species in solution at the low chelate concentrations used here (0.007 to 0.009 M).

A detailed analysis of the CPE results reported in this study must await further theoretical development of f-f optical activity in dissymmetric lanthanide complexes. Almost nothing is known about the mechanisms whereby lanthanide ion f-f transitions acquire optical activity in a chiral ligand environment nor about the influence of specific chelate-solvent interactions on optical activity in metal complexes in general. It seems clear that both TE and CPE are very sensitive probes for investigating chelate-solvent interactions and that they can provide a great deal of qualitative (and, in some cases, semiquantitative) structural information not readily obtainable by other techniques. However, the development of detailed and specific spectra-structure relationships must await further experimental and theoretical study.

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# Proton Magnetic Resonance Study of Divalent Metal Ions Binding to Adenosine 5′-Triphosphate

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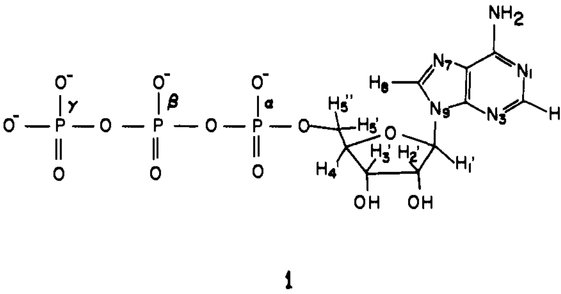
**Abstract**: The monometallic $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, and $Ca^{2+}$ complexes with adenosine 5′-triphosphate have been investigated by proton magnetic resonance. The experimental pD values were adjusted so as to have the adenine ring either protonated or unprotonated, and the phosphate chain triple or fully ionized. The effect of ring protonation or the secondary phosphate hydrogen ionization on the metal-ring and metal-chain interactions has been studied. The formation of a bis $M^{2+}$-ATP complex was established. The equilibria equations involving the formation of 1:1 and 1:2 metal-ligand complexes and the nucleotide molecules self-association are discussed, and a method for calculating the formation constants from the NMR shifts is presented. A model for the bis complex, compatible with the experimental results, is proposed.

Nuclear magnetic resonance has been extensively used in the study of adenine nucleotides and their metal complexes.[2] Concerning the complexes of adenosine 5′-triphosphate (**1**) and divalent metal ions, the main results may be summarized as follows. The paramagnetic ions ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$) bind simultaneously to the three ($\alpha$, $\beta$, $\gamma$) phosphates and to the adenine ring.[3-8] The binding to the ring is not direct but *via* a water molecule which is coordinated to the metal ion and hydrogen bonded to $N_7$ of the adenine ring.[9,10] In addition, the metal complex contains another two water molecules in the inner sphere of coordination.[9-11] A more recent $^{13}C$ NMR study of the $Mn^{2+}$ complex of ATP indicated however a direct binding of the manganese ion to the $N_7$ ring nitrogen.[13] At high total ATP concentration it was found that 1:2 metal-ligand complexes are formed.[12,14] The diamagnetic ions ($Mg^{2+}$, $Ca^{2+}$) were found not to interact with the adenine ring[6,15] and their binding is to the $\beta$ and $\gamma$ phosphates[6] or to the $\beta$ phosphate alone.[16]



**1**

ATP has two major ionization sites: the phosphate moiety and the adenine ring. The three primary phosphate hydrogens were estimated to have $pK \lesssim 2$,[17] and the final hydrogen was found to have $pK \sim 7$.[18-23] Hence the major part of ATP will be fully ionized ($ATP^{4-}$) at $pH \gtrsim 8$. At a lower pH both $ATP^{4-}$ and $ATP^{3-}$ will be present, and at $pH \lesssim 5$ the major part will be $ATP^{3-}$. $^{31}P$ NMR studies[3,6,16] revealed almost no difference in the metal-phosphate interaction in the pH range 5.5–8.5. In the present work we show that the same is true also regarding the metal-ring interaction. This metal-ring interaction, however, may be expected to depend on the ionization state of the ring. The p$K$ associated with the ionization from the protonated ring of ATP was found to be ~4.[20-22] The problem was to determine the site of protonation/ionization in the adenine ring. Although some workers have claimed that the ionization is from the protonated $C_6$-amino group,[20,21,25-27] it is commonly agreed that the proton ionization site is the $N_1H^+$ group.[28-31] We now present a study on the effect of the ring protonation on the metal-ring interaction in divalent metal complexes of ATP. The possibility of formation of 1:2 metal-ligand complexes has been taken into account (for the paramagnetic ions) and formation constants for the various processes have been calculated.

