A Proton Magnetic Resonance Study of Metal Ion–Adenine Ring Interactions in Metal Ion Complexes with Adenosine Triphosphate*

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ABSTRACT: Two types of nuclear magnetic resonance measurements have led to the conclusion that the metal ion (Co^{2+}, Mn^{2+}, and Ni^{2+})–ATP complex contains a water molecule that is simultaneously coordinated to the metal ion and hydrogen bonded to N-7 of the adenine ring. In the first series of experiments a comparison was made of the effects of Mn^{2+} and Ni^{2+} on ATP and the analog tubercidin triphosphate (TuTP, 7-deazaadenosine 5′-triphosphate) in which N-7 of the adenine ring is replaced by a CH group. If there are no differences between the metal–TuTP and metal–ATP complexes other than the altered ring, then comparing the results obtained with these two complexes should provide a definitive test for determining whether the metal specifically interacts with the N-7 of the adenine ring. Mn^{2+} produces approximately equivalent broadening of each of the four proton signals (H-2, H-7, H-8, H-1′) of TuTP in contrast to a much greater effect on H-8 of ATP. Ni^{2+} produces significantly greater broadening and shifting of the signal from H-2 as compared to H-7, H-8, and H-1′; this is in contrast to a predominant effect on H-8 with ATP. This comparison leads to the conclusion that in both the Mn^{2+}–ATP and Ni^{2+}–ATP complexes the metal ion is held in the vicinity of N-7 of the adenine ring because of metal ion–ring binding. The second series of experiments involved measuring the T_1 and T_2 relaxation times of solvent water. If the ring does not enter the inner coordination sphere of the metal ion, then the ion would retain three water molecules in its inner sphere. This can be measured indirectly by comparison to a standard, in this study CTP, containing a known number of complexed water molecules.

For the Mn^{2+} and Co^{2+} complexes the water relaxation times of the standard were found to be identical with those of ATP at all temperatures studied. This shows that the Mn^{2+}–ATP and Co^{2+}–ATP complexes do not contain a metal ion–nitrogen coordinate bond and that they are outer sphere complexes. Evidence for the specific structure of the outer sphere complex comes from the comparison of water T_2 values for Ni^{2+}–ATP and Ni^{2+}–CTP. Over a wide range of temperatures the ratio of rapidly exchanging water molecules in Ni^{2+}–ATP to Ni^{2+}–CTP is 2:3. This ratio can be accounted for in two ways: (a) an inner sphere metal–nucleotide complex is formed with the elimination of a coordinated water molecule or (b) a water molecule forms a bridge between the metal and nucleotide and exchanges too slowly to contribute to the overall water relaxation. Supporting evidence for an outer sphere type of metal–ATP complex comes from an examination of the relatively small chemical shift of H-8 in Ni^{2+}–ATP in contrast to the very large shifts observed with a known inner sphere complex of adenosine with bis(acetylacetonato)-nickel(II) at 100 MHz. The former shows an estimated maximum shift of approximately 510 Hz while the latter has a shift of 5800 Hz.

Enzymatic reactions involving nucleoside triphosphates all require a divalent cation. Cohn (1963) has suggested two roles for the cation: (a) to form a metal–nucleotide complex that is the substrate for the reaction and (b) to form a metal–enzyme complex that is the active form of the enzyme. The structure of the metal–nucleotide complex in solution has not been unequivocally defined and there is very little definitive information available concerning the relationship between the conformation of the nucleotide complexes formed with different divalent metals and the relative enzymatic activities of these complexes.

The major site of interaction of the divalent metal ion and nucleotide is between the cation and the phosphate chain. Cohn and Hughes (1960, 1962) and Sternlicht *et al.* (1965a) have shown that divalent metal ions may be classified into two groups, those which bind to the β and γ phosphates of ATP (Mg^{2+}, Ca^{2+}, Zn^{2+}), and those which bind to all three phosphates of ATP (Mn^{2+}, Ni^{2+}, Co^{2+}). This binding accounts for the strong interaction of the metal and nucleotide, but does not explain the specificity or advantage of this type of complex in enzymatic reactions. It has been proposed (Szent-Györgyi, 1957) that in addition to metal ion–phosphate binding in ATP there is also a metal ion–adenine binding. The most direct way of examining this possibility is to determine the effects of divalent cation on the proton magnetic resonance spectrum of ATP.

For the diamagnetic ions, Mg^{2+} and Ca^{2+}, both proton (Cohn and Hughes, 1962), and ^{15}N magnetic resonance (Happe and Morales, 1966) studies have failed to detect any metal ion–ring interaction. This would seem to indicate that no direct metal ion–nitrogen bond or inner sphere complex is involved. These experiments however do not rule out the possibility of having an outer sphere complex with these metals. The magnetic effects of a diamagnetic metal ion such as Mg^{2+} on proton or ^{15}N chemical shifts are very short ranged and binding other than a direct metal ion–nitrogen bond might not be detected.

When the paramagnetic metal ions, Mn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} are added to an aqueous solution of ATP, the proton

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signals of the adenine ring are extensively broadened and/or shifted depending on the particular metal ion added (Cohn and Hughes, 1962; Sternlicht *et al.*, 1965b; Sternlicht *et al.*, 1968). There is no question that these metal ions are interacting magnetically with the ring. However, these experiments by themselves do not indicate whether this interaction arises from a 1:1 metal–nucleotide complex in which the metal ion binds simultaneously to both the ring and the phosphate groups, whether the metal ion binds at a preferred site on the ring and whether the metal ion is binding to the ring through an inner or outer sphere coordination complex. One major problem encountered in trying to answer these questions is that both a 1:1 and a 1:2 metal ion–ATP complex may form in the presence of a large excess of ligand (Sternlicht *et al.*, 1968). This complication can be eliminated by examining the simultaneous binding of the metal ion to the phosphates and ring under conditions which do not favor the formation of the 1:2 metal–nucleotide complex. This can be done by using solutions which have the metal:nucleotide ratio approaching unity or which have the nucleotide concentration so low that the metal–$(ATP)_2$ species is only a minor component. Line-broadening measurements under the latter conditions were used by Sternlicht *et al.* (1968) to approximate the distance of Mn^{2+} from H-2 and H-8 of the adenine ring in ATP. They found that the metal ion was 3.8 ± 0.6 Å from H-8 and 5.5 ± 0.8 Å from H-2. The relatively short distance to H-8 suggests that the Mn^{2+} is binding to the ring at or near N-7. However, no definitive conclusions regarding binding to the adenine ring can be drawn from the line-broadening data since the broadening of the ring signals is effected by magnetic dipolar coupling and requires only that the metal ion be in the vicinity of the ring and necessarily bound to it (Solomon, 1955). The Mn^{2+} may be held near the ring for steric and conformational reasons not associated with any specific binding.

