# Interaction of Divalent Manganese Ion with Adenosine Triphosphate and Related Compounds*

Michael J. Heller, Alan J. Jones, and Anthony T. Tu†

ABSTRACT: Complexes of divalent manganese with adenosine triphosphate and other nucleotides have been investigated by magnetic resonance techniques. Proton magnetic resonance broadening techniques have been used to quantitatively determine the spin–spin relaxation time ($T_2$), for water protons in $Mn^{2+}$–nucleotide solution. $T_2$ relaxation times were also measured for the proton resonances of the purine ring in ATP, ADP, AMP-5′, and polyadenylic acid, and for the pyrimidine ring protons of CTP and CMP-5′ in $Mn^{2+}$ solutions. Electron spin resonance studies gave equilibrium constants for $Mn^{2+}$–nucleotide complexes and enabled observation of the electron spin relaxation of the $Mn^{2+}$ ion when complexed with nucleotides. From water proton $T_2$ relaxation times, it has been possible to calculate the number of binding sites of $Mn^{2+}$ on the nucleotide. A value of between two and three was obtained for the 5′-triphosphates, whereas, for the corresponding 5′-monophosphates, a value of one is obtained. It has been concluded that in the $Mn^{2+}$–ATP complex and probably the other nucleoside triphosphate complexes, the binding of the metal ion to ring positions is negligible.

The problem of the interaction of metal ions with nucleotides and nucleic acids has been extensively studied (Weser, 1968). The adenosine triphosphate–metal complex, because of its importance in a great many biological reactions, has been of particular interest.

Two of the important metal ions associated with ATP are divalent magnesium and manganese. Since $Mn^{2+}$ is capable of replacing $Mg^{2+}$ in many biological reactions of the metal–ATP complex (Vallee, 1960), it would seem reasonable to expect both to have a similar structure. However, the structures reported in several recent nuclear magnetic resonance studies are quite different for each metal ion. Cohn and Hughes (1962) have shown that $Mg^{2+}$ ion does not affect the proton resonances in the purine ring, but observation of $^{31}P$ resonance for the $\beta$ and $\gamma$ phosphate groups in ATP indicates that this is the position of binding. Happe and Morales (1966), using $^{15}N$ nuclear magnetic resonance, provided further evidence for the absence of ring interaction by showing $Mg^{2+}$ ion had no effect on the nitrogen resonances in ATP. However, Sternlicht *et al.* (1965a,b) using proton and $^{31}P$ nuclear magnetic resonance to observe relaxation effects due to paramagnetic metal ion have concluded that $Mn^{2+}$ in the ATP complex is equally bound to the $\alpha$, $\beta$, and $\gamma$ phosphate groups, and to the adenine ring, between the N-7 and $NH_2$-6 position.

Independent studies, using alternative experimental procedures, support the nuclear magnetic resonance results for the $Mg^{2+}$–ATP complex, but conflict with the $Mn^{2+}$–ATP structure, where strong interaction at the adenine ring is suggested. Thus, Taqui Kahn and Martell (1962) using potentiometric techniques have shown that the stability constants greatly increase for both $Mg^{2+}$ and $Mn^{2+}$ in going from the AMP-5′ to the ATP complex. These workers propose that the phosphate groups are the major binding sites for the metal. This proposal is further supported by Walaas (1958), whose results show no change in the stability constant for either metal ion, if uridine or guanosine triphosphate replaces ATP. In addition, Martell and Schwarzenback (1956) suggest little or no interaction of either $Mg^{2+}$ or $Mn^{2+}$ with adenosine, since the observed stability constants are very small. Schneider *et al.* (1964), using ultraviolet difference spectra (a direct method of ring observation), have reported negligible ring binding for both $Mn^{2+}$ and $Mg^{2+}$ in the ATP complex.

These conflicting data illustrate the ambiguity concerning the problem of metal ion interaction with nucleotides; in particular, in the $Mn^{2+}$–ATP complex. We believe a detailed knowledge of the structure for $Mn^{2+}$–ATP is a prerequisite to understanding its role in various enzymatic reactions. Therefore, we have further examined the interaction of $Mn^{2+}$ with ATP, as well as various other nucleotides, using proton nuclear magnetic resonance and electron spin resonance spectroscopy.

## Experimental Section

Proton spectra of solutions of $Mn^{2+}$ and various nucleotides were recorded on a Varian A-60A nuclear magnetic resonance spectrometer. The instrument was equipped with a Varian V-6040 temperature control apparatus.

Electron spin resonance spectra were obtained on an Alpha Scientific Laboratories Model ALX-10, X-Band spectrometer. A special flat rectangular quartz cell of dimensions 4.0 × 0.75 × 0.030 cm was used for the aqueous samples.