## Experimental Section

**Materials.** ATP (disodium salt) and adenosine (A) of highest grade were obtained from Sigma Chemical Co. and were used without further purification. The metal salts $Co(NO_3)_2{\cdot}6H_2O$ and $MgCl_2{\cdot}6H_2O$ were obtained from Merck and $Ni(NO_3)_2{\cdot}6H_2O$ and $CaCl_2{\cdot}2H_2O$ from BDH Chemicals. The salts were dried overnight by slight heating in vacuo (to prevent decomposing). Stock solutions (2-3 M) of the nickelous and cobaltous salts were prepared with $D_2O$ (99.7%) as solvent and standarized by EDTA complexometry with murexide as indicator. All other concentrations were determined gravimetrically. The ATP concentrations used were in the range 0.06-0.09 M, the $Ni^{2+}$ and $Co^{2+}$ concentrations varied from $1 \times 10^{-3}$ to $5 \times 10^{-1}$ M, and those of $Mg^{2+}$ and $Ca^{2+}$ from $8 \times 10^{-2}$ to $6 \times 10^{-1}$ M. In the higher $Ca^{2+}$ concentrations a small volume of precipitate appeared in the APT solution and was dissolved after slight heating of the solution (to about 50 °C). The pH was adjusted, using concentrated acid or base solutions in $D_2O$.

**NMR Spectra.** Proton magnetic resonance measurements were recorded on a Bruker HFX-10 spectrometer operating at 90 MHz. A trace of *p*-dioxane in the experimental solutions served as internal reference for shift measurements. All the measurements were performed at an ambient probe temperature of 27 °C, except for one case (see below) when a temperature study was made. The temperatures were stabilized by a Bruker B-ST 100/760 control unit and were calibrated by measuring the peak separation of an ethylene glycol sample. The experimental error in the field shift measurements was taken as about 5% of the line width values but not less than 1 Hz. An average of three or four measurements has been taken in order to improve the overall accuracy.

**Experimental pD.** Considering the following equilibria between ATP and $D^+(H^+)$ in $D_2O$ solutions:

$$^{+}\mathrm{DATP}^{3-} \rightleftarrows \mathrm{ATP}^{3-} + \mathrm{D}^+ \qquad \mathrm{p}K' \sim 4$$

$$\mathrm{ATP}^{3-} \rightleftarrows \mathrm{ATP}^{4-} + \mathrm{D}^+ \qquad \mathrm{p}K'' \sim 7$$

where $^{+}DATP^{3-}$ denotes a protonated ring state, we have

$$\frac{[\mathrm{ATP}^{3-}]}{[^{+}\mathrm{DATP}^{3-}]} = 10^{\mathrm{pD}-\mathrm{p}K'}$$

$$\frac{[\mathrm{ATP}^{4-}]}{[\mathrm{ATP}^{3-}]} = 10^{\mathrm{pD}-\mathrm{p}K''}$$

Accordingly, the experimental pD values, which are the pH meter readings +0.4,[32] were chosen to be: pD ~ 3 for the study of the $^{+}DATP^{3-}$ species, pD 5-6 for the study of $ATP^{3-}$, and pD ~ 8 for the study of $ATP^{4-}$.

## Calculation of Formation Constants

Taking a solution containing divalent metal ions and ATP, in appropriate pH (pD), so that the ATP-proton reactions can be neglected, the major processes that take place are the formation of 1:1 and 1:2 metal-ligand complexes and dimerization of ATP molecules due to self-association.[12,33,34] (The complexation and association of higher orders, i.e., $ATP_n$ and $M^{2+}$-$ATP_n$, $n > 2$, are neglected.) The equilibria and the formation constants involved may be expressed as

$$\mathrm{M} + \mathrm{L} \overset{K_1}{\rightleftarrows} \mathrm{ML} \qquad K_1 = \frac{[\mathrm{ML}]}{[\mathrm{M}][\mathrm{L}]} \tag{1}$$

$$\mathrm{ML} + \mathrm{L} \overset{K_2}{\rightleftarrows} \mathrm{ML}_2 \qquad K_2 = \frac{[\mathrm{ML}_2]}{[\mathrm{ML}][\mathrm{L}]} \tag{2}$$

$$\mathrm{M} + \mathrm{L}_2 \overset{K_3}{\rightleftarrows} \mathrm{ML}_2 \qquad K_3 = \frac{[\mathrm{ML}_2]}{[\mathrm{M}][\mathrm{L}_2]} \tag{3}$$

$$\mathrm{L} + \mathrm{L} \overset{K_4}{\rightleftarrows} \mathrm{L}_2 \qquad K_4 = \frac{[\mathrm{L}_2]}{[\mathrm{L}]^2} \tag{4}$$