This study has two objectives. The first is to decide whether the results of line-broadening and -shifting studies are measuring a specific metal ion–ring interaction in the 1:1 metal–ATP complex; the second is to determine if this interaction involves an inner or outer sphere complex. The experimental approach used for answering the first question was based on the fact that if the ions bind specifically to the ring, then there must be a site or sites on the ring associated with this attraction. If this site is altered, then the binding of the ion to the ring should also be changed and these changes should be reflected in the proton spectrum of the metal-containing solution. On the other hand, if no specific binding between the metal ion and the ring occurs, then substantial changes in the ring structure should result in little or no change in the proton spectrum. Previously published results have strongly implicated N-7 as the likely binding site of the metal ion (Sternlicht *et al.*, 1968). Tubercidin triphosphate differs structurally from ATP by having a carbon atom substituted for N-7. If there are no other differences in the metal–TuTP[1] complex other than the altered ring, then comparing the effects of a paramagnetic metal ion on the proton spectrum of TuTP to its effects on the ATP proton spectrum should provide a definitive test for the occurrence of a specific metal–ring interaction in metal–ATP complexes.

The second question was examined by comparing the longitudinal (T_1) and transverse (T_2) relaxation times of the solvent water in metal–ATP complexes to the relaxation times of a suitable standard. This measures the number of rapidly exchanging water molecules bound to the metal in the metal–nucleotide complex. The metals studied (Co^{2+}, Mn^{2+}, and Ni^{2+}) bind to all three phosphates and if there is no interaction with the ring the metal should also have three water molecules complexed to it. If the metal forms an inner sphere complex with the ring, then there should only be two water molecules bound to the metal. If the complex is of the outer sphere type and involves a water molecule coordinated to the metal ion and hydrogen bonded to the ring, then the number of observed water molecules can vary between two and three depending on the exchange rate of the bridging water molecule.

Experimental Procedures

Adenosine and the disodium salts of ATP and cytidine triphosphate (CTP) were purchased from Sigma Chemical Co. TuTP was prepared from tubercidin by modifications of Tener's (Tener, 1961) method for monophosphorylation and Moffatt and Khorana's (1961) method for triphosphorylation. Bis(acetylacetonato)nickel(II) was purchased from MacKenzie Chemical Works.

Metal ion solutions were prepared from certified atomic absorption standards (Fisher Scientific Co.). The solutions were concentrated by evaporation and their concentrations determined by a Beckman atomic absorption spectrometer.

A Chelex-100 column (200–400 mesh, 1.5 × 10 cm, Na^+ form) was used to remove trace metals from approximately 1 mmole of sample. The compounds were eluted with triply distilled water and this solution was evaporated to dryness at 30° under reduced pressure. The sample was redissolved, the pH adjusted with 0.5 N HCl and the concentration determined from the ultraviolet absorbance. Samples used for obtaining proton magnetic resonance spectra were lyophilized overnight and then lyophilized three additional times from D_2O (Columbia Organic Chemicals). The samples were dissolved in D_2O and the pH and ultraviolet absorbance again determined. The appropriate amount of metal ions was added to the nucleotide solutions and then the pH was adjusted to 5.5 ± 0.2 with a small volume of NaOH. Because small volumes of metals and nucleotides were used for T_1 and T_2 measurements, all deliveries were made with glass micropipettes that were calibrated prior to use.

Proton magnetic resonance spectra of ATP and TuTP were recorded on a Varian HA-100 spectrometer. A trace of tetramethylammonium nitrate was added to the solutions to provide a lock signal and shift standard.

Relaxation times, T_1 and T_2, were determined by the spin-echo technique. Measurements were made on a Nuclear Magnetic Resonance Specialties PS-60 instrument at 15 MHz. The null method was used to measure T_1. Although the conclusions from both the T_1 and T_2 determinations were the same, only T_2 values are reported because of the experimental uncertainty of the T_1 measurements.

The stability constant of the adenosine complex with the bis(acetylacetonato)nickel(II) in dimethyl sulfoxide was calculated by the Benesi-Hildebrand method (1949). All solutions were 0.05 M bis(acetylacetonato)nickel(II) and contained varying concentrations of adenosine (0.05–0.25 M). A solution of 0.05 M bis(acetylacetonato)nickel(II) was used as a reference and measurements were recorded on a Gilford 2000 spectrometer at 580–600 mμ.

Theory of Chemical Exchange. When the paramagnetic ions, Mn^{2+} or Ni^{2+}, are added to a solution of ATP, two effects are seen on the proton magnetic resonance spectrum. While both

[1] Abbreviations used are: TuTP, tubercidin 5′-triphosphate; 7-deazaadenosine 5′-triphosphate; pD, observed pH plus 0.4.

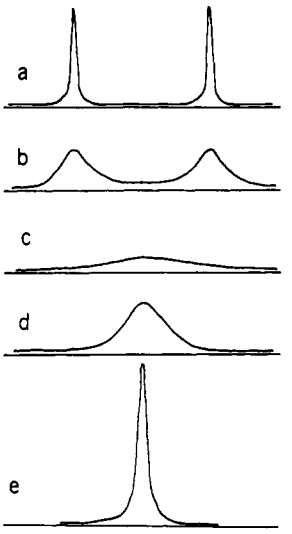


FIGURE 1: Shapes of nuclear magnetic resonance peaks as a function of exchange rate.

