added over a 45-min period. During this addition, the mixture was maintained at pH 5.0–5.5 with glacial acetic acid. The mixture was stirred for 1 hr at 0° to destroy any $NaBH_4$ that might have been present, the pH was adjusted to 6.8 with ammonium hydroxide, and the solvents were removed *in vacuo*. The resulting oil was dissolved in 30 ml of 1-butanol and 6 ml of benzene. A 2-ml aliquot was placed on a 64 × 2.8 cm column of Sephadex G-25 (100–200 mesh) that had been equilibrated with the lower and upper phases of the solvent system 1-butanol–benzene–3.5% acetic acid in 1.5% aqueous pyridine (5:1:6) according to the method of Yamashiro.[14] The product was eluted with the upper phase, and 60 7.2-ml fractions were collected. Folin-Lowry color values[18] were determined and the contents from the major peak (R_f 0.42) were pooled. Then 200 ml of distilled water was added and the mixture was evaporated to dryness *in vacuo* to give a viscous oil (~45 mg). The entire procedure was repeated on a large scale with the remaining 1-butanol–benzene solution to give 0.820 g of the product as a semi-solid foam. All attempts to crystalline the material failed. A sample was prepared for elemental and amino acid analysis by dissolving a 100-mg portion of the product in 3 ml of methylene chloride, filtering the solution, removing the solvent *in vacuo*, and drying the residue at 25° for 24 hr *in vacuo* to give the product as a foam, mp 55–58°, $[\alpha]^{22}D$ −59.3° (*c* 1.0, ethanol).

Anal. Calcd for $C_{26}H_{41}N_3O_4S$: C, 60.1; H, 7.95; N, 13.5. Found: C, 59.5; H, 7.95; N, 13.2.

A sample was hydrolyzed for 24 hr in 6 *N* HCl at 110° and analyzed[19] on a Beckman-Spinco amino acid analyzer. The following molar ratios were obtained with the value of glycine taken as 1.0: proline, 1.0; glycine, 1.0; leucine, 1.0; and ammonia, 1.0. Authentic N-isopropyl-S-benzyl-L-cysteine has no ninhydrin color value.[13]

Acknowledgments. We wish to thank Mr. Joseph Albert for the elemental analyses and Mr. Roger Sebbane for the amino acid analysis.

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Metal Ion Binding to Adenosine Triphosphate. III. A Kinetic Analysis[1]

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Abstract: Previous attempts to elucidate the mechanism of metal–adenosine triphosphate (ATP) binding have resulted in apparent disagreement between the results of two fast reaction techniques, namely, nmr and temperature jump. We have extended the nmr measurements on manganese(II) to low ATP concentrations (*ca.* 5×10^{-4} *M*). Competition studies in mixtures of adenosine monophosphate (AMP), ATP, and Mn^{2+} have also been carried out. The results of these measurements confirm the existence of a 1:2 metal–ligand complex at high total ATP. The low concentration studies support the assignment of temperature-jump spectra to the formation of a 1:1 complex. The kinetic scheme at room temperature reconciling the results of the different experimental techniques is shown in Scheme I. By using the rate constants for step $1 \rightleftharpoons 2$ obtained by temperature jump, and an estimate of the equilibrium quotient for the metal-independent step $1 \rightleftharpoons 4$, we have been able either to determine or to estimate the remaining rate constants and equilibrium quotients for the metal-dependent steps. These constants are shown for Mn^{2+}. This scheme predicts that the phosphorus and proton magnetic resonance line broadening studies should reach a low concentration asymptote consistent with pathway $1 \rightleftharpoons 2$. This limit was experimentally observed in the proton case, where ATP concentrations as low as 5×10^{-4} *M* could be studied using computer enhancement. In the MA_2 complex the metal ion simultaneously binds to the phosphate moiety of one nucleotide and to the adenine ring nitrogen of the second nucleotide. The MA_2 complexes in which the metal ion binds to the N-7 position predominate. At low nucleotide concentration where the MA complex becomes accessible to the nmr, we show that the metal ion is *ca.* 3.8 Å from the H_8 proton. This distance could arise either (1) from the metal ion binding predominantly to the N-7, or (2) from the metal ion being near the H_8 but separated from the adenine ring by a coordination shell water molecule. An earlier uv difference study strongly suggests the second alternative.

In recent years there have been a number of studies of transition metal binding to adenosine triphosphate (ATP). Nuclear magnetic resonance,[3] temperature jump,[4,5] ultraviolet spectroscopy,[6] and electron spin resonance[7] have all been used to determine the nature of the metal–ATP interaction. The conclusions of these studies have apparently been in disagreement with respect to the detailed kinetics, and to whether the metal does[3,5] or does not[6,7] simultaneously bind to the phosphates and the adenine ring of the ATP molecule as first proposed by Szent-Györgyi.[8] We have reexamined the problem of metal binding to ATP

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(2) U. S. Public Health Service Special Fellow, 1967–1968.

(3) (a) H. Sternlicht, R. G. Shulman, and E. W. Anderson, *J. Chem. Phys.*, 43, 3125 (1965); (b) H. Sternlicht, R. G. Shulman, and E. W. Anderson, *ibid.*, 43, 3133 (1965). These papers are, respectively, I and II in the series. The interested reader is referred to these two papers for further references, particularly to the pioneering nmr studies of metal ion binding to ATP done by M. Cohn.

(4) G. G. Hammes and S. A. Levison, *Biochemistry*, 3, 1504 (1964).

(5) G. G. Hammes and D. L. Miller, *J. Chem. Phys.*, 46, 1533 (1967).

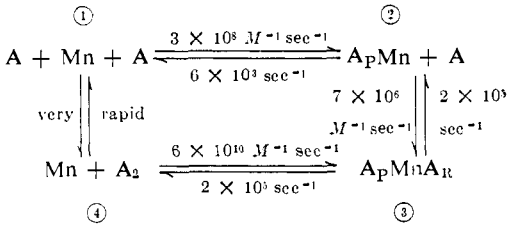
(6) P. W. Schneider, H. Brintzinger, and H. Erlenmeyer, *Helv. Chim. Acta*, 47, 992 (1964).

(7) H. Brintzinger and G. Palmer, private communication.

(8) A. Szent-Györgyi, "Bioenergetics," Academic Press, New York, N. Y., 1957.

including the carrying out of new kinetic experiments. As a consequence of the new data and a reconsideration of the previous results, a mechanism consistent with all the kinetic details of the binding is postulated. (This is shown specifically for Mn^{2+} in Scheme I). Further-

Scheme I



more, the conformational nature of the complexation and the published discrepancies are also explained.

Attention has been focused on manganese(II), cobalt(II), and nickel(II), since these paramagnetic ions are involved in both the nmr and temperature-jump experiments. In essence, a proper interpretation of the magnetic resonance and temperature-jump data requires a recognition of the existence of multiple equilibria and a correct identification of the higher order complexes present. The treatment presented below is general, and should serve as a model for other kinetic studies of metal binding involving multiple equilibria.