All the nucleotides, nucleosides, and the polyadenylic acid used in our study were purchased from Sigma Chemical Co. and were of the highest grade obtainable. All materials were run through a Dowex 50 ion-exchange column to remove any background metal ions. Stock solutions of $Mn^{2+}$ ion were made from Baker Analyzed Reagent $MnCl_2 \cdot 4H_2O$ crystals. The concentrations of nucleotide and nucleoside sample solutions were measured by standard ultraviolet methods.

* From the Departments of Biochemistry and Chemistry, Colorado State University, Fort Collins, Colorado 80521. *Received June 22, 1970.* This investigation was supported by an NIH Career Development Award, No. 1 K4 6M41 786, from the National Institute of General Medical Sciences to A. T. Tu.

† To whom correspondence should be addressed.

In nuclear magnetic resonance studies, where proton relaxation of the base portion of the nucleotide was observed, samples were lyophilized twice from $D_2O$ to remove most of the background water and then redissolved in $D_2O$. In all nuclear magnetic resonance work, the pH was adjusted to 7.8 ± 0.1, using concentrated NaOH (or NaOD) and $HNO_3$, except for polyadenylic acid samples where the pH was 6.2 ± 0.1. In electron spin resonance studies, samples were made up in 0.1 M Tris buffer of pH 7.8 ± 0.1.

*Theoretical Basis.* The magnetic resonance lines of ligand nuclei, which are near the binding sites of paramagnetic metal ions, are found to broaden because of the ability of the metal ions to shorten the relaxation time of the nuclei. The theory of this phenomena has been discussed by McConnell (1958), McConnell and Berger (1957), and Swift and Connick (1962).

In our studies, measurements of spin–spin relaxation time ($T_2$), for ligand protons, are obtained from the nuclear magnetic resonance line widths by the following equation (Shulman *et al.*, 1965):

$$T_2 = \frac{1}{\pi\Delta\nu} \tag{1}$$

where $\Delta\nu$ represents the frequency separation of the peak at one-half amplitude.

Data obtained from these experiments are reported in terms of $fT_2p$, where $f$ is given by:

$$f = \frac{(Mn^{2+})\text{total concentration}}{(\text{Nucleotide})\text{total concentration}} \tag{2}$$

$f$ is usually of the order of $1.0 \times 10^{-4}$, since in most experiments $Mn^{2+}$ concentration is $5 \times 10^{-5}$ M. $T_2p$ is the actual contribution of $Mn^{2+}$ ion to the broadening of the proton resonance. It is given by:

$$T_2p = 1/[\Delta\nu(MN) - \Delta\nu(N)]\pi \tag{3}$$

where $T_2N$ is the relaxation time of the nucleotide proton when no $Mn^{2+}$ is present in solution, and $MN$ is the relaxation time of the same proton with $Mn^{2+}$ present in solution. Multiplication of $f$ by $T_2p$ gives the normalized value (Shulman *et al.*, 1965), which is used to report the data.

The proton resonance of water, usually observed as a single sharp peak, is found to broaden (relax) upon addition of paramagnetic metal ions. The theory of this effect has been discussed by Bloembergen and Morgan (1961).

When a ligand molecule is added to a solution of paramagnetic metal ions, $Mn^{2+}(H_2O)_6$, each ligand binding site should, in theory, prevent a water molecule from entering the coordination sphere of the metal ion and being relaxed. The number of ligand binding sites for $Mn^{2+}$ ion can be obtained from water proton relaxation times ($T_2$), using the following equation (Eisinger *et al.*, 1965):

$$T_2^{-1} = {}^1/_3S(S + 1)(A^2/h^2)\tau_ePN \tag{4}$$

where $S$ refers to electron spin; $A$ is the isotropic indirect hyperfine interaction; $\tau_e$ is the correlation time for the isotropic hyperfine interaction; $P$ is the probability that a proton is in the hydration sphere of the paramagnetic ion; $N$ is the molar concentration of paramagnetic ion.

In our studies, the number of primary binding sites is obtained using a simplified form of eq 4, proposed by Sternlicht *et al.* (1965a):

$$T_2 \propto (A^2P\tau_e) \tag{5}$$

It was also proposed that $\tau_e$ and $A$ remain constant for the $Mn^{2+}$–ligand complex. Therefore, the relationship $T_2 \propto P$ is obtained. This means that the change in $T_2$, due to the ligand binding $Mn^{2+}$ ion, is directly proportional to the number of binding sites on the ligand molecule which can effectively eliminate one or more of the six coordinated water molecules of the $Mn^{2+}$ ion.