$$K_1K_2 = K_3K_4 \tag{5}$$

where M represents a divalent metal ion, L represents ATP in any one of it's ionization states (i.e., $^{+}DATP^{3-}$, $ATP^{3-}$, or $ATP^{4-}$), and [M] and [L] denote free concentrations. The total concentrations $L_0$ and $M_0$ are given by

$$L_0 = [\mathrm{L}] + 2[\mathrm{L}_2] + [\mathrm{ML}] + 2[\mathrm{ML}_2] \tag{6}$$

$$M_0 = [\mathrm{M}] + [\mathrm{ML}] + [\mathrm{ML}_2] \tag{7}$$

Taking M to be a paramagnetic ion then under conditions of fast chemical exchange (in the NMR scale) between free and bound ligands, the following relation holds[35]

$$\Delta H_\mathrm{P} = (1 + P_\mathrm{M}^{-1})\Delta H_\mathrm{a} \tag{8}$$

where $\Delta H_P$ is the field shift between the signals of the free and bound ligands, $\Delta H_a$ is the observed coalescent signal shift with respect to metal free solution, and $P_M$ is the mole ratio of bound and nonbound ligands. In our case, having two types of complexes, $P_M$ is given by

$$P_\mathrm{M} = \frac{[\mathrm{ML}] + 2[\mathrm{ML}_2]}{L_0 - [\mathrm{ML}] - 2[\mathrm{ML}_2]} \tag{9}$$

Substituting eq 9 in 8 we get

$$\Delta H_\mathrm{a} = \frac{[\mathrm{ML}] + 2[\mathrm{ML}_2]}{L_0}\Delta H_\mathrm{P} \tag{10}$$

It should be noted that in eq 10 an assumption has been made that the ligands bound either in the 1:1 or the 1:2 complexes undergo the same field shift. This assumption will be discussed later on. In principle, by solving simultaneously eq 1-4 one can get expressions for [ML] and $[ML_2]$ in terms of the formation constants. Then, by fitting eq 10 to the experimental results the formation constants may be calculated. Since eq 1-4 cannot be solved analytically, an approximate solution is required.

Equations 1 and 2 give

$$[\mathrm{ML}] = K_1[\mathrm{M}][\mathrm{L}] \tag{11}$$

$$[\mathrm{ML}_2] = K_1K_2[\mathrm{M}][\mathrm{L}]^2 \tag{12}$$

Substituting (11) and (12) in (7) gives

$$M_0 = [\mathrm{M}](1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2) \equiv [\mathrm{M}] \cdot Q(K_1,K_2,[\mathrm{L}]) \tag{13}$$

The concentrations of the various types present may be written now as

$$[\mathrm{M}] = M_0/Q \tag{14}$$

$$[\mathrm{ML}] = K_1M_0[\mathrm{L}]/Q \tag{15}$$

$$[\mathrm{ML}_2] = K_1K_2M_0[\mathrm{L}]^2/Q \tag{16}$$

$$[\mathrm{L}_2] = K_4[\mathrm{L}]^2 \tag{17}$$

$$L_0 = [\mathrm{L}] + 2K_4[\mathrm{L}]^2 + M_0(K_1[\mathrm{L}] + 2K_1K_2[\mathrm{L}]^2)/Q \tag{18}$$

**(i) Linear Approximation Solution.** In solutions where the paramagnetic ion concentrations are small relative to the ligands, i.e., $M_0 \ll L_0$, eq 18 reduces to the form

$$L_0 = [\mathrm{L}] + 2K_4[\mathrm{L}]^2 \tag{19}$$

Hence $[L] = L_1(L_0,K_4) \simeq$ constnt. If $K_4$ is independently

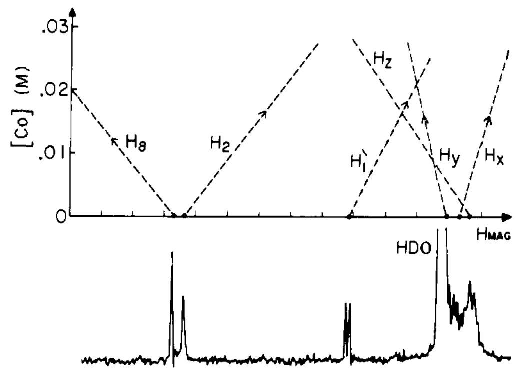


Figure 1. Proton magnetic resonance spectrum of ATP (0.081 M, pD 3, 27 °C) in metal free $D_2O$ solution. In the upper part the paramagnetic shifts induced by adding $Co^{2+}$ ions are shown. $H_x$, $H_y$, and $H_z$ indicate ribose hydrogens. The uncertainty in the metal-free shifts of these protons is ±15 Hz. The scale markers are at 40-Hz intervals.