The relatively high ATP concentration nmr results[3] revealed a simultaneous binding of the paramagnetic ion to a phosphate moiety and adenine ring which was suggestive of a back-bound complex. This conclusion was in disagreement with an earlier uv spectral study of metal ion binding to ATP at low total ATP concentration ($\sim 1 \times 10^{-4}$ *M*) carried out by Brintzinger, *et al.*,[6] which showed either no or only a small degree of back-binding for complexes of Ni^{2+}, Co^{2+}, and Mn^{2+}. Furthermore, the nmr values for τ_M, the length of time the metal ion remained bound to ATP before chemical exchange with another ATP occurred, were ~30 times shorter than the dissociation lifetimes, $1/k_{21}$, reported by Hammes and Levison[4] from temperature-jump studies of reaction a ([ATP] $\sim 1 \times 10^{-4}$ *M*). The reasons

$$M^{2+} + A^{4-} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} MA^{2-} \qquad \text{(a)}$$

for the lifetime discrepancies were not clear at that time.[3]

Brintzinger and Palmer[7] subsequently proposed that the reason for the discrepancy was that the temperature-jump and uv measurements[6] were done on a 1:1 MA complex, whereas the nmr study was done on a 1:2 MA_2 complex because of the relatively high ATP concentrations. These authors further showed from an electron spin resonance study of Mn^{2+} and Cu^{2+} at high ATP concentrations (0.05–0.5 *M*) that an MA_2 complex probably does exist. They estimated that in the case of Mn^{2+}

$$K_{23} = \frac{[Mn(ATP)_2^{6-}]}{[MnATP^{2-}][ATP^{4-}]} \approx 10 \qquad \text{(b)}$$

Their estimate, although only approximate, certainly showed that a 1:2 MA_2 complex might well be the dominant species in the nmr study. Also in support of higher order complexes are ultracentrifuge experiments with the potassium(I)[9a] and magnesium(II)[9b] salts of adenosine monophosphate (AMP), which show evidence of $M(AMP)_2$ complexes, and Cl^{35} nmr studies[9c] of adenosine diphosphate, which show evidence for $Zn(ADP)_2$.

In a second temperature-jump study, Hammes and Miller[5] examined the kinetics of metal binding in the ATP concentration range of 0.01–0.1*M*. They obtained relaxation times that were essentially independent of the ATP concentration and which were now similar to the nmr results, clearly indicating the need for further experimental study.

(9) (a) G. P. Rossetti and K. E. Van Holde, *Biochem. Biophys. Res. Commun.*, **26**, 717 (1967); (b) G. P. Rossetti, unpublished results; (c) R. L. Ward and J. A. Happe, *Biochem. Biophys. Res. Commun.*, **28**, 785 (1967).

Experimental Section

Materials. ATP and AMP were obtained as the disodium and monosodium salt, respectively, from the Sigma Chemical Co. Solutions of the nucleotides (pH ≈ 7) were passed through a Chelex 100 (Calbiochem) resin bed to remove background metal ion impurities. The treated solutions were lyophilized and the residues dissolved in D_2O for the proton resonance experiments. The lyophilization and addition of D_2O were repeated to reduce the H_2O background peak in the proton resonance study. The nucleotide concentrations were varied form 5×10^{-4} *M* to 0.5 *M*. The pD of the solutions was adjusted to ≈7. The ionic strength of the low concentration nucleotide solutions was maintained at ≈0.3 *M* using recrystallized $NaClO_4$. G. Frederick Smith Co. $Mn(ClO_4)_2 \cdot 6H_2O$ was recrystallized from ethyl acetate upon addition of small amounts of toluene to the solution. The total Mn^{2+} concentration varied from 2×10^{-7} to 1×10^{-4} *M*.

Nmr Spectra. The ^1H and ^{31}P studies of competition binding were done at 60.0 and 24.3 MHz, respectively, on an extensively modified Varian HR-60 using a side-band lock for field stabilization. The field was "locked" to a trace amount of *p*-dioxane added to the solutions in the ^1H study, and to an internal capillary of P_4O_6 or 85% phosphoric acid in the ^{31}P study. The Varian preamplifier of the phosphorus probe in the competition studies was replaced by a microwave planar triode preamplifier designed and loaned to us by Dr. Melvin Klein (this latter preamplifier gave an improved signal-to-noise ratio).

The low concentration studies were subsequently done on a Varian HA-100 spectrometer using a Technical Measurement Corp. C-1024 time-averaging computer.

Competition Study

The nmr measurements are generally done on solutions with a large excess of nucleotide to metal ion such that $[M^{2+}]_0/[\text{nucleotide}]_0 \equiv f \ll 1$. The ratio *f* must be small compared with unity otherwise excessive magnetic broadening of the ATP resonances by the paramagnetic ion occurs. The symbol $[M^{2+}]_0$ denotes the *total* metal ion concentration, and includes the "free" metal ion in solution and metal ion complexed by the nucleotide. The symbol $[\text{nucleotide}]_0$ is similarly defined. If the earlier nmr measurements were indeed done on a 1:1 MATP complex in which the metal ion is simultaneously bound to the phosphate moiety and the adenine ring, then repeating the measurements on mixtures of ATP and AMP, where $[AMP]_0 \approx [ATP]_0 \gg [M^{2+}]_0$, should reveal little or no broadening of the AMP resonances by the paramagnetic ion. In addition, the AMP resonances should remain sharp, essentially the same as in an AMP solution free of paramagnetic metal ion even when the metal ion concentration is sufficiently large to broaden the ATP resonances beyond detection. This behavior would be a consequence of the fact that the ATP should compete more than an order of magnitude more favorably than AMP for the small amount of metal ion present in solution.

In the case of Mn^{2+}–AMP, the proton line widths at room temperature are dipolar determined, and are the same as that found for solutions of Mn^{2+}–ATP (*f*

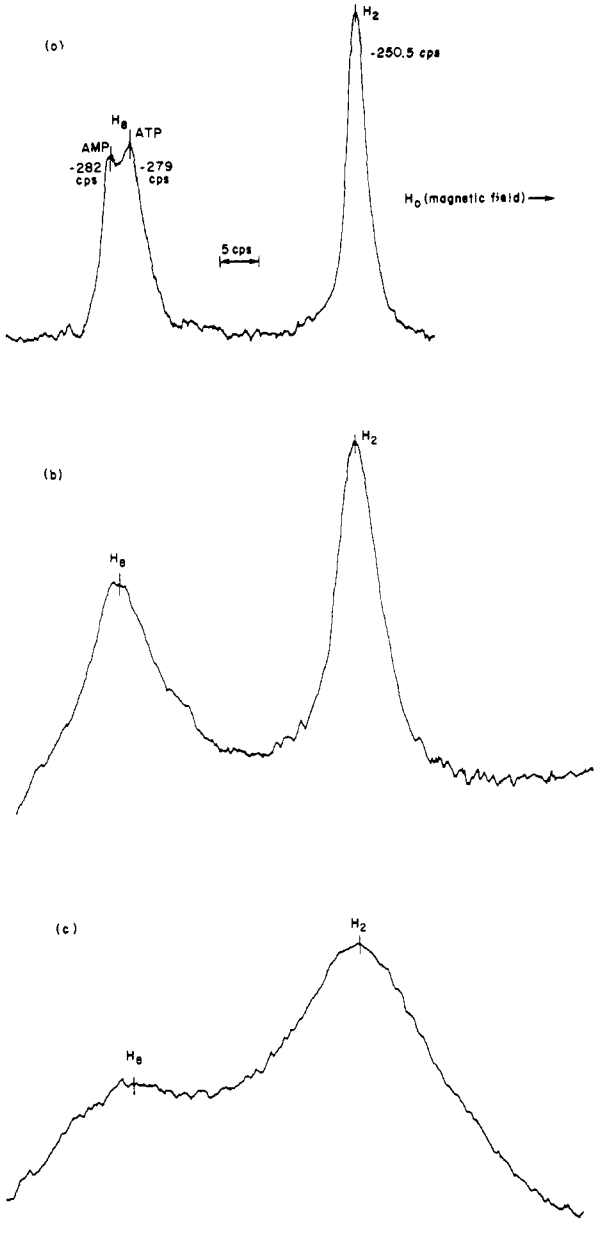


Figure 1. ATP–AMP competition study, room temperature, pD 7.6, $[ATP]_0 = [AMP]_0 = 0.25\ M$: (a) $[Mn^{2+}]_0 = 0$; (b) $[Mn^{2+}]_0 = 10^{-4}\ M$; (c) $[Mn^{2+}]_0 = 10^{-3}\ M$. The H_2 and H_1 (not shown) protons of ATP and AMP coincide. Shifts in cps are downfield of the *p*-dioxane "lock" reference. The intensities of H_2 and H_8 resonances are not commensurate.