In our electron spin resonance work, stability constants for various $Mn^{2+}$–nucleotide complexes are obtained from electron spin resonance spectra using the procedures given by Cohn and Townsend (1954) and Larsson-Raznikiewicz and Malmström (1961), where the amplitude of the observed spectrum is a measure of the uncomplexed $Mn^{2+}(H_2O)_6$. The stability constant is calculated using the equation:

$$K = \frac{(Mn^{2+}\text{–ligand concn})}{(Mn^{2+}\text{ concn})(\text{ligand concn})} \tag{6}$$

The value for ($Mn^{2+}$–ligand) is readily obtained, since original concentration of ($Mn^{2+}$) and (ligand) are known, and uncomplexed $Mn^{2+}$ is determined from the electron spin resonance results.

## Results

The effect of paramagnetic $Mn^{2+}$ ion on the $H_8$, $H_2$, $H_1'$ proton resonances of ATP, ADP, AMP-5′, and polyadenylic acid is shown in Figure 1. It can be seen that the $H_8$ resonances of the adenosine compounds are broadened much more than are the $H_2$ or $H_1'$ resonances. For ATP and ADP, this has previously been interpreted to indicate that $Mn^{2+}$ ion interacts to some degree with the adenine ring portion of these molecules, most probably at the N-7 and/or $NH_2$-6 position (Cohn and Hughes, 1962).

It should be further noted that the broadening of the $H_8$ resonance increases as the phosphate residues decrease in going from ATP to AMP-5′. This broadening is presented quantitatively in the relaxation times ($fT_2p$) given in Table I. These values represent the normalized actual contribution of the $Mn^{2+}$ ion ($4 \times 10^{-5}$ M) to the broadening of the nucleotide ($5 \times 10^{-1}$ M) proton resonances at 37° and pH 7.8 ± 0.1. In agreement with Sternlicht *et al.* (1965a,b) results based on the $^{31}P$ resonances of the phosphate groups, the $fT_2p$ for ATP is ~7 times larger than for AMP-5′. At the temperature of 37°, $fT_2p$ should be equal to the chemical exchange time ($\tau_M$) of the metal ion between ligand molecules. The $fT_2p$ values are actually an indication of the amount of time the ligand molecule effectively binds the $Mn^{2+}$ ion. Thus, the results show ATP to have the longest binding time and AMP-5′, the shortest. However, it is important to note that since other binding sites are present (phosphate groups) and resonance line broadening is $\tau_M$ determined, the larger $fT_2p$ value observed for ATP does not necessarily mean that the intramolecular binding strength or time at the N-7 position has increased.

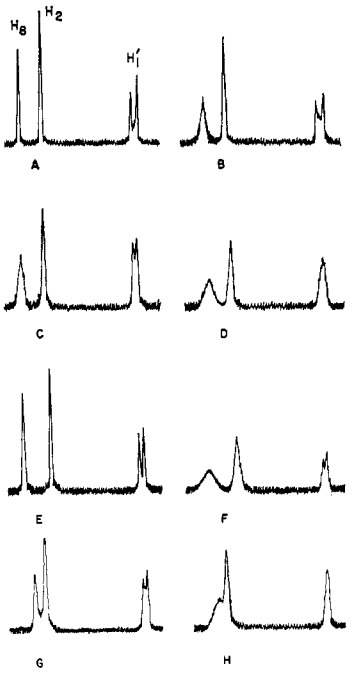


FIGURE 1: Proton nuclear magnetic resonance spectra (60 MHz) showing the $H_8$, $H_2$, and $H_1'$ resonances for solutions of ATP (A), ADP (C), and AMP-5′ (E) without $Mn^{2+}$ ion; and (B, D, and F) with $Mn^{2+}$ ion present. The conditions were $5 \times 10^{-1}$ M nucleotide, $4 \times 10^{-5}$ M $Mn^{2+}$ ion, 37°, and pH 7.8 ± 0.1. The spectra for solutions of $3 \times 10^{-1}$ M polyadenylic acid (G) without and (H) with $3 \times 10^{-5}$ M $Mn^{2+}$ ion were at pH 6.2 ± 0.1. All samples were in $D_2O$.

Figure 2 shows the effect of $Mn^{2+}$ ion on the $H_4$ and $H_5$ resonances of CTP and CMP-5′. The slight amount of broadening most probably indicates that there is no interaction of $Mn^{2+}$ with the cytosine ring portion, rather than a very slow $\tau_M$.