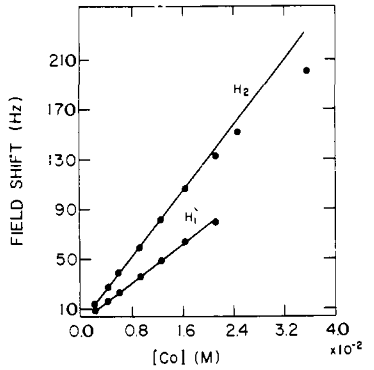


Figure 2. Linear fit of measured field shifts ($H_2$, $H_1'$) for the $Co^{2+}$-ATP system at pD 3 ($[ATP]_0$ = 0.081 M) in the low metal concentration range.

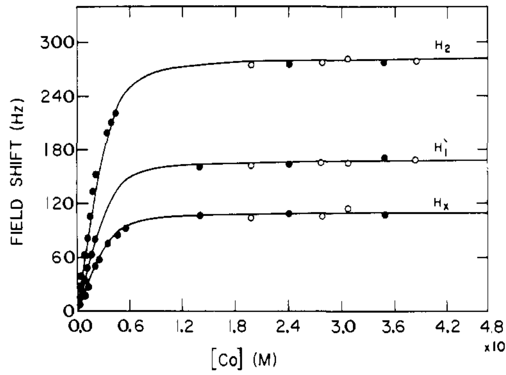


Figure 3. Measured proton shifts in the $Co^{2+}$-ATP system at pD 3 ($[ATP]_0$ = 0.081 M (●), $[ATP]_0$ = 0.070 M (○)). The data were fitted by means of the nonlinear approximation method described in the text.

**Table I.** Formation Constants (in $M^{-1}$) of the 1:1 ($K_1$) and 1:2 ($K_2$, $K_3$) Metal-ATP Complexes and of ATP Self-Association ($K_4$) at 27 °C

| | log $K_1$ | log $K_2$ | log $K_3$ | log $K_4$ |
|---|---|---|---|---|
| $Co^{2+}$-ATP[a] | 2.40 ± 0.20<br>(2.32[c]) | 1.95 ± 0.15 | 3.60 ± 0.20 | 0.80 ± 0.20<br>(0.7[d]) |
| $Ni^{2+}$-ATP[a] | 2.85 ± 0.25 | 1.20 ± 0.08 | 3.28 ± 0.20 | e |
| $Ni^{2+}$-ATP[b] | 2.85 ± 0.25<br>(2.72[c]) | 1.32 ± 0.06 | 3.38 ± 0.20 | e |

[a] pD 3. [b] pD 5.5. [c] From ref 21. [d] From ref 12. [e] The value of $K_4$ obtained from the CoATP study was used.

known, then $L_1$ can be calculated. If not, $K_4$ may be taken as zero and then $L_1 \sim L_0$. We now get

$$Q_1 = 1 + K_1L_1 + K_1K_2L_1^2 \qquad (20)$$

$$[ML] = K_1M_0L_1/Q_1 \qquad (21)$$

$$[ML_2] = K_1K_2M_0L_1^2/Q_1 \qquad (22)$$

Substituting eq 20-22 in eq 10 we obtain

$$\Delta H_a = \frac{(K_1 + 2K_1K_2L_1)}{1 + K_1L_1 + K_1K_2L_1^2} \cdot M_0\Delta H_P \equiv \alpha(\Delta H_P, K_1, K_2, L_1)M_0 \qquad (23)$$

Plotting $\Delta H_a$ vs. $M_0$ will give a straight line with a slope $\alpha$. Applying this method for several solutions of different $L_0$ will enable evaluation of $K_1$ and $K_2$. Another experimental method is to choose $L_0$ such that $K_1L_1 \gg 1$, then eq 23 will reduce to

$$\Delta H_a = \frac{(1 + 2K_2L_1)M_0}{(1 + K_2L_1)L_1}\Delta H_P \qquad (24)$$

and $K_2$ may be derived. On the other hand, under the condition $K_2L_1 \ll 1$, eq 23 will reduce to

$$\Delta H_a = \frac{K_1M_0}{1 + K_1L_1}\Delta H_P \qquad (25)$$

As may be expected the interpretation of eq 24 and 25 is that in solutions dilute in the metal ions, but concentrated in ligand molecules, the 1:2 metal-ligand complex forms the major part. When the ligand is also dilute, the 1:1 complex is the more abundant.