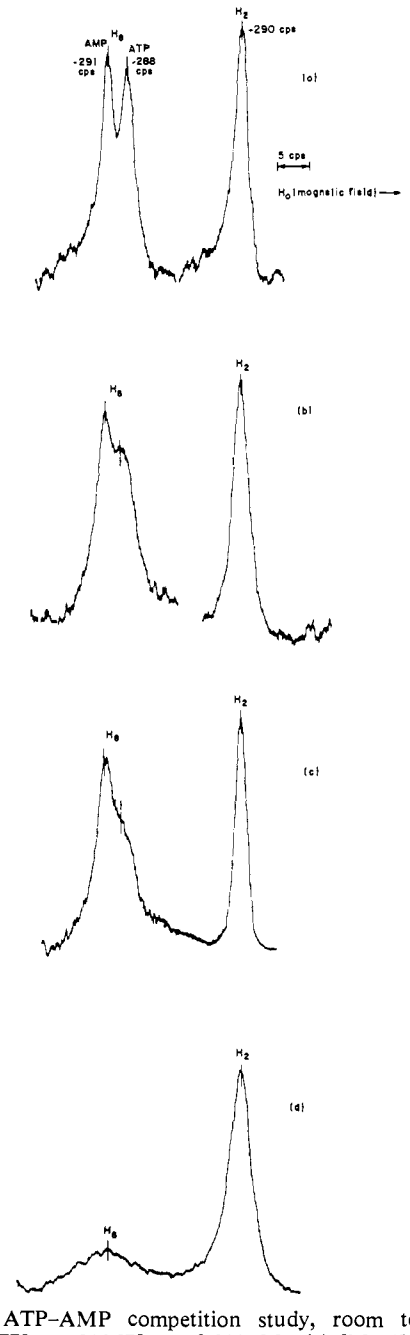


Figure 2. ATP–AMP competition study, room temperature, pD 7.6, $[ATP]_0 = [AMP]_0 = 0.011\ M$: (a) $[Mn^{2+}]_0 = 0$; (b) $[Mn^{2+}]_0 = 2 \times 10^{-6}\ M$; (c) $[Mn^{2+}]_0 = 4 \times 10^{-6}\ M$; (d) $[Mn^{2+}]_0 = 4 \times 10^{-5}\ M$. The H_2 and H_1' (not shown) protons of ATP and AMP coincide. Shifts in cps aredown field of the *p*-dioxane "lock" reference. The intensities of the resonances are not commensurate.

held constant) once exchange rate corrections are made.[10] That is, the Mn^{2+}–proton distances in Mn^{2+}–AMP solutions are the same as the proton–metal ion distances reported[3] for Mn^{2+}–ATP. The results of a proton magnetic resonance study of solutions containing Mn^{2+} and equimolar mixtures of ATP and AMP (pD $\approx$ 7) are shown in Figure 1 ($[ATP]_0 = [AMP]_0 = 0.25\ M$) and in Figure 2 ($[ATP]_0 = [AMP]_0 = 0.011\ M$). These results are in clear disagreement with the assumption that a 1:1 metal–ligand complex is the dominant[3] species in the nmr study between 0.02 *M* and 0.5 *M* total nucleotide. At the high nucleotide concentration the ATP and AMP proton resonance lines of the equimolar mixture are, within experimental error, broadened the same by the Mn^{2+} despite the fact that the binding constant for MnATP is $\approx$300 times greater than MnAMP. Furthermore, the line broadening which occurs upon adding $10^{-4}\ M$ Mn^{2+} to the equimolar mixture of ATP and AMP (Figure 1c) is the same, within $\approx$50%, as occurs[3] when $10^{-4}\ M$ Mn^{2+} is added to 0.35 *M* ATP. In the latter case the Mn^{2+} was shown[3] to be $\approx$3.5 Å from the H_8 proton, and 4.8 Å from the H_2 proton. The competition study clearly indicates that at the high nucelotide concentration the Mn^{2+} divides approximately equally between the ATP and AMP ring sites, and that when it is at an ATP or AMP site it is $\approx$3.5 Å from a H_8 and 4.8 Å from a H_2 proton.

(10) R. G. Shulman and H. Sternlicht, unpublished results.

The Mn^{2+} ion broadens the proton resonances more than it shifts them. The Fermi contact shift consequently cannot be obtained.[3] In the case of Ni^{2+}, where the induced shifts at high temperature are larger than the induced broadening, the contact shift can be obtained. The competition studies were therefore repeated at 80° with Ni^{2+}. Again, the AMP and ATP protons of the equimolar nucleotide mixture were broadened the same extent by the Ni^{2+}. In addition, the Fermi contact shifts observed for the AMP protons in the equimolar mixture were the same as observed for the ATP protons. Since the relative magnitudes of the Fermi contact shifts in the Ni^{2+}–ATP solution are strongly indicative[3] of binding to the N-7 position, we must conclude that binding to the N-7 position of the AMP in the equimolar nucleotide mixture also occurs.

The simplest complex consistent with the nmr results is the complex MA_2 (A = nucleotide ligand), where the metal ion binds to the ATP phosphorus moiety and to the adenine ring of a second nucleotide. In mixtures of ATP and AMP the second nucleotide may be ATP or AMP. Below, we will often designate this complex as A_PMA_R to denote simultaneous binding to the phosphates and ring. Complexes of the type A_PMA_P where the metal ion is simultaneously bound to the phosphate moieties of two different nucleotides are in the minority. This statement is based on electrostatic grounds, and is experimentally confirmed by the ^{31}P competition studies done at room temperature.

The results of the ^{31}P AMP–ATP competition study using Mn^{2+} are summarized in Figure 3 in terms of a log fT_{2p} *vs.* (temperature)$^{-1}$ plot. The competition results have been superimposed on the earlier ATP and AMP study of Shulman, Sternlicht, and Anderson.[3] For purposes of comparison, we have arbitrarily defined f in these competition studies, where $[ATP]_0 = [AMP]_0$, to be $f \equiv [M^{2+}]_0/ATP]_0$. Here T_{2p} is the transverse relaxation time of the nucleotide ^{31}P spin in the presence of the metal ion, after all background impurity contributions have been subtracted, with the definition

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_{2o}} \tag{1a}$$

where

$$\frac{1}{T_2} = \pi\Delta\nu \qquad \frac{1}{T_{2o}} = \pi\Delta\nu_o \tag{1b}$$

In the above T_{2o} is the (^{31}P) transverse relaxation time of the nucleotide solution before adding the paramagnetic metal ion; T_2 is the transverse relaxation time after adding the paramagnetic ion; $\Delta\nu_o$ and $\Delta\nu$ are the respective line widths as measured at half-height.