The relaxation time ($fT_2p$) was studied for the $H_8$ resonance of AMP-5′ in a competing system of 0.4 M AMP-5′, 0.1 M ATP, and $4.0 \times 10^{-5}$ M $Mn^{2+}$ at pH 7.8. Determination of $fT_2p$ was made over a temperature range of 0–70°, in order to vary the chemical exchange time ($\tau_M$), and to observe $fT_2p$ at high temperature. The results are shown in Figure 3, along with $fT_2p$ curves for separate solutions of $Mn^{2+}$ with AMP-5′ and ATP. The curve for AMP-5′ in the competing system is observed to lie above the ATP curve, and is largely displaced from the regular AMP-5′ curve over the complete temperature range studied. Since it is known that ~20% ATP is present in the competing system, it is assumed that the "ATP competing" is slowing the exchange for $Mn^{2+}$ ion through solution, and therefore the $fT_2p$ observed for the $H_8$ resonance of "AMP-5′ competing" is now much larger than in regular solutions of AMP-5′ and $Mn^{2+}$.

In further experiments, it was found that when a small amount of inorganic pyrophosphate (<0.1 M) is present in a solution of either 0.5 M ATP or 0.5 M AMP-5′ with $5 \times 10^{-5}$ M $Mn^{2+}$ ion, $fT_2p$ for the $H_8$ resonance was observed to greatly increase. The increase was large enough that the observed resonance line appeared almost as sharp as when no $Mn^{2+}$ ion

TABLE I: Normalized Relaxation Times.[a]

| Compounds | $fT_2p$ (sec) | |
|---|---|---|
| | $H_8$ | $H_2$ |
| ATP | $1.0 \times 10^{-5}$ | $6.0 \times 10^{-5}$ |
| ADP | $8.0 \times 10^{-6}$ | $3.0 \times 10^{-5}$ |
| AMP-5′ | $3.0 \times 10^{-6}$ | $1.0 \times 10^{-5}$ |
| Poly(A)[b] | $4.0 \times 10^{-6}$ | $1.0 \times 10^{-5}$ |
| | $H_4$ | $H_5$ |
| CTP | $1.0 \times 10^{-4}$ | $1.0 \times 10^{-4}$ |
| CMP-5′ | $3.0 \times 10^{-5}$ | $5.0 \times 10^{-5}$ |

[a] Spin–spin relaxation time for proton resonances of nucleotides ($5 \times 10^{-1}$ M), with $Mn^{2+}$ ion ($4 \times 10^{-5}$ M), at a temperature of 37°, pH 7.8, and in $D_2O$ solvent. [b] Polyadenylic acid concentration was $3.0 \times 10^{-1}$ M, with $Mn^{2+}$ ion ($3.0 \times 10^{-5}$ M), and pH 6.2–6.5.

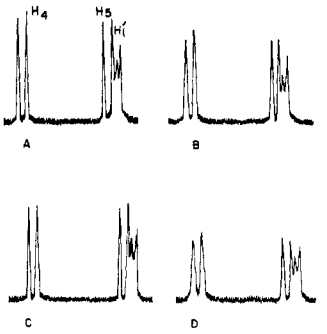


FIGURE 2: Proton nuclear magnetic resonance spectra showing the $H_4$, $H_5$, and $H_1'$ resonances for solutions of CTP (A) and CMP-5′ (C) without $Mn^{2+}$ ion; and (B and D) with $Mn^{2+}$ ion present. Conditions were $5 \times 10^{-1}$ M nucleotide, $4 \times 10^{-5}$ M $Mn^{2+}$ ion, 37°, pH 7.8 ± 0.1, and samples were in $D_2O$.

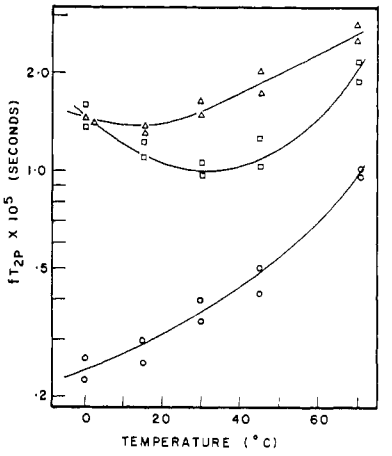


FIGURE 3: Normalized relaxation times $fT_2p$ for the $H_8$ resonance of (○) $5 \times 10^{-1}$ M AMP-5′, (□) $5 \times 10^{-1}$ M ATP, and competing system (△) of $1 \times 10^{-1}$ M ATP and $4 \times 10^{-1}$ M AMP-5′ with $4 \times 10^{-5}$ M $Mn^{2+}$ ion. $fT_2p$ values were determined in duplicate and over the temperature range 0–70°, the pH was 7.8 ± 0.1, and samples were in $D_2O$.

