAMP{\cdot}ATP^{3-}$, respectively. For phosphorus, the lifetimes (τ_M) in the complexes are measured rather than the transverse relaxation times. (This is demonstrated by data presented in the next section.) Again $1/T_{2D}$ is the average of the reciprocal relaxation times or lifetimes of the two nuclei in the $(cAMP)_2$ complex, $1/2\ (1/T_{201} + 1/T_{202})$. The fractions of the total cAMP in the three metal complexes are f_M, f_D, and f_D'. The data obtained for both $H_{1'}$

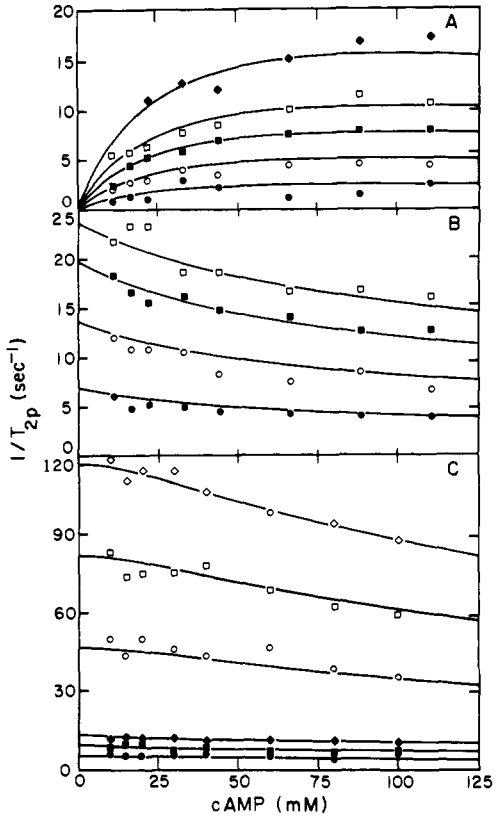

Figure 2. Plots of the effect of Mn^{2+} on the reciprocal transverse relaxation times, $1/T_{2P}$, of cAMP nulcei at 25 °C. (A and B) Measurements made on the $H_{1'}$ nucleus in the absence and presence of 0.93 mM ATP, respectively; the Mn^{2+} concentrations are (●) 0.25 mM, (○) 0.5 mM, (■) 0.75 mM, (□) 1.0 mM, and (♦) 1.5 mM). (C) Measurements made on the ^{31}P nucleus in the absence (open symbols) and presence (closed symbols) of 0.81 mM ATP; the Mn^{2+} concentrations are (○,●) 10 μM, (□,■) 17.5 μM, and (◇,♦) 25 μM). The Na^+ concentration was 0.1 M. The lines were obtained by a nonlinear least-squares fit of the data to eq 5.

and ^{31}P were fit simultaneously to eq 5. The dimerization constants in Table I and a value of 56 250 M^{-1} for the formation constant of $MnATP^{2-}$ [24] were used in this procedure. Values of the association constants characterizing the formation of the complexes $MncAMP^+$, $Mn(cAMP)_2$, and $MncAMP{\cdot}ATP^{3-}$ are given in Table I; the transverse relaxation times of the protons and the lifetimes of the ^{31}P nuclei are given in Table II. The lines in Figure 2 have been calculated with the constants in Tables I and II according to eq 5.