**(ii) Nonlinear Approximation Solution.** In order to solve eq 15 and 16 for [ML] and $[ML_2]$ in terms of the formation constants, the value of [L] is needed. This can be done numerically in the following way. A computer program calculates for given $M_0$ and $L_0$ and for initial values for $K_1$, $K_2$, and $K_4$ a value for [L] which fulfills eq 18. This value is used to calculate [ML] and $[ML_2]$ (eq 15 and 16) which subsequently are substituted in eq 10. This is repeated for all the metal ion concentrations, and the calculated field shifts are compared with the experimental results. The program then changes in a systematic way the values of the formation constants and repeats the above process until a best fit (in a least-squares sense) between the calculated and experimental data is obtained. This method is expected to be very fruitful if the metal ion concentration range is suitably chosen, i.e., changing the values of $M_0$ so that the relative concentrations of the mono and bis ATP complexes vary from $[ML] \ll [ML_2]$ to $[ML] \gg [ML_2]$. It is important to note that while a priori knowledge of $\Delta H_P$ is needed in the nonlinear approximation, this may not be required in the linear approximation (eq 23). Moreover, the linear approximation may also be used to derive the values of $\Delta H_P$. In the case of ATP complexes with $Co^{2+}$ and $Ni^{2+}$, values of $\Delta H_P$ could have been directly measured and hence the nonlinear apprximation could have been applied.

## Results and Interpretation

**$Co^{2+}$-ATP.** The proton spectrum of ATP at pD 3.0 is shown in Figure 1. In the course of the experiment which consisted of titration of ATP with $Co^{2+}$.ions, the shifts of $H_8$, $H_2$, and $H_1'$, and also those of other ribose protons, which we denote as $H_x$, $H_y$ (a double line), and $H_z$, were observed. (Another ribose proton signal was obscured by the water signal.) Some of the shifts were upfield and others downfield, relative to their

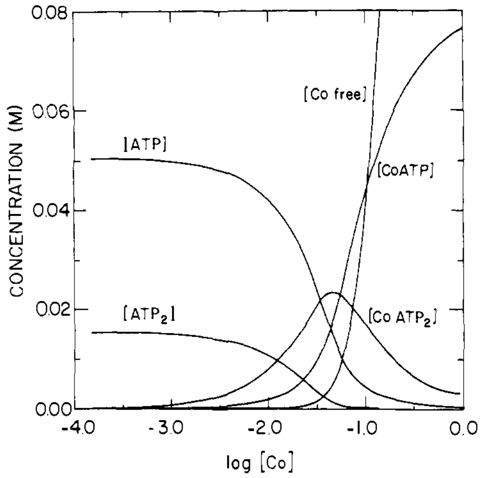


**Figure 4.** Calculated concentrations of the various species present in the ATP (0.081 M) solution as a function of added $Co^{2+}$ ion concentrations. The formation constants from Table I were used.

**Table II.** Bound State Field Shifts ($\Delta H_P$), Line Widths ($\Delta\nu_{2M}$), and Metal-Proton Distances ($R$) for the CoATP (1:1) Complex at pD 3[a]

| | $\Delta H_P$, Hz | $\Delta\nu_{2M}$, Hz | $R$, Å |
|---|---|---|---|
| $H_2$ | 170 ± 5 | 25 ± 3 | 5.0 |
| $H_8$ | −350 ± 20 | 450 ± 50 | 3.1 |
| $H_1'$ | 285 ± 5 | 30 ± 3 | 4.8 |
| $H_x$ | 111 ± 5 | 24 ± 3 | 5.1 |
| $H_y$ | −60 ± 20 | 24 ± 3 | 5.0 |
| $H_z$ | −250 ± 20 | 100 ± 5 | 4.0 |

[a] $H_x$, $H_y$, and $H_z$ denote ribose hydrogens. Negative shifts are to low field.

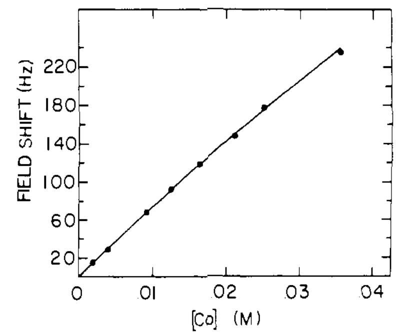


**Figure 5.** Measured $H_8$ field shifts (to low field) in the $Co^{2+}$-ATP system at pD 3 ($[ATP]_0 = 0.081$ M).

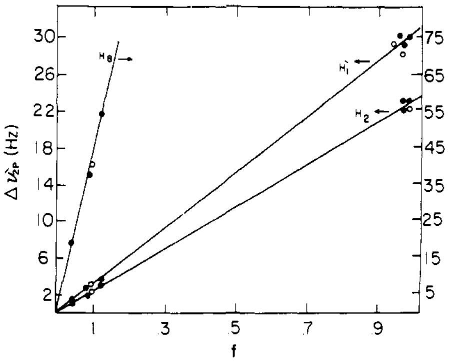


**Figure 6.** Measured paramagnetic line width broadening in the $Co^{2+}$–ATP system at pD 3 ($[ATP]_0 = 0.081$ M, $[ATP]_0 = 0.070$ M (○)). $f = ([ML] + 2[ML_2])/L_0$. For $f \ll 1, f \simeq P_M$ (see text). For $f \sim 1$, the bound line widths were directly measured.