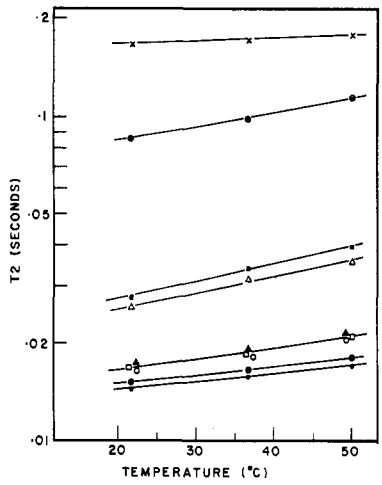


FIGURE 4: The effect of various ligands at concentration of $1 \times 10^{-3}$ M on the water proton relaxation time ($T_2$) for solution of $1 \times 10^{-3}$ M $Mn^{2+}$ ion. $T_2$ values were determined over a temperature range of 20–50°. The curves are as follows: $H_2O$ without $Mn^{2+}$ (×), $1 \times 10^{-3}$ M $Mn^{2+}$ ion solution (●), ATP (■), ADP (△), AMP-5′ (□), AMP-2′ (▲), ribose 5′-monophosphate (○), adenosine (⊗), and $PP_i$ (◒); all ligand solutions with $Mn^{2+}$ ion present.

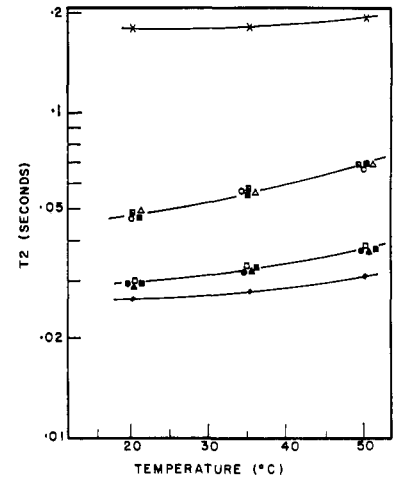


FIGURE 5: The effect of various nucleoside monophosphates and triphosphates at concentration of $1 \times 10^{-3}$ M on the water proton relaxation time ($T_2$) of solutions of $5 \times 10^{-4}$ M $Mn^{2+}$ ion. $T_2$ values were determined over a temperature range of 20–50° and at pH 7.8 ± 0.1. The curves are as follows: $H_2O$ without $Mn^{2+}$ ion (×), $5 \times 10^{-4}$ M $Mn^{2+}$ ion solution (●), ATP (■), CTP (△), GTP (○), UTP (◪), AMP-5′ (□), CMP-5′ (▲), UMP-5′ (⊠), GMP (⊗); all ligand solutions with $Mn^{2+}$ ion present.

was present in solution. The results of the experiments demonstrate that compounds with the ability to bind $Mn^{2+}$ ion for relatively long times can also increase the $fT_2p$ of molecules whose binding times are ordinarily short.

From the determination of spin–spin relaxation time ($T_2$) for water protons in $Mn^{2+}$–nucleotide solutions, it was possible to calculate the number of primary metal ion binding sites on the nucleotide ligand. In these experiments, the following compounds were studied: adenosine, cytidine, guanosine, uridine triphosphates and 5′-monophosphates; and also, adenosine nucleoside, ribose 5′-monophosphate, and inorganic pyrophosphate. The effect of temperature on $T_2$ relaxation was also observed.

Figure 4 shows the results of $T_2$ measured for solutions of $1 \times 10^{-3}$ M ligand and $1 \times 10^{-3}$ M $Mn^{2+}$. The $T_2$ curve for a standard solution of $1 \times 10^{-3}$ M $Mn^{2+}$ ion was found to agree reasonably well with results of Bloembergen and Morgan (1961). The number of binding sites was calculated from eq 5, using values of $1.0 \times 10^{-8}$ sec for $\tau_e$, and $3.3 \times 10^6$ cps for $A$ (Sternlicht *et al.*, 1965a). The experimental $T_2$ values were reproducible to about ±10%, and were within ±25% of the theoretical $T_2$ values calculated for each integer number of binding sites. The compounds ATP and ADP were found to have three binding sites; AMP-5′, AMP-2′, and ribose 5′-monophosphate, one site; adenosine, less than one; and inorganic pyrophosphate ($PP_i$), five sites.

The particularly low value of less than one site for adenosine is believed due to the fact that this compound binds weakly, and therefore, water molecules can effectively compete with it for the $Mn^{2+}$ ion. In the case of inorganic pyrophosphate, the exceptionally high value of five binding sites is due to the particularly strong interaction with the metal, which causes $\tau_e$ in eq 5 to change.[1]

[1] The fact that $\tau_e$ is not constant and changes through a shortening of electron spin relaxation time ($\tau_s$) is demonstrated by the electron spin resonance results to be discussed.