Relaxation Time Measurements. If a nucleus is assumed to exist in two environments, i.e., unbound and bound to the paramagnetic species, Mn^{2+}, the reciprocal magnetic relaxation times for that nucleus can be written as:

$$\left(\frac{1}{T_{1,2obsd}} - \frac{1}{T_{1,20}}\right) = \frac{f_M}{T_{1,2M} + \tau_M} \equiv \frac{1}{T_{1,2P}} \qquad (6)$$

where $T_{1,2obsd}$ is the observed longitudinal or transverse relaxation time, $T_{1,20}$ and $T_{1,2M}$ are the corresponding relaxation times for the free and unbound nucleus, respectively, τ_M is the residence time (lifetime) of the nucleus in the bound state, and f_M is the fraction of the nucleus bound to Mn^{2+}. (This equation assumes no contact or pseudocontact shift contribution to $1/T_{2P}$, which is reasonable for Mn^{2+} complexes.[25]) Two limiting cases can be delineated which lead to a simplification of eq 6: fast exchange and slow exchange of the nucleus between the two environments. In the former case T_{1M} or $T_{2M} \gg \tau_M$, while in the latter case $\tau_M \gg T_{1M}$ or T_{2M}. (The meaning of fast and slow exchange must be defined separately for each relaxation time.) For fast exchange $(f_M T_{1,2P})^{-1}$ should de-

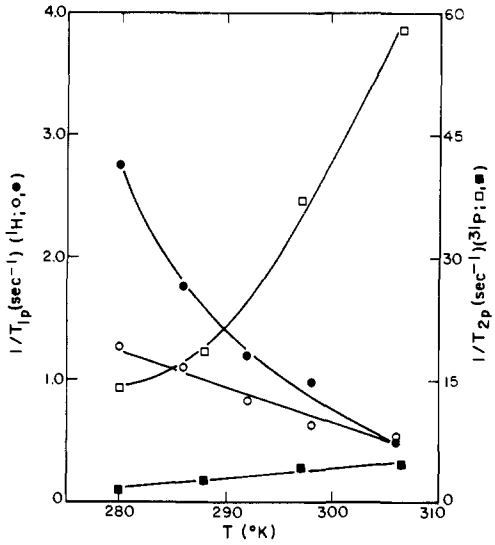

Figure 3. Plots of the temperature dependences of the relaxation times of cAMP nuclei. (○,●) $H_{1'}$ nucleus reciprocal longitudinal relaxation times, $1/T_{1P}$, in the absence and presence of 0.92 mM ATP, respectively; the Mn^{2+} and cAMP concentrations were 50 μM and 86 mM. (□,■) The ^{31}P nucleus reciprocal transverse relaxation times, $1/T_{2P}$, in the absence and presence of 1.0 mM ATP, respectively; the Mn^{2+} concentration was 10 μM and the cAMP concentration was 89 mM. The Na^+ ion concentration was 0.1 M.

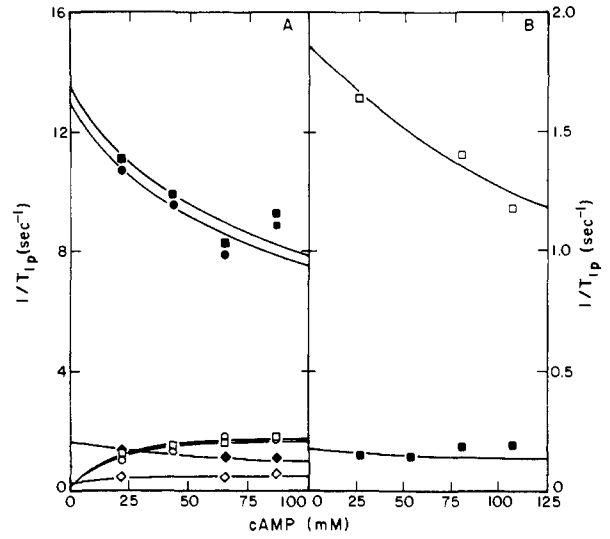

Figure 4. Plots of the concentration dependences of the reciprocal longitudinal relaxation times, $1/T_{1P}$, of cAMP nuclei at 25 °C. (A) Proton measurements made in the absence (open symbols) and presence (closed symbols) of 0.86 mM ATP; the Mn^{2+} concentration was 60 μM; (○,●) H_8, (□,■) H_2, and (◇,◆) $H_{1'}$. (B) ^{31}P measurements made in the absence (□) and presence (■) of 1.0 mM ATP; the Mn^{2+} concentration was 25 μM. The Na^+ concentration was 0.1 M. The lines were obtained by a nonlinear least-squares fit of the data to eq 7.