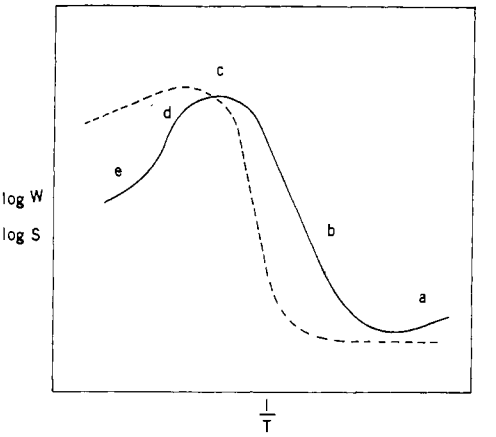


FIGURE 2: Hypothetical plot of log W (—) and log S (---) *vs.* $1/T$ plot for an exchanging diamagnetic–paramagnetic system.

metal ions increase the line widths, Ni^{2+} produces a measurable change in the chemical shifts. Data from the line broadening can give the position of the metal ion in the complex while the chemical shifts may be related to the degree of direct metal ion–ring binding. The important parameters to be obtained are the line widths and chemical shifts characteristic of the 1:1 metal–ATP complex in solution. It is often not possible to examine a 1:1 metal ion–ligand complex directly. For example, the proton spectrum of a solution of equimolar Mn^{2+} and ATP is obscured by extensive overlapping of peaks arising from the broadening caused by the metal. The following discussion of chemical exchange in nuclear magnetic resonance will explain how information about the 1:1 metal ion–ligand complex can be derived from solutions containing excess ligand.

If a solution contains two diamagnetic solutes, X and Y, each containing a single proton, then in general the magnetic environments of the protons in X and Y will not be the same and this will be reflected in different peak positions and line widths of the nuclear magnetic resonance signals. The X and Y peaks may be viewed as a report on the "characteristic behavior" of the corresponding protons in X and Y. In the absence of proton exchange between X and Y, all protons behave in a way characteristic of either X or Y and the proton spectrum is a simple superposition of the X and Y spectra (Figure 1a).

If X $\rightleftarrows$ Y proton exchange is allowed, how long does it take a proton passing from X to Y to assume the behavior characteristic of Y? For protons in a typical diamagnetic environment this relaxation time (T_2) is of the order of seconds. When the average residence time of the exchanging protons in Y is of this order, then these protons will include some which have been in Y for a long time and are behaving in a manner characteristic of Y, some which have just entered and are still behaving in a way characteristic of X, and others which have intermediate behavior. This distribution of behavior will be reflected in a broadening of the proton resonance signal (Figure 1b).

If the X $\rightleftarrows$ Y exchange is very rapid, essentially none of the protons will be behaving in a manner which is characteristic of either X or Y. Very few will have been in either environment long enough to reflect its characteristics. In fact, essentially all of the protons behave in the same manner, which is an average of the behaviors characteristic of X and Y. Because these protons are acting like a single species, the spectrum will consist of a single peak with a position and width which are the weighted averages of the X and Y peaks (Figure 1e). Other intermediate spectral shapes are shown in Figures 1c and 1d. The treatment of X $\rightleftarrows$ Y exchange is given in mathematical form by Gutowsky *et al.* (1953).

This same treatment holds when X is diamagnetic and Y is paramagnetic. In this case uncomplexed ATP would be X and complexed ATP would be Y. Exchange is accomplished by the exchange of whole ATP ions between the free and complexed states. A pure spectrum of the paramagnetic species Y cannot be observed because the peaks are generally so broad they cannot be resolved and in the absence of exchange, the observed proton spectrum of a mixture of X and Y is essentially that of pure X. As the rate of X $\rightleftarrows$ Y exchange increases, the spectral peaks broaden as in Figure 1b, reach some maximum width as in Figure 1c, then narrow to become the weighted average as in Figure 1e. One convenient way to vary the X $\rightleftarrows$ Y exchange rate over a large range is to observe the spectrum as a function of temperature. A typical plot of the log of observed line width (W) measured at half-height and shift (S) of peak position as a function of reciprocal temperature for a diamagnetic–paramagnetic X–Y system is shown in Figure 2. The temperature regions associated with the corresponding spectra of Figure 1 have been labeled. In the absence of exchange broadening, nuclear magnetic resonance signals will in general narrow as the temperature increases. This explains why regions a and e of Figure 2 exhibit line narrowing with increasing temperature.

The chemical shift of the observed peak as a function of exchange rate is also of interest. Figure 2 shows that the observed shift is essentially that of pure X until the X and Y spectra have almost completely coalesced; it then changes very quicky to the weighted average of the shifts of X and Y. The discussion above is, in qualitative form, the essence of the more rigorous treatment of paramagnetic broadening and shifting (Swift and Connick, 1962).

The line width and chemical shift of the paramagnetic Y species are of primary interest and therefore the highest temperature region of Figure 2, where the observed line width and shift are weighted averages, is most important. The line width of X, W_X, is known from the spectrum of pure ATP and the concentrations of X and Y can be calculated from the stoichio-

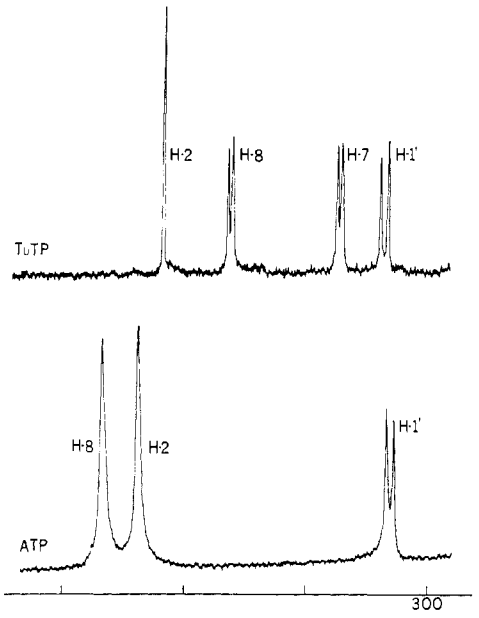


FIGURE 3: Lower field nuclear magnetic resonance spectra of ATP and TuTP in D_2O. ATP spectrum of 0.15 M solution of pD 5.6 at 30°. TuTP spectrum of 0.089 M solution of pD 5.9 at 30°. Scale markers are at 100-Hz intervals. The designation 300 refers to position in Hz below tetramethylammonium ion.

metric concentrations of ATP and metal ion together with the stability constant for complex formation. This leaves the Y line width, W_Y, as the only unknown. These parameters are related to the experimental line width, W_0, by

$$W_0 = \frac{[Y]W_Y}{[X] + [Y]} + \frac{[X]W_X}{[X] + [Y]} \quad (1)$$

For the common case where $[X] \gg [Y]$, eq 1 becomes

$$W_0 - W_X = W_P = fW_Y = \frac{f}{\pi T_{2M}} = \frac{1}{\pi T_{2P}} \quad (2)$$

where the W's are line widths in Hertz at half-maximum peak intensity and T_{2P} and T_{2M} are the transverse relaxation times employed by Sternlicht *et al.* (1965b). The term f is the ratio of metal ion to nucleotide and it is chosen so that fW_Y can be measured as precisely as possible.