The ^{31}P spin relaxation time, T_{2p}, is exchange controlled at room temperature (Figure 3). That is, the rate-determining step for relaxation is the rate at which the nucleotide binds to the metal ion. (Once binding occurs, relaxation is very rapid.) This means that T_{2p} is given by [11]

$$1/T_{2p} = f/\tau_M \tag{2}$$

and corresponds to the time the nucleotide remains in solution before chemically exchanging with another nucleotide bound to the metal ion. As the temperature is raised, the exchange rate increases, until at sufficiently high temperature where $\tau_M < T_{2M}$ the rapid exchange limit is reached and now

$$1/T_{2p} = f/T_{2M} \tag{3}$$

as occurs in the case of AMP (curve II, Figure 3). In eq 3 T_{2M} is the relaxation time of the ATP when in the coordination shell of the metal ion.

The larger fT_{2p} is, the smaller is the broadening produced by the metal ion for a fixed f value. In the ^{31}P competition study fT_{2p} for ATP is the same, within experimental error, as that reported by Shulman and Sternlicht for ATP alone (curve I, Figure 3). On the other hand, fT_{2p} for the AMP in the equimolar ATP–AMP mixture is 30 times larger than that reported for AMP alone (curve II). In other words, the line widths of the ATP ^{31}P in the competition study are, within experimental error, unaffected by the presence of the AMP whereas the AMP ^{31}P line width is narrower by a factor of $^1/_{30}$ as a result of ATP being present. These observations are consistent with the proposed binding model (A_PMA_R as the dominant, A_PMA_P as the minority species), although some questions remain. On the basis of the stability constant ratios for the MA complexes one might have expected a narrowing of the AMP ^{31}P line in the competition study by a factor of $^1/_{300}$ rather than $^1/_{30}$. This factor of $^1/_{10}$ discrepancy may mean that (1) although the Mn^{2+} in A_PMA_R is bound to the triphosphate moiety and the adenine ring of AMP there is still a long-range dipolar interaction ($r \sim 4$ Å) between the Mn^{2+} and the phosphorus of the AMP, or (2) there is a minority species of the order of 10% in which the Mn^{2+} is simultaneously bound to the triphosphate of ATP and the monophosphate of AMP, or (3) the ratio of the stability constants for the MA_2 conplexes may actually be much less than the ratio of 300 for the MA complexes. These three possibilities could be distinguished, in principle, by a temperature and binding study, but these effects are of no significance to the kinetic analysis.

ATP has been shown to stack in aqueous solution at high nucleotide concentration.[3] The stacking is anal-

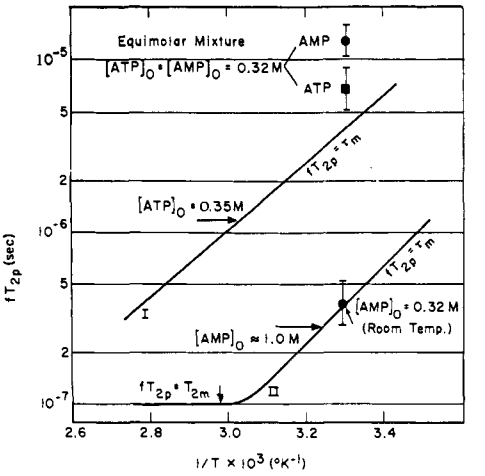


Figure 3. The room temperature phosphorus-31 competition study, pH ≈ 7. The nucleotide composition of the various ATP–AMP solutions are indicated. Curves I and II were determined by Shulman, *et al.*[3] The fT_{2p} values of the α-, β-, γ-phosphorus resonances of ATP, are within experimental error, the same.

(11) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 37, 307 (1962).

ogous to that reported by Chan, *et al.*,[12] in a large number of 3′,5′-dinucleotides, and by Schweitzer, *et al.*, in AMP.[13] The presence of the triphosphate groups apparently does not significantly affect the stability of the stack since $(AMP)_2$ and $(ATP)_2$ appear to be approximately of the same stability. This is suggested by the fact that the AMP and ATP resonances, in particular the H_2 and H_1', are virtually superimposable over the nucleotide range of 0.02–0.5 *M* (Figures 1 and 2). The degree of stacking of ATP is extensive ($\gtrsim 50\%$) at the high nucleotide concentration, while at low concentration, $[ATP]_0 = 0.02M$, $\lesssim 15\%$ of the nucleotides are stacked. The binding constant, K_D, can be estimated from the ring proton shift dependence on $[ATP]_0$ reported in the earlier nmr study[3] if one neglects higher order associations, $[ATP]_n$, $n > 2$. Within this approximation

$$K_D = [A_2]/[A]^2 \approx 5\ M^{-1} \qquad (4a)$$

We propose, therefore, that the dominant conformer of MA_2 consistent with the nmr would have the -4 negatively charged triphosphate moieties as far apart as possible to minimize electrostatic repulsion, while the metal ion binds to the triphosphate of one ATP and to N-7 of the second ATP of the stack.

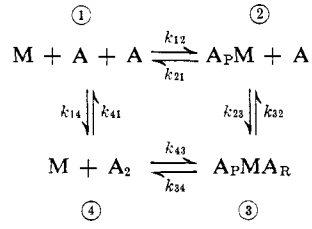
The competition results suggest that the stability constant for MA_2 formation, K_{43}, may be as much as five to ten times greater than that for MA. This is expected because of the additional binding to the ring in the MA_2 case. Consider $K_{43} = [MA_2]/[M][A_2]$; $K_{12} = [MA]/[M][A]$, then $[MA_2]/[MA] = B[A_2]/[A]$ where

$$B = K_{43}/K_{12} \qquad (4b)$$

At $[A]_0 = 0.02\ M$, the competition study indicates that $[MA_2] \approx [MA]$. Using $K_D \approx 5\ M^{-1}$ we would estimate that $[A_2]/[A] \sim 0.1$–0.2. Therefore B must be ≈ 5–10. Furthermore, one can easily show that $K_{23} = BK_D \approx 25$–$50\ M^{-1}$. The lower estimate for K_{23} is in reasonably good agreement with Brintzinger and Palmer's approximate value.[7]

Kinetic Rate Constants

The kinetic scheme proposed for the binding of Co^{2+}, Ni^{2+}, and Mn^{2+} to ATP is shown in modified Scheme I. In this section we will derive estimates of the rate



constants. In a later section we use this scheme to predict the nmr line widths for a fixed f as the nucleotide concentration is decreased from 0.35 *M* to $5 \times 10^{-4}\ M$. The observed results turn out to be in good agreement with the predicted behavior.

At high ATP concentrations, and $f \ll 1$, where MA_2 is the dominant metal ion complex, only the kinetics for $4 \rightleftharpoons 3$ is accessible to nmr. At low ATP concentrations ($[ATP]_0 \approx 0.001\ M$), where MA is the principal metal ion complex, only $1 \rightleftharpoons 2$ is accessible to nmr. In general the nmr results turn out to be relatively insensitive to the kinetic details of the $2 \rightleftharpoons 3$ process. However, the temperature-jump measurements by Hammes and Miller in the ATP concentration range 0.01–0.1 *M*, $f \approx 1$, is sensitive to the $2 \rightleftharpoons 3$ process, in addition to $1 \rightleftharpoons 2$. The nmr and temperature-jump techniques thus complement each other and, in principle, provide rather complete kinetic information for Scheme I.

The kinetic rate constants of Scheme I can be readily filled for the following reasons. (a) The first- and second-order rate constants k_{21} and k_{12}, respectively, were determined by Hammes and Levison.[4] (b) Upper bounds for k_{23} and k_{32} can be derived from the nmr and the temperature-jump measurements of Hammes and Miller.[5] (c) The rate constant, k_{34}, can be determined from the nmr measurements of Shulman, *et al.*[3] (d) The value of k_{43} can be readily estimated in terms of the parameter B previously discussed. (e) We estimate that k_{41}, the rate at which the stacked A_2 dimer dissociates at room temperature, is $\approx 1 \times 10^9$ to $1 \times 10^{10}\ sec^{-1}$ at room temperature, analogous to the times found by Chan and coworkers for the 3′,5′-dinucleotides.[14] In order to have $K_D \approx 5\ M^{-1}$, k_{14} would have to have a value of $\approx 5 \times 10^9$ to $5 \times 10^{10}\ M^{-1}\ sec^{-1}$.