Table II. Longitudinal and Transverse Relaxation Rates of cAMP Nuclei[a]

Complex	cAMP nucleus	T_{1M}, s	T_{2M}, s	τ_M, s
$MncAMP^+$	H_8	$\geq 8.8 \times 10^{-3}$		
	H_2	8.7×10^{-3}		
	$H_{1'}$	3.8×10^{-3}	$\geq 3.0 \times 10^{-3}$	
	^{31}P	1.9×10^{-4}		3.0×10^{-6}
$Mn(cAMP)_2$[b]	H_8	7.2×10^{-5}		
	H_2	7.0×10^{-5}		
	$H_{1'}$	3.0×10^{-4}	1.8×10^{-4}	
	^{31}P	6.8×10^{-5}		9.1×10^{-7}
$MncAMP \cdot ATP^{3-}$	H_8	3.3×10^{-6}[c]		
	H_2	3.2×10^{-6}[c]		
	$H_{1'}$	2.65×10^{-5}	2.6×10^{-5}	
	^{31}P	1.07×10^{-4}		1.6×10^{-6}

[a] 25 °C and pH 7.6 (uncorrected meter reading). [b] The relaxation times are averages of those for the two equivalent nuclei in the dimers. [c] These relaxation rates are almost equal to the values for τ_M obtained for this complex and therefore are equal to $(T_{1M} + \tau_M)$.

crease as the temperature increases, while for slow exchange it should increase with increasing temperature.[25,26]

Both longitudinal and transverse relaxation times of the H_8, H_2, $H_{1'}$, and ^{31}P nuclei of cAMP in aqueous solutions of cAMP, ATP, and Mn^{2+} were determined. Since cAMP can exist in more than two environments, eq 6 is inadequate for describing the reciprocal relaxation times of the nuclei. The appropriate equation is:

$$\left(\frac{1}{T_{1,2obsd}} - \frac{1}{T_{1,20}}\right) = \frac{f_M}{T_{1,2M} + \tau_{MM}} + \frac{f_D}{T_{1,2D} + \tau_{MD}}$$

$$+ \frac{f_D'}{T_{1,2D'} + \tau_{MD'}} \equiv \frac{1}{T_{1,2P}} \quad (7)$$

where $T_{1,2obsd}$ and $T_{1,20}$ are defined as above and are determined by measuring the relaxation rates in the presence and absence of metal, respectively. $T_{1,2M}$, $T_{1,2D}$, and $T_{1,2D'}$ are the relaxation times; τ_{MM}, τ_{MD}, and $\tau_{MD'}$ are the residence times; and f_M, f_D, and f_D' are the fractions of cAMP in $MncAMP^+$, $Mn(cAMP)_2$, and $MncAMP\tau ATP^{3-}$, respectively. Again $T_{1,2D}$ and τ_{MD} are averages for the two sets of equivalent nuclei in $Mn(cAMP)_2$. (The assumption is made in treating the data that both of the equivalent nuclei are either in the fast or slow exchange region.) Plots of $(T_{1P})^{-1}$ and $(T_{2P})^{-1}$ against temperature indicate that all of the relaxation times measured in this work are in the limit of fast exchange with the exception of the transverse relaxation times for the ^{31}P nucleus which are in the region of slow exchange. The results obtained are summarized in Table II, and examples of the temperature dependence of the relaxation times are shown in Figure 3. In determining if the relaxation times are in the limit of fast or slow exchange the temperature dependences of the fractions f_M, f_D, and f_D' were not considered. An exact treatment would require knowledge of the temperature dependence of all of the association constants. Since the results obtained agree with those of several other investigations,[2,3,27] this matter was not investigated further. Also, no effort was made to differentiate between the various relaxation times involved to determine whether or not the mixture of environments was also a mixture of exchange limits. The self-consistency of the results indicates that any such breakdown of the overall relaxation rate is unnecessary.