An equation entirely analogous to eq 1 can be written for the experimental shift S_0 with respect to some suitable standard.

$$S_0 = \frac{[Y]S_Y}{[X] + [Y]} + \frac{[X]S_X}{[X] + [Y]} \quad (3)$$

Again under conditions where $[X] \gg [Y]$, eq 3 becomes

$$S_0 - S_X = S_P = fS_Y \quad (4)$$

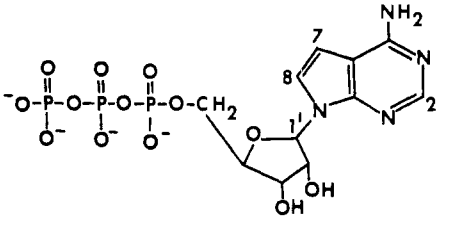
When the spectrum represents a weighted average of the spectra of free and metal-complexed nucleotide and when the contribution of each environment is calculated from the concentration of the metal and nucleotide, then line widths for the metal–nucleotide complex can be measured directly from the spectrum. These widths, in the case of complexed ATP, result from paramagnetic electron–proton magnetic coupling (Solomon, 1955). This is the same type of classical magnetic coupling that occurs between two compass needles placed near each other. Equation 5 gives the relationship between W_Y and r, the distance between the metal ion and the particular proton of interest.

$$W_Y = \left[\frac{7S}{15\pi}(S + 1)g^2\beta^2\gamma_I^2\tau_c\right]r^{-6} = Kr^{-6} \quad (5)$$

This equation is an approximation to the more general Solomon–Bloembergen equation (Solomon, 1955) and it applies to the particular Mn^{2+} system and conditions employed here. In eq 5, S is the spin quantum number, $g\beta$ is the electron magnetic moment of the paramagnetic complex, γ_I is the gyromagnetic ratio of the proton, and τ_c is the correlation time for the tumbling of the complex in solution. For purposes of comparison we have employed the same values of $g\beta$ and τ_c used in the calculation of r by Sternlicht *et al.* (1965b, 1968). In general, the constant K will vary with the metal ion, magnetic field, and temperature.

Results

Figure 3 shows the low-field portion of the 100-MHz proton magnetic resonance spectra of TuTP and ATP. From the structure of TuTP it can be seen that H-2 will yield the only



tubercidin 5′-triphosphate

singlet in the TuTP spectrum. The doublet to the highest field in the TuTP spectrum clearly arises from H-1′ of TuTP since it has the same chemical shift and line separation (6 Hz) as the corresponding proton of ATP. The signals from H-7 and H-8 of TuTP should be doublets. The doublet at lower field is assigned to H-8, because the latter may be described as an NCH proton, whereas H-7 is a CCH proton (Jackman and Sternhell, 1969). This assignment is the one most consistent with the fact that replacement of N-7 of ATP by carbon leads to an upfield shift for the signal from H-8.

Figures 4 and 5 show how the spectrum of TuTP is altered when a paramagnetic ion, Mn^{2+} or Ni^{2+}, is added to the solution. All the signals of TuTP are broadened by Mn^{2+} (Figure 4b). This broadening decreases as the temperature is increased above 30°, showing that in this case the spectrum represents a weighted average of the spectra of free TuTP and the Mn^{2+}–TuTP complex. Since the concentration of metal ion and TuTP are known, it is easy to calculate the contributions of each environment.

Line widths for H-2, H-7, H-8, and H-1′ of TuTP can be measured directly from the spectrum and the distance, r, between the metal ion and respective proton, may be calculated from eq 5. A comparison of the values for Mn^{2+}–TuTP and Mn^{2+}–ATP is given in Table I. These relatively large distances for Mn–TuTP are not consistent with metal ion–ring binding. In fact, they indicate a structure in which the Mn^{2+} is bound only to the phosphates. Since there is no apparent ring binding in Mn^{2+}–TuTP, no species analogous to Mn^{2+}–

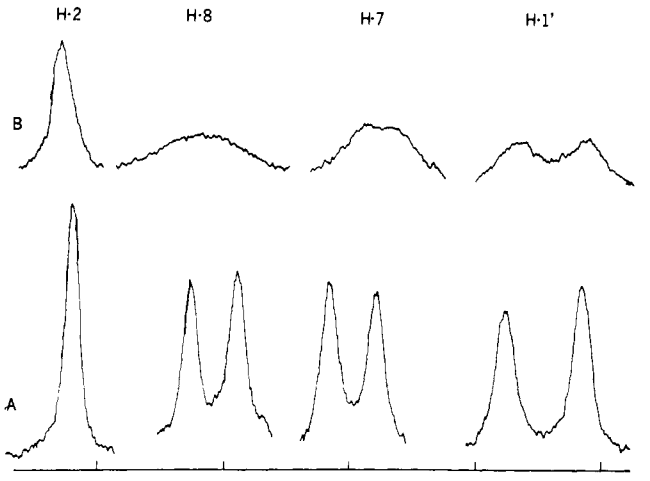


FIGURE 4: Effect of Mn^{2+} on spectrum of TuTP. Spectrum A depicts the expanded peaks of a 0.089 M solution of pD 5.9 at 30° in D_2O. Spectrum B depicts the effect of 1×10^{-4} M Mn^{2+} on the same TuTP solution ($f = 1.13 \times 10^{-3}$). Scale marker intervals are 10 Hz.