Proceeding with the analysis in detail, Shulman and Sternlicht's nmr measurements were done at high ATP concentrations, and $f \ll 1$. The MA_2 complex dominates as shown by the competition study (Figure 1). These investigators reported for Mn^{2+} and Ni^{2+} that the length of time, $\tau_M{}^P$, the metal ion remained bound to the phosphate moiety was the same as the length of time, $\tau_M{}^R$, the metal ion remained bound to the ring before chemical exchange occurred. (In case of Co^{2+} there was considerable evidence that Co^{2+} binding to the ring was thermolabile, and weaker than in the Mn^{2+} and Ni^{2+} complexes.) The observed equalities $\tau_M{}^R = \tau_M{}^P = \tau_M$ signify that either the chemical exchange of the bulk ATP with $M(ATP)_2$ proceeds *via* $3 \rightarrow 4$ rather than $3 \rightarrow 2$ (*i.e.*, $k_{32} < k_{34}$), or that the exchange proceeds *via* $3 \rightarrow 4$ and $3 \rightarrow 2$ with equal probability (*i.e.*, $k_{32} \approx k_{34}$). If $k_{32} > k_{34}$ so that dissociation of $M(ATP)_2$ proceeded *via* $3 \rightarrow 2$, $\tau_M{}^R < \tau_M{}^P$ would have been reported. We are thus able to put an upper bound on k_{32}: $k_{32} \lesssim k_{34}$ where $k_{34} = 1/\tau_M$. We use the k_{34} values based on the room temperature τ_M values reported by Shulman and Sternlicht for 0.35 *M* ATP. A value for k_{23} follows from $k_{23} = k_{32}K_{23} \leq K_{23}/\tau_M$.

Continuing, k_{43} is given by $k_{43} = k_{34}K_{43} = BK_{12}/\tau_M$. Since all the terms in the k_{43} expression are known or estimable (*i.e.*, $B \gtrsim 5$–10, K_{12}, the stability constant, is given in the literature, and τ_M is determined from the high concentration nmr study) k_{43} can be obtained (*vide* Table I).

Comparison of the two sets of association rate constants k_{12} and k_{43} show the latter to be larger in every case. This increase is due to the increased electrostatic attraction resulting from the higher charge on the dimer. The effective charge on the ligand is certainly

(12) S. I. Chan, B. W. Bangerter, and H. H. Peter, *Proc. Natl. Acad. Sci. U. S.*, 55, 720 (1966); S. I. Chan and J. H. Nelson, to be submitted for publication.

(13) M. P. Schweitzer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Am. Chem. Soc.*, 90, 1042 (1968).

(14) M. Eigen, W. Kruse, G. Maass, and L. DeMaeyer, *Progr. Reaction Kinetics*, 2, 287 (1964).

Table I. Rate Constants[f] or Scheme I[a]

	k_{12},[b] M^{-1} sec^{-1}	k_{21},[b] sec^{-1}	K_{12}[c] $= k_{12}/k_{21}$, M^{-1}	k_{34},[d] sec^{-1}	k_{43}, M^{-1} sec^{-1}	k_{32}, sec^{-1}	k_{23}, M^{-1} sec^{-1}
Ni^{2+}	4×10^6	4×10^1	1×10^5	1.2×10^3	4×10^8	1.2×10^3	2×10^4
Co^{2+}	9×10^7	2×10^3	4.6×10^4	5×10^4	1.5×10^{10}	5×10^4	1×10^5[e]
Mn^{2+}	$\sim 3 \times 10^8$[f]	$\sim 6 \times 10^3$[f]	5×10^4	2×10^5	6×10^{10}	2×10^5	3.5×10^6

[a] The equilibrium constants K_D, K_{23}, and K_{43} are set equal to 6 M^{-1}, 36 M^{-1}, and $6K_{12}$, respectively. [b] Reference 4. [c] E. Wallaas, *Acta Chem. Scand.*, **12**, 528 (1968). [d] Reference 3. [e] The Co^{2+} ring binding appears weaker than in the case of Ni^{2+} and Mn^{2+}. [f] Estimated from $k_{21} \approx {}^1/_{30}k_{34}$, as observed in Ni^{2+} and Co^{2+} cases, and $k_{12} = K_{21}k_{21}$.

Table II. A Comparison of the Concentrations[a] of the Various Species in Solution (pH $\approx$7)[b]

f	$[M]_0$	$[ATP]_0$	[MA]	[ATP]	[M]	$[M(ATP)_2]$	$[(ATP)_2]$
1	1×10^{-1}	1×10^{-1}	$\approx 1 \times 10^{-1}$	5×10^{-4}	2×10^{-3}	1.7×10^{-3}	1.2×10^{-6}
	1×10^{-2}	1×10^{-2}	$\approx 1 \times 10^{-2}$	2.5×10^{-4}	3.5×10^{-4}	8.7×10^{-5}	3.4×10^{-7}
1×10^{-4}	1×10^{-5}	1×10^{-1}	3.6×10^{-6}	5×10^{-2}	Negligible	6.4×10^{-5}	2.5×10^{-2}
	1×10^{-6}	1×10^{-2}	7.5×10^{-7}	8.5×10^{-3}	Negligible	2.5×10^{-7}	7.5×10^{-4}

[a] Equilibrium constants same as in Table I. Concentrations are molar. [b] When $f = 1$, as in temperature jump, and $f = 1 \times 10^{-4}$, as in nmr ($f = [M]_0/[ATP]_0$).

less than the formal charge of -8, however, as a result of the proposed dimer geometry in which the two phosphate moieties are at opposite sides. Because of the uncertainty in true charge, the diffusion-controlled limit for these reactants cannot be explicitly calculated.[14] However, it would appear safe to say that this limit is approached only by the pair $Mn^{2+} + A_2^{8-}$.

As a result of the kinetic analysis presented here, it is also possible to understand the temperature-jump experiments[5] in the intermediate nucleotide concentration range of 0.01–0.10 M, where f, the ratio of total metal to total ATP concentration, varied from 0.5 to 1.0. Let us consider the 1:1 metal–nucleotide solution. According to the equilibrium quotients derivable from or presented in Table I, the following inequalities hold for the intermediate concentration range ($f = 1$): $[ATP]_0 \approx [MATP] > [M]$; $[M] > [ATP]$; $[M] \gtrsim [M(ATP)_2]$; $[ATP] \gg [(ATP)_2]$. In Table II we tabulate the approximate concentrations of the various species (pH $\approx$ 7) in the temperature-jump experiment[5] where $f \approx 1$, and compare these values with the nmr case where $f \ll 1$.

Hammes and Miller[5] reported two relaxation times in the intermediate nucleotide concentration range for Ni^{2+} and Co^{2+}. One of the relaxation times, which we designate as τ_s, was short and relatively insensitive to $[ATP]_0$ and pH; the other, which we designate as τ_1, was long and highly dependent on $[ATP]_0$ and pH. In the cases of Ni^{2+} (25°) and Co^{2+} (11°), τ_s was approximately 3–6 $\times$ 10^{-4} sec and 2–3 $\times$ 10^{-5} sec respectively, whereas τ_1 was $\approx 10^{-1}$ sec in the case of Ni^{2+} and 10^{-2} in the case of Co^{2+}.