Longitudinal relaxation times of the H_8, H_2, $H_{1'}$, and ^{31}P nuclei of cAMP in aqueous solutions of cAMP ± Mn^{2+} and in solutions of cAMP and ATP ± Mn^{2+} were measured. These measurements were repeated at several concentrations of cAMP and the results are shown in Figure 4. The values of $(1/T_{1obsd} - 1/T_{10})$ obtained were fit to eq 7 using the association constants given in Table I. The values obtained for T_{1M}, T_{1D}, and $T_{1D'}$ are included in Table II. The transverse relaxation times of the $H_{1'}$ and ^{31}P nuclei of cAMP were determined by the line broadening experiments described previously, and the values of T_{2M}, T_{2D}, and $T_{2D'}$ for the proton and τ_{MM}, τ_{MD}, and $\tau_{MD'}$ for the ^{31}P nucleus are also in Table II. The values of the residence times are comparable with the published values of 1.1×10^{-6} s for MnAMP[27] and 5×10^{-6} s for $MnATP^{2-}$.[3]

The longitudinal and transverse relaxation rates of the bound ligand nuclei due to its interaction with a paramagnetic ion, Mn^{2+}, at a distance r, are given by:[28,29]

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)\left[\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right] \quad (8)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2 \times \left[\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right] \quad (9)$$

where S is the electron spin quantum number for the Mn^{2+} ion ($\frac{5}{2}$), g is the g factor of the electron, β is the Bohr magneton of the electron, γ_I is the gyromagnetic ratio of the nuclei interacting with Mn^{2+} (either ^1H or ^{31}P in this study), ω_I is the nuclei resonance frequency, ω_S is the electron resonance frequency, τ_c is the dipolar correlation time given by $1/\tau_c = 1/\tau_R + 1/\tau_M + 1/\tau_S$ (τ_R is the rotational correlation time and τ_S is the electron spin relaxation time), $1/\tau_e = 1/\tau_M + 1/\tau_S$, and $A/\hbar$ is the isotropic hyperfine interaction constant. For the case under consideration $\omega_S \gg \omega_I$ so that the second term in the brackets of eq 8 can be neglected. Also, since $\tau_S \simeq 10^{-8}$ s, $\tau_M \simeq 10^{-6}$ s (Table II), and $\omega_S = 3.72 \times 10^{11}$ s^{-1}, the hyperfine coupling term can also be neglected in eq 8, which can then be rearranged to give:

$$r = B\left(\frac{3\tau_c T_{1M}}{1+\omega_I^2\tau_c^2}\right)^{1/6} \quad (10)$$

where $B = 815$ for protons and 601 for phosphorus nuclei. Simplification of eq 9 is not as straightforward as for eq 8 since the hyperfine coupling term cannot be readily neglected. However, eq 9 can be simplified to give:

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\tau_e \quad (11)$$

A comparison of the T_1 and T_2 values for the $H_{1'}$ proton in Table II shows $T_{1M} \simeq T_{2M}$. This indicates that the isotropic hyperfine interaction term is negligibly small for this proton (if this is true, $T_{1M}/T_{2M} \simeq 7/6$).