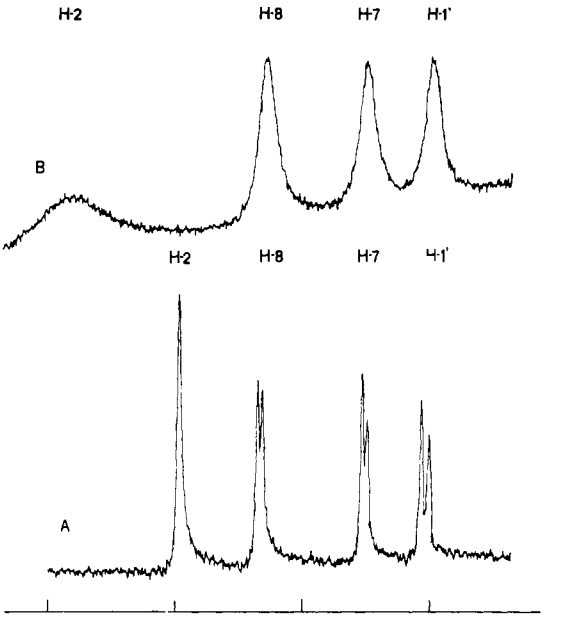


FIGURE 5: Effect of Ni^{2+} on spectrum of TuTP. Spectrum A depicts the peaks of a 0.10 M solution of pD 6.1 at 70° in D_2O. Spectrum B depicts the effect of 0.036 M Ni^{2+} on the same solution ($f = 0.36$). Scale marker intervals are 100 Hz.

$(ATP)_2$ forms. Because altering the ring dramatically changed the ability of the metal to bind to the ring and interact at a preferential site, the Mn^{2+}–ATP complex probably involves metal ion–ring binding at or near N-7.

Figure 5 contains a comparison of the spectrum of an aqueous solution of TuTP with the spectrum of the same solution to which Ni^{2+} had been added. Because Ni^{2+} is not as effective a broadener as Mn^{2+}, a spectrum could be recorded when the metal:nucleotide ratio approached unity. Although the relative line widths and shifts are preserved at a ratio of unity, a ratio of 0.36 was chosen because it provides the best graphic display of the relative shifting and broadening effects on the various peaks.

The most striking contrast between the effect of Ni^{2+} on TuTP and on ATP is the relative broadening of the H-8 and H-2 peaks. In the Ni^{2+}–ATP system the H-8 signal is much broader than the H-2 signal and as the metal:nucleotide ratio approaches unity the H-2 signal remains clearly discernible while the H-8 signal is so broad that it can no longer be seen. A different situation holds with Ni^{2+}–TuTP, where the H-2 signal is significantly broader than any of the others. If Ni^{2+} were not specifically bound to the adenine portion of ATP, then changing the structure of the ring would not be expected to change the magnetic interaction between the metal ion and the ring. The best explanation for this change in position is that Ni^{2+} binds specifically to ATP at or near N-7. When this site is removed, the ion seeks out the next most favorable binding site, probably N-1 and/or N-3. We conclude that an association does exist between the phosphate-complexed metal ion and the adenine ring of ATP and that the predominant conformation involves the N-7 position.

The next question is whether this association involves an inner or outer sphere complex. The metal ions, Ni^{2+}, Co^{2+}, and Mn^{2+} are presumed to have primary coordination numbers of six and to form direct coordinate bonds with each of the three phosphates of ATP. Because sizeable line broadening can occur in outer sphere coordination, the large broadening observed for all three phosphorus signals upon the addition of Mn^{2+}, Co^{2+}, or Ni^{2+} to ATP in aqueous solution cannot be taken as conclusive evidence for simultaneous inner sphere binding involving all three phosphates (Sternlicht *et al.*, 1965a). This points merits further investigation, and, although we have tentatively accepted the conclusion of Sternlicht *et al.* (1965a) concerning the phosphate coordination, our conclusions do not rely on this assumption.

If the ring does not enter the inner coordination sphere of the metal ion, then each metal ion would retain three water molecules in its inner sphere. This amount of water in the inner sphere would be reflected in the T_1 and T_2 relaxation times of the solvent water. There are three factors which determine the effect of the metal ion on the relaxation times of the solvent water: (1) the number of water molecules in the first coordination sphere of the metal; (2) the influence of other ligands on the interaction of the metal and the remaining coordinated water molecules; and (3) the rate of chemical exchange of the water molecule from the environment of the metal to the bulk water. A water molecule bound tightly to the metal will be relaxed, but unless it is exchanging rapidly enough with the solvent water, it will not contribute to the broadening and relaxation of the solvent water peak. Unfortunately the effect of the metal on the individual ligands is too complex a phenomenon to predict precisely. Because of this it is impossible to determine with any degree of confidence the number of water molecules in the first coordination sphere of the metal by a single direct measurement of T_1 or T_2 for the bulk water. It should be possible to determine indirectly

TABLE I: Manganous Ion to Proton Distances in Ångströms from Proton Line-Broadening Data for TuTP and ATP Complexes.

Complex	H-2	H-7	H-8	H-1′
TuTP	6.6 ± 0.7	5.8 ± 0.6	5.3 ± 0.5	5.8 ± 0.6
ATP[a]	5.5 ± 0.8		3.8 ± 0.6	

[a] Sternlicht *et al.* (1968).

TABLE II: Effect of Paramagnetic Ions on CTP and ATP.

Metal Ion	[Metal]: [Nucleotide]	CTP Δ Shift[a] H-6	CTP Δ Shift[a] H-5	CTP Δ Shift[a] H-1′	CTP Δ Width H-6	CTP Δ Width H-5	CTP Δ Width H-1′	ATP Δ Shift[a] H-8	ATP Δ Shift[a] H-2	ATP Δ Shift[a] H-1′	ATP Δ Width H-8	ATP Δ Width H-2	ATP Δ Width H-1′
Co^{2+}	1.1	24	36	*b*	13	4	*b*	−950	90	22	120	23	24
Ni^{2+}	1.04	−8	−36	8	30	34	8	≤510[c]	−110	−150	≤1600[c]	200	70
Mn^{2+}	0.026	0	0	0	64	76	22						
	0.003[d]										170	7	7

[a] Negative sign indicates downfield shift. [b] Peak obscured by water signal. [c] Peak obscured by H-2 signal; values obtained by linear extrapolation. Shift is downfield. [d] Sternlicht *et al.* (1965b).

the number of water molecules in the metal–ATP complexes by comparing the relaxation rate of a metal–ATP complex with that of a standard having a known number of water molecules in its first coordination sites and which has nearly the same effects on its individual water ligands as does the metal–ATP complex. The standard must have three phosphates which bind to the metal in the same manner as ATP, it must be approximately the same size and shape as ATP and it must not have any other significant interaction with the metal. The standard chosen was CTP. Complexes of CTP with Ni^{2+}, Co^{2+}, and Mn^{2+} have approximately the same stability constants as the corresponding ATP complexes (Walaas, 1958) and thus presumably involve three direct metal ion–phosphate bonds. Table II compares the line widths and chemical shifts for CTP and ATP protons in aqueous solutions containing either Mn^{2+}, Co^{2+}, or Ni^{2+}. The ring protons of CTP are shifted and broadened about equally. The interaction of these protons with the metals is much less than that between the metals and H-8 of ATP, indicating very little interaction between the metal ion and the cytosine ring. Cytidine triphosphate therefore appears to fulfill all the requirements that were set up for a standard.