Hammes and Miller did not assign either of the observed relaxation processes to the reaction $1 \rightleftharpoons 2$, as had hitherto been the case in the previous temperature-jump experiment. Instead, they used the following information to make a new assignment. (1) The relaxation time for the $1 \rightleftharpoons 2$ process would be too short to observe in the 0.01–0.1 M concentration range. (2) τ_s was approximately equal to τ_M. (3) The ATP adenine ring absorption and the M^{2+} absorption relaxed with the same τ_s.

These observations together with the relative insensitivity of τ_s to $[ATP]_0$ as $[ATP]_0$ varied from 0.01 to 0.1 M suggested τ_s corresponded to an intramolecular process. They proposed as two likely possibilities for τ_s that in the MA species there may be (a) a tautomerism of the metal ion between the adenine ring and the triphosphate chain, or (b) an opening and closing of a folded backbound complex,[8] $MA_{P,R}$ ($MA_R \rightleftharpoons MA_{P,R} \rightleftharpoons MA_P$). Mechanisms a or b would be reasonably consistent with the nmr as reported in the literature.[3]

The nmr competition study above, however, clearly shows that τ_M is associated with the MA_2 complex, *i.e.*, with the $4 \rightleftharpoons 3$ process. The temperature-jump experiment must be reopened for interpretation.

Consider the entire reaction mechanism I. This system possesses five reaction variables and two conservation equations (*i.e.*, mass balances for metal and ligand, respectively); hence, it possesses three relaxation times.[15] Computation of these times involves the diagonalization of a 3 $\times$ 3 matrix. Before undertaking such an evaluation, it would be well to indicate the major uncertainties in this process. First, a simple computation of the relaxation time for the isolated process $1 \rightleftharpoons 4$ indicates a time on the order of nanoseconds. Consequently, in principle, all of the other processes will be influenced by the values we choose for k_{14} and k_{41}, that is, for the equilibrium quotient K_D. In practice, the calculations are insensitive to K_D because of its relatively small value. A factor of 2 to 3 error in estimating K_D has no significant effect. Second, it is necessary to estimate the concentrations of all the species in I.

In carrying out the evaluation, however, we are seeking the answers to two simple questions. (1) Will one of the relaxation times for Scheme I be in the observed time range (*e.g.*, 3–6 $\times$ 10^{-4} sec for nickel(II))? (2) Will this relaxation time be relatively independent of concentration as Hammes and Miller observed? Our Scheme I provides an affirmative answer to these two questions. We find, based on the concentrations listed in Table II, that the secular determinant possesses a unique solution for $k_{32} = k_{34}$ of $\tau_i \sim 7 \times 10^{-10}$ sec, $\tau_{ii} = 6 \times 10^{-5}$ sec, and $\tau_{iii} = 6 \times 10^{-4}$ sec at 0.1 M. At the lower concentration limit of

(15) M. Eigen and L. DeMaeyer in "Techniques of Organic Chemistry," Vol. VIII, Part 2, A. Weissberger, Ed., John Wiley & Sons, New York, N. Y., 1963, p 895, also references therein.

0.01 M, τ_{iii} has the value 7×10^{-4} sec, whereas τ_{ii} changes appreciably with concentration.

On the basis of this interpretation of the relaxation data, we propose that the observed τ_s be assigned to τ_{iii}. We further propose, in agreement with Hammes and Miller, that the experimentally observed τ_1, which varied appreciably with $[ATP]_0$ and pH, arises from hydrolysis and the formation of polynuclear species. (An exact treatment would, of course, necessitate inclusion of this process into the complete reaction manifold.) Stated simply, we conclude that τ_s arises from the relaxation of $2 \rightleftharpoons 3$, coupled to the other steps of reaction mechanism I in a known way.

The temperature-jump measurements indicate that $k_{32} \sim \tau_M$, and the nmr measurements indicate that $k_{32} \lesssim \tau_M$. In Table I we have set $k_{32} = 1/\tau_M = k_{34}$. We estimate that $k_{32}, k_{34} \approx 30k_{21}$. This type of rate behavior has been exhibited by many other ligands. For bidentate amino acids complexed to nickel(II) or cobalt(II), for example, the dissociation constant for the bis complex is often 10 to 50 times greater than the dissociation constant of the mono complex.[16-18] The reasons underlying this kinetic behavior are not entirely clear at present. However, ligand charge[17] and structure[19] play an important role in determining the lability of coordination positions in substituted aquo complexes. In view of this fact, the approximate equality of k_{32} and k_{34} also seems reasonable.

It is also desirable to compare the estimated value of k_{23} to the much more precisely determined k_{12}. In order to do so it will be useful to know how the first- and second-formation rate constants for other ligands are related. For neutral and most -1 ligands, the second rate constant is observed to be comparable to the first.[16] For ligands of charge -2, and/or for large ligands (peptides, for example[16]), however, the second rate constant is less than the first. This effect has been ascribed primarily to decreased electrostatic attraction between the reaction partners[17] and to steric blocking by the bound ligand.[18,19] For ATP, both effects would be more pronounced in step $2 \rightleftharpoons 3$. Indeed, there is actually electrostatic repulsion between the negatively charged mono complex and the incoming ligand. The bulky ATP molecule also hinders attack at the remaining unsubstituted coordination positions. Therefore, even if attachment of a negatively charged ATP molecule labilizes the water molecules which remain coordinated to the metal ion, as has been postulated,[15] the bimolecular complexation rate constant would be expected to be smaller for formation of the second, as opposed to the first, complex (*cf.* Table I).

A word of caution is perhaps in order. Ionic strength corrections have been neglected as well as higher order associations of the type $[ATP]_n$, $n > 2$. Below we measure BK_D to be approximately 50. Values of 20–80 for BK_D and 2–10 for K_D would not be outside the range of uncertainty considering the complexity of the problem.

(16) G. Davies, K. Kustin, and R. F. Pasternack, *Intern. J. Chem. Kinetics*, in press.
(17) G. G. Hammes and J. I. Steinfeld, *J. Am. Chem. Soc.*, **84**, 4639 (1962).
(18) J. I. Steinfeld and G. G. Hammes, *J. Phys. Chem.*, **67**, 928 (1963).
(19) (a) A. Kowalak, K. Kustin, R. F. Pasternack, and S. Petrucci, *J. Am. Chem. Soc.*, **89**, 3126 (1967); (b) D. W. Margerum and H. Rosen, *ibid.*, **89**, 3126 (1967).

The Nmr Line Widths as a Function of ATP Concentration

In this section we will show how $K_{23} = BK_D$ can be determined from line-width measurements of the nmr resonances, and will discuss the conformational aspects of MATP, as obtained from nmr measurements done at low $[ATP]_0$.

Simple expressions for the phosphorus and proton line widths as a function of nucleotide concentration can be derived provided the spin relaxation times are exchange controlled as a result of a sufficiently slow chemical exchange time. At high ATP concentration where the $3 \rightleftharpoons 4$ process dominates kinetically, the phosphorus nuclear relaxation rates $1/T_{2p}$, for Ni^{2+}, Co^{2+}, and Mn^{2+}, were shown to be exchange controlled below 100° (eq 2). At the high nucleotide concentration limit $1/\tau_M = 1/\tau_M^{\infty} = k_{34}$. As $[ATP]_0$ is decreased, one predicts that, in the limit of low concentration where the $1 \rightleftharpoons 2$ process dominates, the apparent exchange rate $1/\tau_M$ at room temperature would have decreased by a factor of $^1/_{30}$ to the limiting value $1/\tau_M = 1/\tau_M^0 = k_{12}$. The ^{31}P relaxation rate remains exchange controlled (eq 2) as $[ATP]_0$ is decreased. An expression for $1/\tau_M$ as a function of $[ATP]_0$, B, and K_D can be readily derived.