In order to calculate the distances of the nuclei from the paramagnetic center, using eq 10 and the T_1 values in Table II, the value of τ_c must be known for each complex. Although the values of τ_c were not determined experimentally, estimates of the values of τ_c are available. Since $\tau_S \simeq 10^{-8}$ s, $\tau_M \simeq 10^{-6}$ s, and $\tau_R \simeq 10^{-11}$–10^{-9} s for similar complexes,[28] the dipolar correlation times are essentially equal to the rotational correlation times of the complexes ($\tau_c \simeq \tau_R$). If it is assumed that in the $MncAMP^+$ complex the metal is inner-sphere coordinated to the phosphate group (which is true for other adenine nucleotides[2,7]), then using a value of 3.3 Å for the expected ^{31}P to Mn^{2+} distance[2,7] a value of 5×10^{-11} s is obtained for the τ_c of this complex. This value agrees well with a published value for $MnAMP^+$ of 6×10^{-11} s.[27] The longitudinal relaxation times measured for the $Mn(cAMP)_2$ complex are the average T_1 values for the pairs of equivalent nuclei in the complex. Therefore, the distance, r, calculated from T_{1D} is related to the true distances by $1/r^6 = 1/2\,(1/r_1^6 + 1/r_2^6)$. Hence, if $r_1 = r_2$, the measured distance is the real distance, and if $1/r^6 \gg 1/r_2^6$ then $1/r^6 \simeq 1/(2r_1^6)$. In the $Mn(cAMP)_2$ complex steric hindrance prevents simultaneous stacking of the adenine rings and interaction of both ^{31}P nuclei with the Mn^{2+}.

Table III. Distances between Mn^{2+} and cAMP Nuclei in Metal Nucleotide Complexes[a]

	Complex			
		$Mn(cAMP)_2$		
Nucleus	$MncAMP^+$	*b*	*c*	$MncAMP{\cdot}ATP^{3-}$
H_8	8.5	4.5	5.1	≥2.8
H_2	8.5	4.6	5.1	≥2.7
$H_{1'}$	7.4	5.8	6.5	≥4.4
^{31}P	3.3[d]	3.3[d]		≥4.1
τ_c	5×10^{-11} [e]	3×10^{-10} [e]		≥3×10^{-10} [f]

[a] The distances calculated from the T_{1M} values in Table II are given in angstroms. [b] Assuming $1/r_1^6 \gg 1/r_2^6$. [c] Assuming $1/r_1^6 = 1/r_2^6$. [d] Assumed distances; ref 2 and 7. [e] τ_c calculated from the expected ^{31}P to Mn^{2+} distance. [f] Assuming the same τ_c as for $Mn(cAMP)_2$.

Therefore, for this complex, it is likely that for the ^{31}P nuclei $1/r_1^6 \gg 1/r_2^6$. Using this assumption, the value of T_1 from Table II, and $r_1 = 3.3$ Å, a value for τ_c of 3×10^{-10} s is obtained. This gives a value of 6 for the ratio of dimer to monomer correlation times. This is similar to the value of 8 proposed for the ratio of AMP dimer to monomer correlation times.[15] (Both these values are higher than the expected value of $\simeq 2$,[30] possibly indicating the formation of higher aggregates.) Since the Mn^{2+} would be expected to bind predominantly to ATP rather than to cAMP, a direct estimate of τ_c for the mixed dimer is not possible as with $MncAMP^+$ and $Mn(cAMP)_2$. Instead, we assume τ_c is the same for the mixed dimer as for $Mn(cAMP)_2$. Since the mixed dimer is slightly larger, 3×10^{-10} s can be regarded as a lower bound for τ_c. Using the values of τ_c deduced above, the T_1 values in Table II, and eq 10, the distances given in Table III were calculated. Two sets of distances are given for the protons of the complex $Mn(cAMP)_2$: the assumptions used in calculating these distances were that the distances for the equivalent pairs of protons were related by (i) $1/r_1^6 \gg 1/r_2^6$ or by (ii) $1/r_1^6 = 1/r_2^6$. In reality the true relationship is probably somewhere between these two limits.