Table III shows the data for the water T_2 studies of the metal ion nucleotide solutions. The ratio of relaxation rates, R, of the metal–ATP:metal–CTP complexes gives the ratio of rapidly exchanging water molecules of the two complexes. The water relaxation times, T_1 and T_2, for Mn^{2+}–ATP and Mn^{2+}–CTP are identical at all accessible temperatures. The average value of R for all measurements is 0.97 ± 0.05 and similar results were obtained in the case of Co^{2+}. From the line broadening studies, it is known that these metals interact strongly with H-8 of ATP being 3.3–3.8 Å from it, and that they interact weakly with the ring of CTP. However this marked increase in metal ion–ring interaction of ATP complexes compared to CTP complexes is not correlated with a decreased number of water molecules in the first coordination sphere of the metal. This indicates that complexes of Mn^{2+} and Co^{2+} with ATP are not forming direct metal ion–nitrogen bonds and must therefore be outer sphere complexes.

With Ni^{2+}, the conclusion is not as obvious. The value of R over a wide temperature range is 0.66 ± 0.005 or two-thirds. This value could arise in two ways. The Ni^{2+}–ATP may involve a strong metal ion–nitrogen bond and form an inner sphere complex. There would be two coordinated water molecules in this complex while Ni^{2+}–CTP has three. The other possibility is that this metal complex is similar to those of Mn^{2+} and Co^{2+}, but that the water molecule between the metal and nitrogen is bound so tightly that it is exchanging very slowly with the bulk water. If the assumption that the metal ion is simultaneously bound to the three phosphates is not correct, the conclusions for Mn^{2+} and Co^{2+} are still valid because the number of coordinated water molecules is the same in the ATP and CTP complexes regardless of what the number might be. The two possible explanations for the Ni^{2+} experiments also do not depend on the exact number of coordinated water molecules in the ATP and CTP complexes. The R value is significantly less than 1 and this must be explained by either a direct metal–ring bond or by the trapping of one of the coordinated water molecules.

If Ni^{2+}–ATP forms an inner sphere complex, then the magnitude of the chemical shift of H-8 should reflect this. Figure 6 contains a plot of the shifts of protons H-8, H-1′, and H-2 of Ni^{2+}–ATP solutions *vs.* the metal:nucleotide ratio, f. The H-8 peak cannot be observed at high f values because it is too broad and becomes obscured by the H-2 peak. It is reasonable to assume that the H-8 shift shows a deviation from linearity just as the H-2 and H-1′ shifts do (Figure 6). By linear extrapolation of the low f values of H-8, an upper limit of 510 Hz for the shift of this proton in the Ni^{2+}–ATP complex is obtained. In order to decide whether this is characteristic of a direct Ni^{2+}–(N-7) bond, the shift should be compared to that of a Ni^{2+}–adenosine complex which is known to have a direct metal–nitrogen bond. The effect of Ni^{2+} on the proton spectrum of adenosine in aqueous solution even at a ratio of 1:1 is slight indicating that very little complexation of any sort occurs. A convenient technique for increasing the tendency of Ni^{2+} to coordinate with nitrogen ligands is to prepare the bis(acetylacetonato) complex of the metal (Fack-

TABLE III: Ratio of Relaxation Rates of Metal–ATP:Metal–CTP Complexes.

Metal Ion	Concn (M)	Temp (°C)	R[a]
Mn^{2+}	$\sim 10^{-3}$	0–65	0.97 ± 0.05 (4-II)
Co^{2+}	$\sim 10^{-1}$	−8 to +65	0.94 ± 0.05 (16-III)
Ni^{2+}	$\sim 10^{-1}$	−8 to +65	0.66 ± 0.05 (14-II)

[a] The ratio $R = [T_2(M^{2+}\text{–CTP})]/[T_2(M^{2+}\text{–ATP})]$ is given as mean ± std dev, with the number of measurements (arabic numbers) and number of different solutions (Roman numerals) given in parentheses.

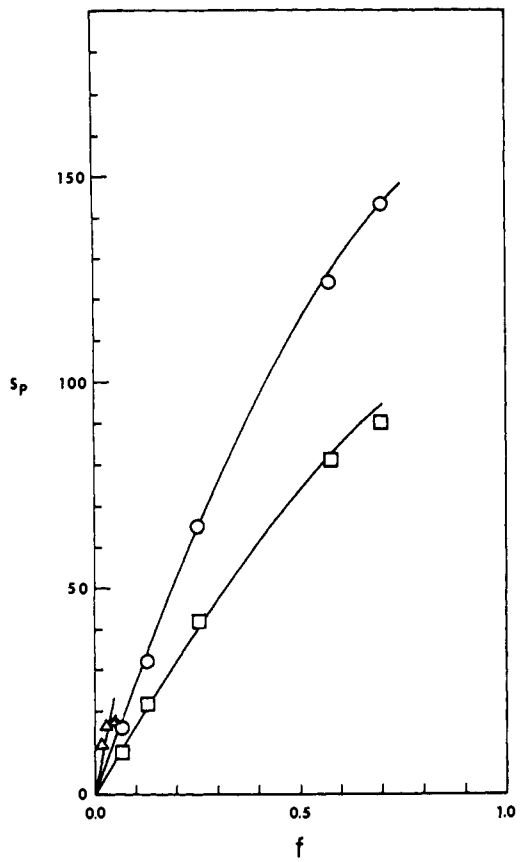


FIGURE 6: Relationship of shifts of Ni^{2+}–ATP and *f*, the ratio of the concentration of Ni^{2+} to ATP: (△) H-8; (○) H-1′; (□) H-2. Spectra recorded at 100 MHz at 70° in D_2O.