There are two spin relaxation modes in the ^{31}P case. In the limit of slow exchange

$$A^* + M \xrightarrow{k_{12}} A^*M \xrightarrow{\text{fast}} AM \qquad (5a)$$

$$A_2^* + M \xrightarrow{k_{43}} A_2^*M \xrightarrow{\text{fast}} A_2M \qquad (5b)$$

We use an asterisk to denote an A or A_2 before it is relaxed. One can manipulate the relaxation modes (eq 5) subject to the inequalities $f \equiv [M]_0/[A]_0 \ll 1$, $[M] < [MA], [MA_2]$, which hold in the nmr case, to obtain eq 6a for the apparent exchange time, τ_M

$$\tau_M^{\infty}/\tau_M = R^P \qquad (6a)$$

where

$$R^P = B(1 + BK_D[A]_0C)^{-1}\left[\frac{1 - C}{2C} + \frac{1}{B}\left(\frac{\tau_M^{\infty}}{\tau_M^0}\right)\right] \qquad (6b)$$

$$C = \frac{[A]}{[A]_0} = (-1 + \sqrt{1 + 8K_D[A]_0})(4K_D[A]_0)^{-1} \qquad (6c)$$

where $(\tau_M^{\infty})^{-1} = k_{34}$; $(\tau_M^0)^{-1} = k_{21}$; $\tau_M^{\infty}/\tau_M^0 \cong {}^1/_{30}$.

A derivation of eq 6a is straightforward. A weighted average over the two relaxation modes (5a) is taken. This is appropriate because of the very rapid chemical exchange between A_2 and A. That is, the rate at which A associates to form A_2 and the rate at which A_2 dissociates to form 2A is very rapid compared with the formation and dissociation rates of MA and MA_2 ($f \ll 1$). One notes, as expected, that $R^P \rightarrow 1$ for high total ATP concentrations where $C \rightarrow (2K_D[A]_0)^{-1/2} \ll 1$, and $R^P \rightarrow \tau_M^{\infty}/\tau_M^0 = {}^1/_{30}$ for low total ATP concentration where $C \rightarrow 1$. Note that R^P is sensitive mainly to the product $BK_D = K_{23}$.

In the proton case the situation is much more complicated. We shall only discuss the Mn^{2+}–ATP system since this was the only one studied. At room temperature and high ATP concentration, the relaxation rates

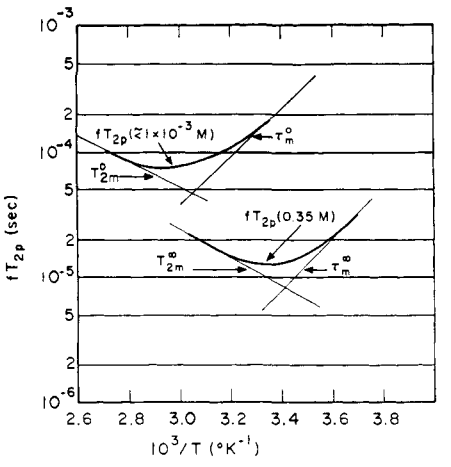


Figure 4. Predicted proton fT_{2p} behavior ($f \ll 1$) for MnATP at low nucleotide concentration ($[ATP]_0 \lesssim 1 \times 10^{-3}$ M) where the $1 \rightleftharpoons 2$ kinetically dominates. The proton is assumed to be a distance of ≈ 3.5 Å from the Mn^{2+} ion.

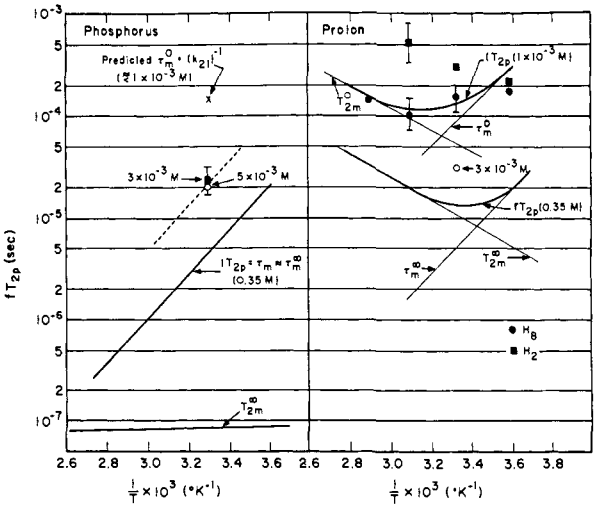


Figure 5. Observed fT_{2p} behavior for Mn–ATP phosphorus and proton resonances as a function of $[ATP]_0$ (pD $\approx$ 7, $f \approx 2$–4×10^{-4}). The expected temperature dependance of the ^{31}P line width at 5×10^{-3} M is shown as a dotted line.

of the H_2 and H_1' protons correspond to the fast exchange value (eq 3) since $1/\tau_M > 1/T_{2M}$. The H_8 proton relaxation rate at room temperature is in an intermediate region where $1/\tau_M \approx 1/T_{2M}$. The fast exchange value, $1/T_{2M}$, of all three protons are dipolar determined[3] and are proportional to τ_c/r^6 where τ_c is a rotational correlation time, and r denotes the distance of the metal ion to the proton of interest. If one lowers the ATP concentration, one reduces the apparent exchange rate, $1/\tau_M$, so that T_{2p} for H_8 should go from the intermediate region into the slow exchange region. The situation is actually still more complicated if the metal–proton distances and/or viscosity change with nucleotide concentration (which means T_{2M} would also be a function of $[ATP]_0$). For example, at high concentration where the MA_2 complex dominates, the metal ion is ≈ 3.5 Å from H_8. At low nucleotide concentration, MA dominates. If MA is not a back-bound complex, the Mn–H_8 distance should increase relative to the MnA_2 case.

In Figure 4 we sketch the predicted fT_{2p} for the low concentration case ($[ATP]_0 \lesssim 0.001$ M) as a log function of $1/T(°K^{-1})$, and compare the predicted fT_{2p} with that observed for H_8 at high nucleotide concentration, where the Mn^{2+}–H_8 distance ≈ 3.5 Å. If backbinding occurs exclusively to N-7 in MnATP, then the fT_{2p} for H_8 would be predicted to be as shown. The room temperature value for $\tau_M{}^0$ was set equal to the room temperature $1/k_{21}$ value reported by Hammes and Levison.[4] We assumed that $\tau_M{}^0$ has the same activation energy as that measured[3] for $\tau_M{}^\infty$. At elevated temperatures the exchange rate is expected to become sufficiently rapid so that the fast exchange value $fT_{2p} = T_{2M}{}^0$ is obtained, where $T_{2M}{}^0$ is proportional to r^6/τ_c. The rotational correlation time, τ_c, is proportionally dependent on viscosity. The $T_{2M}{}^0$ plot of Figure 4 was obtained by setting the metal ion–proton distance, r, equal to 3.5 Å and using a τ_c ($\tau_c \approx 1 \times 10^{-10}$ sec at 25°) for the MnATP complex which is approximately one-half that for $Mn(ATP)_2$ used in the earlier nmr study. The factor of $^1/_2$ is a viscosity correction. (The viscosity of the pH $\approx$ 7, 0.34 M ATP solution relative to water at the same temperature is 2.35 at 25°, and 1.98 at 75° as measured in this laboratory.) If values for $T_{2M}{}^0$ are obtained which are significantly larger than the predicted $T_{2M}{}^0$, Figure 4, the implication is either τ_c is significantly smaller than 1×10^{-10} sec at room temperature, which is unlikely, or that r is larger than 3.5 Å and back-binding does not occur.