metal-free shifts, indicating the presence of both contact and pseudocontact hyperfine interactions contributing to the shifts (compare with the solely downfield shifts observed for the NiATP complex, cf. Table V below). Since the chemical exchange of ATP in the cobaltous ATP system is fast[3,4] the above method for calculation of the formation constants could be applied. Since the $H_2$ and $H_1$ shifts could be observed throughout almost the whole measuring range, they were thus used for the calculation. First we applied the linear approximation solution assuming $L_1 \simeq L_0$. It came out that eq 24 is the appropriate one for this case. A least-squares fit of the experimental results (Figure 2) for $[Co^{2+}]_0 \ll [ATP]_0$ yielded an average value of $K_2 = 75 \pm 20$ $M^{-1}$ ($\log K_2 = 1.88$). The deviation of the higher concentration shifts from the straight line in Figure 2 may be attributed to departure from the linear approximation conditions. Next, the nonlinear approximation was applied to all the $H_2$ and $H_1$ experimental shifts (Figure 3). The formation constants obtained (an average of the two data set results) are given in Table I. The relatively large errors in the formation constants are due to "flatness" and relative low sensitivity of the fitting function for large formation constants. It should be noted, however, that although the published errors for the formation constants determined by pH titration are very small, the distribution of the various results is no better than our error range.[2] Using the derived formation constants, the concentrations of the different species present in the experimental solution were calculated as a function of the $Co^{2+}$ concentrations. The results are presented in Figure 4. It can be seen that in our experimental solution the linear approximation (in the low $Co^{2+}$ concentration range) is partly fulfilled. [L] is indeed approximately constants but $[L_2]$ is not zero, and thus a lower value of $K_2$ was obtained. At low-metal concentrations the $Co(ATP)_2$ complex predominates, but as the concentration rises the CoATP complex becomes dominant. Experimental values obtained for $[Co^{2+}]_0 \gtrsim [ATP]_0$ can thus be assigned to the 1:1 metal-ligand complex.

The proton field shifts in the bound state ($\Delta H_P$) of the 1:1 compex are given in Table II. The $H_2$, $H_1'$, $H_x$, $H_y$, and $H_z$ shifts were measured directly. Since the metal-free shifts of $H_y$ and $H_z$ could not be determined accurately, the errors in their paramagnetic shifts were accordingly noted. The shifts of $H_x$ (Figure 3) and $H_8$ (Figure 5) were determined by a least-squares fitting of eq 10 to their experimental results.

The line widths ($\Delta\nu_{2M}$) of the bound state (1:1 complex) are given in Table II. Except for $H_8$ all the other line widths were measured directly. The $H_8$ line width was calculated in the following way. Let $\Delta\omega_M$ be the shift (in rad/s) between the bound and the fast exchange coalescent signals and $T_{2M}^{-1}$ ($=\pi\Delta\nu_{2M}$) the relaxation rate in the bound state, then for the CoATP complex, $\Delta\omega_M^2 \gg T_{2M}^{-2}$. Hence, the paramagnetic and exchange contributions to the relaxation rate of the nonbound molecules (for $[Co^{2+}]_0 \ll [ATP]_0$) are given by[36]

$$T_{2P}^{-1} = P_M T_{2M}^{-1} + P_M \tau_M \Delta\omega_M^2 \qquad (26)$$

where $\tau_M$ is th mean lifetime in the bound state, $\Delta\omega_M \simeq 2\pi\Delta H_P$, and $P_M \simeq ([ML] + 2[ML_2])/L_0$. Thus if we define

$$\Delta\nu_{2P}' = \Delta\nu_{2P} - 4\pi P_M \tau_M \Delta H_P^2 \qquad (27)$$

we get

$$\Delta\nu_{2P}' = P_M \Delta\nu_{2M} \qquad (28)$$

The appropriate $P_M$ values for any $Co^{2+}$ concentration can be calculated using the formation constants obtained above. Equation 28 was fitted to the line widths of $H_2$ and $H_1'$ (Figure 6) to yield a mean value of $(8 \pm 1) \times 10^{-5}$ s for $\tau_M$. With this