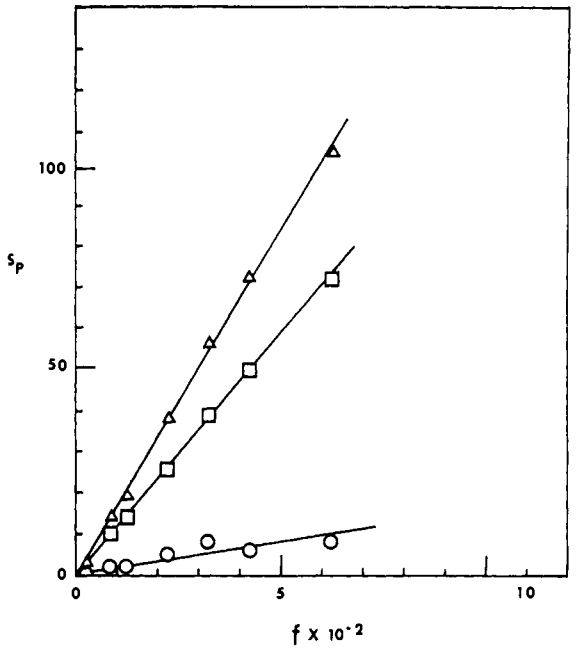


FIGURE 7: Relationship of shifts of bis(acetylacetonato)nickel(II)–adenosine adduct and *f*, the ratio of the concentration of bis(acetylacetonato)nickel(II)–adenosine: (△) H-8; (□) H-2; (○) NH_2. Spectra recorded at 60 MHz at 70° in dimethyl sulfoxide.

ler, 1966). The formation of this complex in dimethyl sulfoxide is accompanied by a distinct color change from the bright green of the bis(acetylacetonato)nickel(II) species to blue for the adenosine adduct and this color change was used to determine the stability constant. The complex appears to be a relatively weak 1:1 adduct in dimethyl sulfoxide with a stability constant at 70° of 19 ± 2 M^{-1}. The shifts of the amino group, H-8 and H-2 of this complex as a function of *f*, the stoichiometric ratio of bis(acetylacetonato)nickel(II) to adenosine, are plotted in Figure 7. Both the H-8 and H-2 peaks are markedly shifted downfield while the amino peak is relatively uneffected. From these shifts it is apparent that base adduct formation is occurring at N-7 and in addition at N-1 and/or N-3. The chemical shift resulting from direct Ni^{2+}–(N-7) binding in this complex can be calculated when the assumption is made that the ratio of the shift of H-8 to the shift of H-2 produced by complex formation directly reflects the ratio of complexation at N-7 to that at N-1 and/or N-3. The shift for H-8 in the N-7 bonded base adduct at 70° and 100 MHz is calculated to be 5800 Hz. In spite of this assumption there is little doubt that the shift is much larger than that observed for H-8 of the Ni^{2+}–ATP complex. This result is very strong evidence against direct Ni^{2+}–(N-7) binding in the Ni^{2+}–ATP complex.

Discussion

The two objectives of this study were to determine whether the interaction of paramagnetic ions with H-8 of ATP arises from a specific metal ion–ring attraction in the 1:1 metal–ATP complex and whether such interaction results either from an inner sphere complex in which the metal ion is directly coordinated to the nitrogen of the ring or from an outer sphere complex in which the binding to the ring is *via* an intervening molecule, solvent water. Our first conclusion is that a specific metal ion–ring interaction exists in the 1:1 metal–ATP complex. This conclusion is valid if comparing metal complexes of ATP to those of TuTP reflects only changes in the adenine ring. Because the glycosidic bond of adenosine is readily hydrolyzed by acid while the glycosidic bond of tubercidin resembles that of the pyrimidine nucleosides in that it is very resistant to hydrolysis, it might be argued that the changes seen with TuTP results from restricted rotation about this glycosidic bond and not from ring changes. If this increased stability reflects double-bond character, then the glycosidic bonds of tubercidin and the pyrimidines should be shorter than that of adenosine. Table IV shows a comparison of glycosidic bond distances which demonstrates that the bond length is not correlated with the nature of the base ring. Whatever causes the increased stability of the glycosidic bond in TuTP presumably does not affect free rotation about this bond

TABLE IV: Glycosidic Bond Distances in Ångströms for Adenosine, AMP, Cytidine, and 5-Bromouracil.

	(N-1)–(C-1′)	(N-9)–(C-1′)
Cytidine	1.497 ± 0.006[a]	
AMP		1.492 ± 0.012[b]
Adenosine		1.51[c]
5-Bromouracil	1.53[c]	

[a] Furberg *et al.* (1965). [b] Kraut and Jensen (1963). [c] Haschemeyer and Sobell (1965).

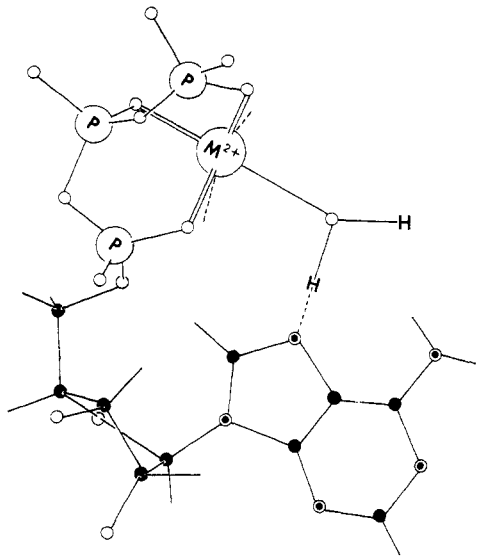


FIGURE 8: A possible structure of a metal–ATP outer sphere complex. The metal ion is octahedrally coordinated and two water molecules not involved in hydrogen bonding to the ring have been omitted.

and therefore those differences seen with TuTP are due to alterations of the ring.