Since the ribose 5′-monophosphate curve fits with AMP-5′ and AMP-2′, this would tend to indicate that the phosphate group, rather than the base portion of the nucleotide, is predominantly responsible for the increase in $T_2$. Therefore, it accounts for the one primary binding site observed for nucleotide monophosphates.

For solutions of $5 \times 10^{-4}$ M $Mn^{2+}$ and $1 \times 10^{-3}$ M nucleotide the number of binding sites determined was the same as for the 1:1 solutions and the temperature dependence of $T_2$ was identical. In experiments with $5 \times 10^{-4}$ M $Mn^{2+}$ and nucleotide concentrations as high as 0.1 M, the $T_2$ values were found to increase slightly, but the values for the number of binding sites did not change significantly.

Studies of water proton relaxation and determination of number of binding sites were also carried out for solutions of cytidine, uridine, and guanosine triphosphates and their corresponding 5′-monophosphates. Figure 5 shows $T_2$ results for solutions of $5 \times 10^{-4}$ M $Mn^{2+}$ and $1 \times 10^{-3}$ M nucleotide. It can be seen that all the nucleoside triphosphates fit with the ATP curve and all 5′-monophosphates fit with AMP-5′. The number of binding sites is again three and one, as determined previously.

*Electron Spin Resonance Results.* In further studies, the effect of various ligands on the electron spin resonance spectrum of $Mn^{2+}$ ion was observed. From this study, it was possible to determine stability constants, and to observe changes in electron spin relaxation time for the metal ion when complexed with ligand.

Electron spin resonance spectra of a $1 \times 10^{-3}$ M $Mn^{2+}$ solution and $Mn^{2+}$ ion with AMP-5′, ATP, and $PP_i$ were determined. The spectrum of $Mn^{2+}$–AMP-5′ is slightly reduced in intensity compared with the pure $Mn^{2+}$ solution. In the spectrum of 1:1 $Mn^{2+}$–ATP, the intensity is reduced to less than 10% of the pure $Mn^{2+}$ solution. For 1:4 $Mn^{2+}$–ATP, the overall spectrum intensity continues to decrease and pronounced broadening of the hyperfine structure is observed.

TABLE II: Stability Constants and Free Energy of Interaction for Various Ligands with $Mn^{2+}$ Ion.

| Compounds | Free $Mn^{2+}$ Ion (M), Determined by Electron Spin Resonance[a] | Stability Constant ($M^{-1}$), $K$[b] | Stability Constant, Potentiometric ($M^{-1}$)[c] | Free Energy of Binding (kcal/mole), $\Delta G°$[d] |
|---|---|---|---|---|
| ATP | $1.0 \times 10^{-4}$ | $7.0 \times 10^{4}$ | $6.03 \times 10^{4}$ | −6.7 |
| ADP | $2.5 \times 10^{-4}$ | $1.2 \times 10^{4}$ | $1.46 \times 10^{4}$ | −5.7 |
| AMP-5′ | $9.1 \times 10^{-4}$ | $1.2 \times 10^{2}$ | $2.53 \times 10^{2}$ | −2.9 |
| Adenosine | $9.7 \times 10^{-4}$ | $2.6 \times 10^{1}$ | | −1.9 |
| $PP_i$ | $2.0 \times 10^{-5}$ | $2.5 \times 10^{6}$ | | −8.5 |

[a] Amount of free $Mn^{2+}$ ion for solutions of $1 \times 10^{-3}$ M $Mn^{2+}$ ion with $1 \times 10^{-3}$ M ligand at a temperature of 37°, 0.1 M Tris buffer, and pH 7.6. [b] Stability constants determined assuming a 1:1 complex formation; see text. [c] Taqui Kahn and Martell (1962). [d] See text.

With $Mn^{2+}$–$PP_i$, the spectrum broadens until the line structure is no longer observable. This extreme broadening and corresponding loss of observable spectrum for $Mn^{2+}$–$PP_i$ are particularly important because they indicate that the electron spin relaxation time ($\tau_s$) is shortening (McGarvey, 1957). The fact that $\tau_s$ shortens is important when considering that determination of the number of metal ion binding sites was based on the assumption that $\tau_e$ (which contains the $\tau_s$ term) was constant. Since $\tau_s$ changes, it is reasonable to suggest that a value of five binding sites for $PP_i$ is too high. The electron spin resonance results also indicate that $\tau_s$ shortens for ATP and ADP solutions. It was also observed that CTP gave spectral changes identical with ATP, indicating a similar $\tau_s$ and stability constant for this system.