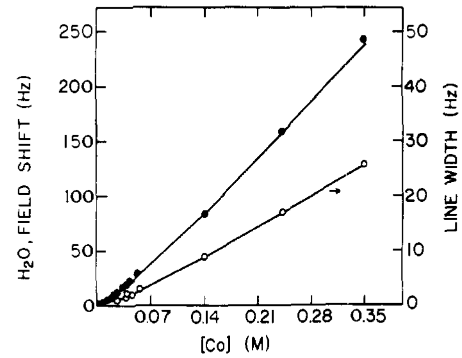


Figure 7. The field shifts (●) and paramagnetic line broadening (○) of the signal of the coordinated water molecules in the $Co^{2+}$-ATP system at pD 3 ($[ATP]_0$ = 0.081 M).

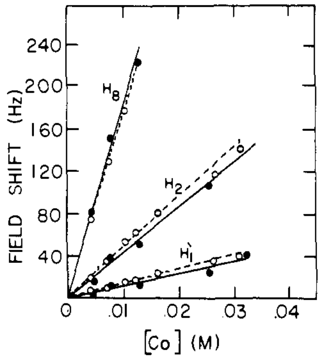


Figure 8. Proton field shifts in the $Co^{2+}$-ATP system at pD 5.8 (●) and pD 8.5 (○).

value, and the same fitting procedure, the $H_8$ line width was determined.

The transverse relaxation rate of the protons in the bound ATP, being of a dipolar character,[4] is given by[37]

$$T_{2M}^{-1} = \tfrac{4}{3}S(S + 1)\hbar^2 g^2 \beta_e^2 \gamma_n^2 R^{-6} T_e \qquad (29)$$

where $R$ is the metal-proton distance and $T_e$ is the metal ion unpaired electronic relaxation time. In eq 29 any anisotropy in the $Co^{2+}$ $g$ factor[38] is neglected and an "effective" $g$ is used. Sternlicht et al.[4] estimated the relative error in the relaxation rates, due to this, to be smaller than the factor 4. In order to calculate the metal-proton distances eq 29 was used, assuming $g$ and $T_e$ to have the cobaltous-aqueous complex values, hence getting the relation,

$$R(\mathrm{ATP}) = R(\mathrm{H_2O}) \left[\frac{\Delta\nu_{2M}(\mathrm{H_2O})}{\Delta\nu_{2M}(\mathrm{ATP})}\right]^{1/6} \qquad (30)$$

The combined uncertainties in this method of calculation may result in about 40-50% error in the metal proton distances. This is quite an apprecible error, but since the relative distances are reasonably reliable itis still worth calculating them. The values obtained are given in Table II. (The parameters for the cobaltous aqueous complex are given in the Appendix.)

The solvent molecules (HDO) may also enter the metal ion coordination sphere either in a pure aqueous complex, with coordination number 6, or in the 1:1 and 1:2 metal-ligand complexes, with coordination numbers $N_1$ and $N_2$, respectively. The number of bound water molecules in either complex could not be determined directly. However, an estimate of this number can be made. Assuming that the coordinated water in the three complex types undergoes the same field shifts ($\Delta H_P(\mathrm{H_2O})$), an assumption that will be considered later on, the shifts of the bulk water molecules may be written as:

Table III. Calculated Number of Coordinated Water Molecules in the 1:1 ($N_1$) and 1:2 ($N_2$) $M^{2+}$-ATP Complexes

| $M^{2+}$ | $N_1$ | $N_2$ |
|---|---|---|
| $Co^{2+\,a,c}$ | 3.6 ± 0.1 | 3.5 ± 0.1 |
| $Co^{2+\,a,d}$ | 3.1 ± 0.1 | 3.3 ± 0.2 |
| $Ni^{2+\,a,d}$ | 4.4 ± 0.2 | 4.0 ± 0.5 |
| $Ni^{2+\,b,d}$ | 4.0 ± 0.2 | 3.8 ± 0.5 |

[a] pD 3. [b] pD 5.5. [c] From shift measurements. [d] From line width measurements.