In the proton case, a temperature study of fT_{2p} at low total nucleotide concentration is therefore necessary to establish whether back-binding occurs in MATP, and to determine $\tau_M{}^0$ and T_{2M}. To a first approximation, T_{2p} of the H_8 proton at room temperature is equal to τ_M over the entire $[ATP]_0$ range of interest. An expression for τ_M can be readily derived. There are three relaxation modes of the proton spin in the limit of slow exchange.

$$A^* + M \xrightarrow{k_{12}} A^*M \xrightarrow{\text{fast}} AM \tag{7a}$$

$$AM + A^* \xrightarrow{k_{23}} AMA_R{}^* \xrightarrow{\text{fast}} A_2M \tag{7b}$$

$$A_2{}^* + M \xrightarrow{k_{43}} A_P{}^*MA_R{}^* \xrightarrow{\text{fast}} A_P{}^*MA_R \tag{7c}$$

The asterisks here are used to denote only unrelaxed protons. (The phosphorus relaxation was discussed above.) We obtain, again, subject to the restrictions, $\ll 1$, $[M] < [MA], [MA_2]$

$$\tau_M{}^\infty/\tau_M = R^H \tag{8a}$$

where

$$R^H = B(1 + BK_D[A]_0C)^{-1} \times \left[\left(\frac{1-C}{2C}\right)(1 + k_{32}\tau_M{}^\infty) + \frac{1}{B}\left(\frac{\tau_M{}^\infty}{\tau_M{}^0}\right)\right] \tag{8b}$$

and $\tau_M{}^\infty = 1/k_{34}$. Aside from the factor $1 + k_{32} \cdot \tau_M{}^\infty$ multiplying $(1 - C)/2C$ in eq 8b, R^H is the same as R^P. We note that $R^H \rightarrow 1 + k_{32}\tau_M{}^\infty \approx 1$–$2$ at high total ATP concentration,[20] and $R^H \rightarrow \tau_M{}^\infty/\tau_M{}^0 \cong {}^1/_{30}$ at low total ATP concentration.

(20) We have also neglected the possibility that when M^{2+} binds to the N-7 of the stack neighbor, it can also relax the protons of the parent A_P. However, the intramolecular proton–M^{2+} distances can be comparable to the intermolecular distances, and therefore relaxation *via* A_P may be important. In that case one would substitute

$$A_2{}^* + M \xrightarrow{k_{43}} A_P{}^*MA_R{}^* \xrightarrow{\text{fast}} A_PMA_R$$

for eq 7c, and R^H would now be essentially the same as (8b) except we

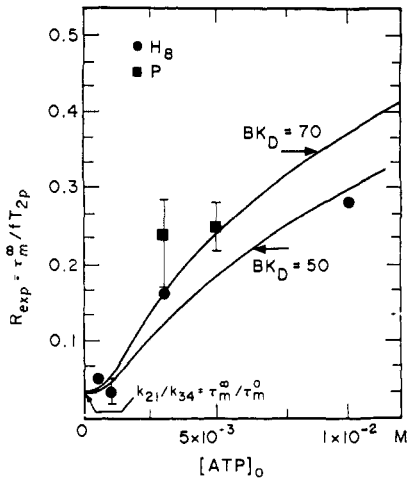


Figure 6. Plot of $R_{exp} = \tau_M^\infty/fT_{2p}$ as a function of $[ATP]_0$. Measurements for $[ATP]_0 = 5 \times 10^{-4}$ M are included.

Experimentally it is very difficult to measure the predicted small relative difference between R^H and R^P at the high concentration limit. We thus expect the observed τ_M from the proton nmr to agree within experimental error with τ_M from the phosphorus measurement over the entire range of $[ATP]_0$. Furthermore, the nmr is predicted to be sensitive to the $3 \rightleftharpoons 2$ process only through the K_{23} equilibrium constant, at best.

The results of our nmr study of Mn^{2+}–ATP are shown in Figures 5 and 6. The phosphorus study, which was done only at room temperature, required overnight signal accumulation, and was restricted to $[ATP]_0 \gtrsim 3 \times 10^{-3}$ M. A limiting value for fT_{2p} is apparently observed in the proton case when $[ATP]_0 \lesssim 1 \times 10^{-3}$ M. We note further that the temperature dependence of fT_{2p} for the H_8 at $[ATP]_0 = 1 \times 10^{-3}$ M, where MA dominates, indicates a crossover region $1/\tau_M \approx 1/T_{2M}$. The value we obtain at room temperature for τ_M when $[ATP]_0 = 1 \times 10^{-3}$ M is in excellent agreement with Hammes and Levison's value for k_{21}. Furthermore, the ratio of τ_M^∞ to the τ_M value of the low concentration sample ($[ATP]_0 = 1 \times 10^{-3}$ M) is $\approx 1/15$ to $1/30$, in excellent agreement with Scheme I.

would have to replace $[(1 - C)/2C](1 + k_{32}\tau_M^\infty)$ by $[(1 - C)/C](1 + (k_{32}\tau_M^\infty/2))$. This results in a 50% increase in R^H at the high total ATP concentration limit, and leaves the low concentration limit $R^H \rightarrow 1/30$ unchanged.

In Figure 6 we show R_{exp} at room temperature as a function of $[ATP]_0$ using both the phosphorus and H_8 proton fT_{2p} values. We have taken $R_{exp} = \tau_M^\infty/fT_{2p}$. We note that R_{exptl} can be fitted by R of eq 6b or 8b. In the case of the H_8 proton, fT_{2p} is only approximately equal to τ_M since T_{2M}, which also is seen to depend on $[ATP]_0$, contributes to fT_{2p} (Figures 4 and 5). We obtain as our "best fit" kinetic paramter $BK_D = K_{23} \approx 50$–70. The nmr low-concentration study is seen to give a value for K_{23} which, within experimental error, is consistent with the competition study ($K_{23} \approx 25$–50).

The results shown in Figures 5 and 6 strongly support Scheme I. We further estimate (Figure 5) for the H_8 proton that $T_{2M} \approx 6 \times 10^{-5}$ sec at room temperature when $[ATP]_0 = 1 \times 10^{-3}$ M. Assuming this value corresponds to the MnATP complex, and setting $\tau_c = 1 \times 10^{-10}$ sec, we obtain $r \approx 3.8$ Å ± 15% for the Mn^{2+}–H_8 distance. In a similar fashion we obtain $r \approx 5.5$ Å ± 15% for the Mn^{2+}–H_2 distance. These values are within experimental error those observed for the $[ATP]_0 = 0.32$ M sample.

The low concentration values correspond to the MnATP complex since it is not possible for the apparent T_{2M} values to arise from the small amount of $Mn(ATP)_2$ present. The close agreement between the Mn^{2+}–proton distances of the MnATP complex and those of the $Mn(ATP)_2$ complex is fortuitous.

The Mn^{2+} distance of ≈3.8 Å to the H_8 in the MnHTP complex could arise either (1) from the metal ion binding predominately to the N-7, or (2) from the metal ion being near the H_8, but separated from the adenine ring by a coordination shell water molecule. Fermi contact shift measurements could distinguish between these two possibilities. Unfortunately, reliable Fermi contact shifts cannot readily be obtained at low nucleotide concentration using Ni^{2+} or Co^{2+} because the chemical exchange rate, $1/\tau_M^0$, is much too slow, even at elevated temperatures. The uv difference study[6] strongly suggests alternative 2.

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