The effect of various concentrations of ligand on spectral intensity for solutions of $1 \times 10^{-3}$ M $Mn^{2+}$ ion is shown graphically in Figure 6. The 100% value of the ordinate represents intensity of $1 \times 10^{-3}$ M $Mn^{2+}$ ion at 37°, 0.1 M Tris buffer, and pH 7.6, with no ligand present. The abscissa represents the concentration of ligand added. The curves readily show that $PP_i$, ATP, and ADP (in that order) have much more effect on reducing the intensity of the electron spin resonance spectrum than do AMP-5′ and adenosine.

Since the residual electron spin resonance spectral intensity represents the amount of $Mn^{2+}(H_2O)_6$, this fact was used to calculate stability constants for the various $Mn^{2+}$–nucleotide complexes. The results given in Table II agree reasonably well with those obtained potentiometrically (Taqui Khan and Martell, 1962). The free energy ($\Delta G°$) of complex formation was also calculated, using the relationship $\Delta G° = -RT \ln K$. The results show that for nucleotides, stability constants and $\Delta G°$ values increase as phosphate residues increase.

## Discussion

The nuclear magnetic resonance studies of Cohn and Hughes (1962) have shown that $Mn^{2+}$ ion interacts with the adenine ring and phosphate groups of ATP. Sternlicht *et al.* (1965) have further suggested that the degree of interaction at both ring position and phosphate groups is equivalent. We believe our studies provide evidence that the interaction of $Mn^{2+}$ ion at the ring position is actually much less than at the phosphate groups.

Our results show $Mn^{2+}$ ion produces broadening in all the adenosine compounds studied. However, broadening and corresponding $fT_2p$ values (Table I) are found to increase as phosphate residues increase. Since $fT_2p$ is equivalent to chemical exchange time ($\tau_M$), this indicates that ATP can effectively bind $Mn^{2+}$ ion longer than AMP-5′. Even though in ATP $fT_2p$ is increased for the $H_8$ resonance, this does not mean that the actual binding time for this intramolecular site becomes longer. Our results for a competing system of ATP and AMP-5′ demonstrate this point more clearly. It is observed (Figure 3) that a small amount of ATP is able to slow the exchange time of $Mn^{2+}$ through solution, and thereby increase the relaxation time $fT_2p$ for the $H_8$ resonance of AMP-5′ present in the same solution. The fact that the $fT_2p$ curve for AMP-5′ is still displaced, even up to 70°, is important because we believe this indicates that $\tau_M$, of $Mn^{2+}$ with ATP, is still relatively slow, and $fT_2p$ is not in a region of fast chemical exchange as had been proposed by Sternlicht *et al.* (1965b). Since ATP can determine $fT_2p$ for other molecules in solution, we propose that its own intramolecular sites are still $\tau_M$ determined. Therefore, in ATP, one or more intramolecular sites (the phosphate groups, most likely) can actu-

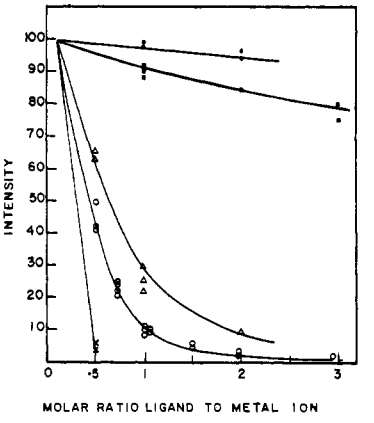


FIGURE 6: The effect of various ligands on the electron spin resonance spectral intensity of solutions of $1 \times 10^{-3}$ M $Mn^{2+}$. The abscissa represents the molar ratio of ligand to $Mn^{2+}$ ion. The curves are as follows: adenosine (●), AMP-5′ (■), ADP (△), ATP (○), $PP_i$ (×). The electron spin resonance spectra were determined for sample solutions at 37°, pH 7.6, and 0.1 M Tris buffer.

ally be the long-time binding sites, with the N-7 and/or $NH_2$-6 position being a short-time binding site.

The water relaxation studies are of particular relevance at this point because they indicate the number of primary or significant binding sites and other important factors concerning the interaction of $Mn^{2+}$ ion with various nucleotide ligands. First, it should be noted (Figure 4) that adenosine is found to have a relatively small influence on $T_2$, and does not account for one complete binding site. Secondly, AMP-5′, AMP-2′, and ribose 5′-monophosphate are all found to have similar $T_2$ values and one primary site. Thirdly, all nucleoside triphosphates and monophosphates are found to have three sites and one site, respectively. Concerning this last statement, it is important to remember that $Mn^{2+}$ ion was observed to have negligible influence on the proton resonances (Figure 2) of the cytidine ring portion of CTP and CMP-5′, thus indicating no interaction. Therefore, even if the calculations for number of binding sites (eq 5) are in error, the observed $T_2$ times for ATP and AMP-5′ should be higher than CTP and CMP-5′, in order to account for the extra site. The fact that the extra site is not accounted for in either ATP or AMP-5′, and since adenosine itself shows only minor influence on $T_2$, indicates that the primary binding sites are actually the phosphate groups.