Discussion

The association constant obtained for the formation of $(cAMP)_2^{2-}$, 4.2 M^{-1}, compares favorably with published values of 5 and 2 M^{-1} for the formation of $(AMP)_2^{4-}$.[12,15] The value obtained for the dimerization of ATP, 0.45 M^{-1}, is a factor of 10 less than the value of 6.3 M^{-1} obtained by Granot and Fiat (1977).[16] However, their value was obtained at pD 3.0 where the ATP is less highly charged than in our study. The value of 3 M^{-1} for the association constant of $cAMP{\cdot}ATP^{5-}$ is consistent with the hypothesis that charge repulsion is an important factor in the dimerization reaction. Although the monomer–dimer model for the stacking of ATP and cAMP adequately explains the data, it is very probable that aggregates larger than the dimer are formed.[11-16] Therefore, the model used should be considered only as a convenient approximation.

The chemical-shift data do not provide any detailed information about the geometry of the nucleotide association. However, on stacking, the H_2 proton of both cAMP and ATP is more shielded than the H_8 proton, indicating that the six-membered rings are more involved in the stacking than the five-membered rings. A study of the intramolecular interactions of AMP by Evans and Sarma[31] showed that the preferred self-association of two nucleotide molecules occurs with the bases aligned face-to-back in vertical stacks involving almost 100% base overlap. In these stacks the ribose groups are close but the phosphate groups are well separated in order to eliminate steric hindrance and reduce electrostatic repulsion. Evidence also indicates this is only a preferred orientation,[31] and the formation of face-to-face dimers as the preferred orientation is suggested by Ts'o et al.[32] and Berger and Eichhorn.[33]

The manipulation of Corey-Pauling atomic models of cAMP and ATP shows the ability of both to form face-to-face and face-to-back dimers.

The interatomic distances measured for $MncAMP^+$ clearly reflect the rigidity of the cAMP molecule (the ribose ring and cyclic phosphate form a planar two-ring system). In this complex the metal is coordinated to the phosphate group, and it has little interaction with the adenine ring. The distances in Table III are the averages of the distances in the various possible orientations and conformations of the complexes. For example, in the $MncAMP^+$ complex the adenine ring can rotate, with respect to the metal ion, around the adenine-ribose bond. The measured average distances are weighted heavily in favor of their shorter components. Therefore, the distances given in Table III most closely resemble the shortest distances associated with all possible conformations and orientations. In the $Mn(cAMP)_2$ complex, the calculated distances indicate the metal ion is coordinated to one of the phosphate groups directly and to the adenine ring of the other cAMP molecule possibly via a water bridge to one of the base ring nitrogens. Stacking of the bases, either face-to-face or face-to-back, is also important to the stability of this complex. The Mn^{2+} in the $MncAMP{\cdot}ATP^{3-}$ complex is coordinated to the triphosphate chain of ATP and to the adenine ring of the cAMP molecule and possibly also the adenine ring of the ATP[16] (water bridges to ring nitrogens may be involved). This is consistent with the broadening of the proton resonances and the narrowing of the phosphorus resonance when ATP is added to Mn^{2+}-cAMP solutions. The greater flexibility of the phosphate chain in ATP is reflected in the fact that the Mn^{2+} is capable of interacting more strongly with the cAMP adenine ring. The phosphorus nuclear resonance of cAMP is also strongly influenced by Mn^{2+} in the $MncAMP{\cdot}ATP^{3-}$ complex suggesting that in one orientation of the complex, at least, the phosphorus is close to Mn^{2+}. Base stacking also occurs in this complex, but again the preferred orientation of the stacking cannot be inferred.

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM 13292). A. C. Storer is a recipient of a Fulbright-Hays travel grant.