Our second conclusion is that the metal and ATP form an outer sphere complex with the metal indirectly bound to N-7 of the ring through a water molecule (Figure 8). This conclusion results from "counting" the water molecules in the first coordination sphere of the metal–ATP complex by comparing the relaxation of solvent water from this complex to that of the metal–CTP complex. The use of CTP is justified for the reasons stated in the Results and because with two metals, Mn^{2+} and Co^{2+}, the relaxation rates of solvent water in CTP and ATP complexes were identical. This is not merely fortuitous because the factors contributing to the relaxation rates, such as the tumbling times of the complexes, are temperature dependent and yet the ratio of these rates, R, is not. It is unlikely that these factors randomly combine to give identical values of R if CTP were not an appropriate standard. The presence of a water molecule between Ni^{2+} and N-7 of ATP was indicated by Ni^{2+}–ATP having only two rapidly exchanging water molecules in its first coordination sphere. The small shift of H-8 in the Ni^{2+}–ATP complex relative to the adenosine adduct of bis(acetylacetonato)nickel(II), shows that an inner sphere complex is not being formed and the water molecule must be trapped between the metal and the ring.

The Ni^{2+}–N=CH interaction has also been studied in some well-characterized Ni^{2+} octahedral inner sphere complexes. For nickel(bipyridine) $Cl_2 \cdot 2H_2O$ the peak of the protons nearest the Ni^{2+} is shifted downfield by 11,300 Hz at 70° and 100 MHz (Wicholas and Drago, 1968) and for nickel(pyridine)$_6^{2+}$ (Cramer and Drago, 1970) the shift of the α proton under these conditions is 5640 Hz downfield. A comparison of chemical shifts arising from interactions in the Ni^{2+}–N=CH skeleton has some validity because the spin delocalizations occur primarily in the σ bonding system (Happe and Ward, 1963) and the shifts will not be as sensitive to the exact nature of the aromatic system as shifts which arise from π-electron delocalization. The large downfield shift, 5800 Hz, calculated for H-8 of the bis(acetylacetonato)nickel(II)–adenosine complex is quite consistent with spin delocalization

TABLE V: Rate Constants for H_2O Exchange at 25°.

	k (sec^{-1})[a]
Co^{2+}	10^6
Ni^{2+}	2.7×10^4
Mn^{2+}	3.7×10^7

[a] Swift and Connick (1962).

through the Ni^{2+}–N=CH skeleton. These studies suggest that the shift of 510 Hz as seen with Ni^{2+}–ATP does not arise from an inner sphere complex and could be due to long range spin polarization from Ni^{2+} being rigidly held with respect to the adenine ring in an outer sphere complex. This interaction due to long-range electron orbital overlap gives rise to the familar long-range peak splitting of electron paramagnetic resonance and nuclear magnetic resonance. Recently it has been shown (Brown and Drago, 1970) that quite sizeable shifts of this type can be observed for ion pairs in solution.

How can a water molecule become "trapped" between the metal and the ring? Our interpretation of the data presented in this paper is consistent with the structure of the metal–ATP complex in Figure 8. A water molecule in the first coordination sphere of the metal ion is hydrogen bonded to the adenine ring at N-7. This complex forms with relatively little strain when made from space-filling models. The driving force for the formation of such a water-bridged complex would be the same entropy increase accompanying any chelate complex formation. The N-7 of the adenine ring is a basic site and will, on the average, have a water molecule associated with it *via* a hydrogen bond. If this water molecule also happens to be coordinated to the metal ion, a potentially bound water is freed when the complex forms, thus resulting in an entropy increase. The coordinated water molecules are the most acidic water molecules in solution (Sillen and Martell, 1964) and this would also contribute to forming a water-bridged complex.

Direct evidence for the trapped water was obtained for the Ni^{2+}–ATP complex. However, it has also been demonstrated that both the Co^{2+}–ATP and Mn^{2+}–ATP complexes involve outer sphere metal ion–adenine ring interactions. In the preceding paragraph we presented two "driving forces" which might be expected to lead to the trapping of a water molecule. These forces should also apply to the Co^{2+}–ATP and Mn^{2+}–ATP systems. It is not unreasonable to propose that the outer sphere association in these other two systems also involves the trapped water molecule.

If this water-bridging model is correct for the ATP complexes of Mn^{2+}, Co^{2+}, and Ni^{2+} why does the Ni^{2+} complex, but not the others, involve a slowly exchanging water molecule? Table V lists the rate constants for water exchange from the inner coordination spheres of $Ni(H_2O)_6^{2+}$, $Co(H_2O)_6^{2+}$, and $Mn(H_2O)_6^{2+}$ to the aqueous solution bulk at 25°. The Ni^{2+} ion exhibits the slowest water exchange of the three metals by a considerable factor. The relaxation of bulk water protons has been studied as a function of temperature by Swift and Weinberger (1968). Below room temperature there is a marked decrease in the relaxation rate due to slow exchange. It is clear from this that it would be relatively easy to "trap" a water molecule in Ni^{2+}–ATP so that it did not contribute appreciably to the relaxation of bulk protons. Because of the inherently more rapid water exchange with Co^{2+}

and Mn^{2+} this trapping is much more difficult to observe by proton magnetic resonance studies.

There is one well-characterized example of a water-bridged metal–nitrogen ligand complex. This is the Fe^{3+}–H_2O–histidine portion of ferrimyoglobin (Nobbs *et al.*, 1966). The ferric ion in the heme is bound *via* a water molecule to the imidazole ring of histidine.

If a water-bridged structure exists for ATP complexes of Mn^{2+}, Co^{2+}, and Ni^{2+}, it seems likely that it could also exist for such diamagnetic ions as Mg^{2+} and/or Ca^{2+}. Because binding of the metal ion to the ring is not direct, investigation based on the change in ultraviolet spectrum (Schneider *et al.*, 1964) or on the effects of diamagnetic ions on the proton (Cohn and Hughes, 1960, 1962) or ^{15}N (Happe and Morales, 1966) magnetic resonance spectrum would quite likely be too insensitive to record an interaction. A challenging problem for further investigation in this area is the development of a technique for the critical examination of possible outer sphere complexing in Mg^{2+}–ATP.

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Added in Proof

A paper has recently appeared (Heller *et al.*, 1970) in which the Mn^{2+} complex of ATP is studied by nuclear magnetic resonance means. We concur with the authors in the conclusion concerning no direct metal–ring bond in this complex. However their data do not bear on the question of the indirect bond proposed here. For reasons presented in this manuscript we do not believe that the authors' method of "counting" water molecules coordinated to Mn^{2+} in the Mn^{2+} complex of ATP in valid. The secondary effect of phosphate coordination on τ_s will invalidate this procedure as the authors themselves have experimentally observed.

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