The electron spin resonance results show that a shortening of $\tau_s$ occurs for $PP_i$, ATP, and ADP. Since the $\tau_e$ term in eq 5 contains $\tau_s$, this would mean $\tau_e$ becomes smaller. Correspondingly, the value determined for primary binding sites would be too high. This fact appears to account for the unrealistically high number of sites found for $PP_i$. Since $\tau_s$ also shortens for ADP and ATP, we would (qualitatively) propose that for both these compounds there are probably two rather than three primary binding sites. Also, it should be noted that our electron spin resonance results for CTP show a spectrum equivalent to ATP. This means that the $\tau_s$ value is the same for both compounds.

It is important to know the difference in the actual degree of interaction between the ring portion of ATP and the phosphate groups. We estimated this several ways. First, $\tau_M$ values at room temperature were almost one order of magnitude greater for ATP than AMP-5′. Our results would suggest this is due totally to the phosphate groups, and as stated above, a larger $\tau_M$ in effect means the metal ion is bound for a longer time. Also, water $T_2$ results indicate that even in AMP-5′, the ring binding probably is not equivalent to the phosphate groups. Secondly, the $\Delta G°$ values (Table II) from our electron spin resonance results show that $\Delta G°$ for the Mn–adenosine complex is about 2 kcal/mole. This complex would be quite easily subject to disruption by thermal motion. However, for ATP, $\Delta G°$ is of the order of 7 kcal/mole. This is a relatively much more stable structure and it appears that this stability is due primarily to the phosphate groups.

In conclusion, we propose a structure for $Mn^{2+}$–ATP in which two or three of the phosphate groups are the primary or long-time binding sites. In this complex, binding to the ring position does take place, but is relatively weak (or short) in comparison to the phosphate groups. The $Mn^{2+}$–ATP structure is probably very similar to the $Mg^{2+}$–ATP complex (Cohn and Hughes, 1962). It also appears that complexes of other nucleoside triphosphates are of similar structure, in that the phosphate groups are the primary binding sites. However, in the study of interaction of guanosine with $Ag^+$ and $Cu^{2+}$, we proved that the base was involved (Tu and Reinosa, 1966; Tu and Friederich, 1968).

## Acknowledgments

The authors wish to express their sincere gratitude to Dr. Thomas N. Solie for his invaluable contributions to this research. Also, Michael J. Heller wishes to express his appreciation to Saint Joseph College, West Hartford, Connecticut, for the use of their electron spin resonance spectrometer, and to Sister Claire Markham, Professor of Chemistry, for her helpful advice and encouragement.

## References

Bloembergen, N., and Morgan, L. O. (1961), *J. Chem. Phys. 34*, 842.

Cohn, M., and Hughes, T. R. (1962), *J. Biol. Chem. 237*, 176.

Cohn, M., and Townsend, J. (1954), *Nature (London) 173*, 1090.

Eisinger, J., Fawaz-Estrup, F., and Shulman, R. G. (1965), *J. Chem. Phys. 42*, 43.

Happe, J. A., and Morales, M. (1966), *J. Amer. Chem. Soc. 88*, 2077.

Larsson-Raznikiewicz, M., and Malmström, B. G. (1961), *Arch. Biochem. Biophys. 92*, 94.

Martell, A. E., and Schwarzenback G. (1956), *Helv. Chim. Acta 39*, 653.

McConnell, H. M. (1958), *J. Chem. Phys. 28*, 430.

McConnell, H. M., and Berger, S. B. (1957), *J. Chem. Phys. 27*, 230.

McGarvey, B. R. (1957), *J. Phys. Chem. 61*, 1232.

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

Shulman, R. G., Sternlicht, H., and Wylunda, B. J. (1965), *J. Chem. Phys. 43*, 3116.

Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965a), *J. Chem. Phys. 43*, 3123.

Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965b), *J. Chem. Phys. 43*, 3133.

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

Taqui Khan, M. M., and Martell, A. E. (1962), *J. Phys. Chem. 66*, 10.

Tu, A. T., and Friederich, C. G. (1968), *Biochemistry 7*, 4367.

Tu, A. T., and Reinosa, J. A. (1966), *Biochemistry 5*, 3375.

Vallee, B. L. (1960), *Enzymes 3*, 225.

Walaas, E. (1958), *Acta Chem. Scand. 12*, 528.

Weser, U. (1968), *Struct. Bonding (Berlin) 5*, 41.