References and Notes

(1) A. T. Tu and M. J. Heller in "Metal Ions in Biological Systems", Vol. I, H. Sigel, Ed., Marcel Dekker, New York, N.Y., 1974, pp 8–10, and references therein.
(2) H. Sternlicht, R. G. Shulman, and E. W. Anderson, *J. Chem. Phys.*, **43**, 3123 (1965).
(3) H. Sternlicht, R. G. Shulman, and E. W. Anderson, *J. Chem. Phys.*, **43**, 3133 (1965).
(4) R. G. Shulman and H. Sternlicht, *J. Mol. Biol.*, **13**, 952 (1965).
(5) M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 176 (1962).
(6) M. J. Heller, A. J. Jones, and A. T. Tu, *Biochemistry*, **9**, 4981 (1970).
(7) F. F. Brown, I. D. Campbell, R. Hensen, C. W. J. Hirst, and R. E. Richards, *Eur. J. Biochem.*, **38**, 54 (1973).
(8) T. A. Glassman, C. Cooper, L. W. Harrison, and T. J. Swift, *Biochemistry*, **10**, 843 (1971).
(9) G. P. P. Kuntz, T. A. Glassman, C. Cooper, and T. J. Swift, *Biochemistry*, 11, 538 (1972).
(10) C. D. Barry, D. R. Martin, R. J. P. Williams, and A. V. Xavier, *J. Mol. Biol.* **84**, 491 (1974).
(11) A. D. Broom, M. P. Schwiezer, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **89**, 3612 (1967).
(12) H. Sternlicht, D. E. Jones, and K. Kustin, *J. Am. Chem. Soc.*, **90**, 7110 (1968).
(13) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Am. Chem. Soc.*, **90**, 1042 (1968).
(14) D. Pörschke and F. Eggers, *Eur. J. Biochem.*, **26**, 490 (1972).
(15) W. Egan, *J. Am. Chem. Soc.*, **98**, 4091 (1976).
(16) J. Granot and D. Fiat, *J. Am. Chem. Soc.*, **99**, 70 (1977).
(17) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **87**, 5241 (1965).
(18) S. I. Chan, M. P. Schweizer, P. O. P. Ts'o, and G. K. Helmkamp, *J. Am. Chem. Soc.*, **86**, 4182 (1964).
(19) P. O. P. Ts'o, I. S. Melvin, and A. C. Olson, *J. Am. Chem. Soc.*, **85**, 1289 (1963).
(20) P. O. P. Ts'o and S. I. Chan, *J. Am. Chem. Soc.*, **86**, 4176 (1964).
(21) A. Cornish-Bowden and D. E. Koshland, Jr., *Biochemistry*, **9**, 3325 (1970).
(22) C. W. Wharton, A. Cornish-Bowden, K. Brocklehurst, and E. M. Crook, *Biochem. J.*, **141**, 365 (1974).
(23) A. C. Storer and A. Cornish-Bowden, *Biochem. J.*, **159**, 1 (1976).
(24) A. T. Tu and M. J. Heller, in "Metal Ions in Biological Systems", Vol. I, H. Sigel, Ed., Marcel Dekker, New York, N.Y., 1974, p 23.
(25) T. J. Swift and R. E. Connick, N.Y., *J. Chem. Phys.*, **37**, 307 (1962).
(26) Z. Luz and S. Melboom, *J. Chem. Phys.*, **40**, 2686 (1964).
(27) A. Bennick, I. D. Campbell, R. A. Dwek, N. C. Price, G. K. Radda, and A. G. Salmon, *Nature (London), New Biol.*, **234**, 140 (1971).
(28) R. A. Dwek, "Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems", Clarendon Press, Oxford, 1973, pp 175–212.
(29) I. Solomon, *Phys. Rev.*, **99**, 559 (1955).
(30) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect: Chemical Applications", Academic Press, New York, N.Y., 1971, pp 26–31.
(31) F. E. Evans and R. H. Sarma, *Biopolymers*, **13**, 2117 (1974).
(32) P. O. P. Ts'o, M. P. Schweizer, and D. P. Hollis, *Ann. N.Y. Acad. Sci.*, **158**, 256 (1969).
(33) N. A. Berger and G. L. Eichhorn, *Biochemistry*, **10**, 1847 (1971).