Table IV. Bound State Field Shifts ($\Delta H_P$), Line Widths ($\Delta\nu_{2M}$), and Metal-Proton Distances ($R$) for the CoATP (1:1) Complexes at pD 5.8 and 8.5

| | $\Delta H_P$, Hz | $\Delta\nu_{2M}$, Hz | $R$, Å |
|---|---|---|---|
| $H_2$ | 230 ± 15[a] | 24 ± 3 | 5.0 |
| | 245 ± 15[b] | 25 ± 3 | 5.0 |
| $H_8$ | −900 ± 80[a] | 210 ± 30 | 3.5 |
| | −870 ± 80[b] | 230 ± 30 | 3.4 |
| $H_1'$ | 51 ± 5[a] | 22 ± 3 | 5.1 |
| | 70 ± 5[b] | 24 ± 3 | 5.0 |

[a] pD 5.8. [b] pD 8.5.

$$\Delta H_a(\mathrm{HDO}) = \tfrac{1}{50}(6[\mathrm{M}] + N_1[\mathrm{ML}] + N_2[\mathrm{ML_2}])\Delta H_P(\mathrm{H_2O}) \qquad (31)$$

A similar relation may be written for $\Delta\nu_{2M}(\mathrm{HDO})$:

$$\Delta\nu_{2M}(\mathrm{HDO}) = \tfrac{1}{50}(6[\mathrm{M}] + N_1[\mathrm{ML}] + N_2[\mathrm{ML_2}]\Delta\nu_{2M}(\mathrm{H_2O}) \qquad (32)$$

The bulk water shifts and line widths were analyzed by means of eq 31 and 32 (Figure 7). The values obtained for the coordination numbers are given in Table III. As a coordination number 2-3 is expected for the water in the CoATP complex,[9-11] our results may indicate: (a) shortening of the cobaltous electronic relaxation time relative to the aqueous complex (a similar result has been obtained from ESR measurements in MnATP complex[39]); (b) a smaller paramagnetic shift in the CoATP complex relative to the aquo complex. This may be attributed to the presence of a pseudocontact contribution in the ATP complex having a symmetry lower than octahedral. But the major significance of the results lies in the equality (within the error range) of the water coordination numbers in the 1:1 and 1:2 complexes. This may be of importance in understanding the structure of the 1:2 metal-ATP complex.

To complete the study of the cobaltous-ATP system at pD 3, additional experiments have been carried out by titrating a $Co^{2+}$ solution (0.027 M) with ATP (in the concentration range $5 \times 10^{-3}$ to $7 \times 10^{-2}$ M). The advantage of carrying out these ATP titrations lies in the ability to measure the lower field part of the CoATP spectra, since owing to the low metal concentration the water signal is almost unchanged. Also line broadenings due to susceptibility effects are absent. Indeed the last ribose proton (denoted as $H_z'$) was observed, its bound state parameters being $\Delta H_P = -170 \pm 20$ Hz, $\Delta\nu_{2M} = 70 \pm 5$ Hz, and $R = 4.2$ Å. In addition, the bound $H_8$ parameters which could be directly measured were found to be $\Delta H_P = 380 \pm 15$ Hz and $\Delta\nu_{2M} = 410 \pm 10$ Hz, in good agreement with our calculated values.

The observed $H_x$, $H_y$, $H_z$, ad $H_z'$ could not be unequivocally identified with the appropriate ribose protons because their metal-free shifts could not be determined accurately enough. However, with reasonable certainty $H_z$ and $H_z'$ may be

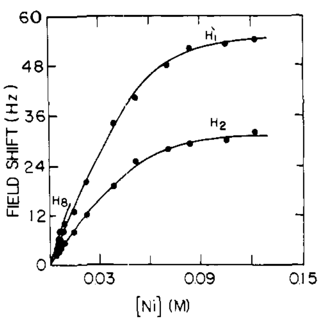


**Figure 9.** Proton field shifts in the $Ni^{2+}$-ATP system at pD 3 ($[ATP]_0$ = 0.082 M). All the shifts are to low field.

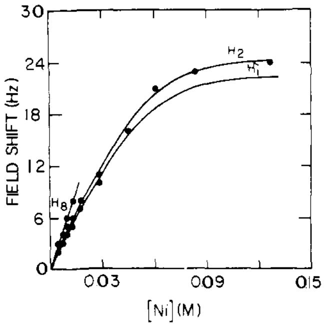


**Figure 10.** Proton field shifts in the $Ni^{2+}$-ATP system at pD 5.5 ($[ATP]_0$ = 0.082 M). All the shifts are to low field.

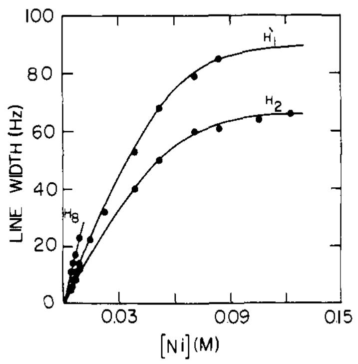


**Figure 11.** Proton paramagnetic line broadening in the $Ni^{2+}$-ATP system at pD 3 ($[ATP]_0$ = 0.082 M).

**Table V.** Bound State Field Shifts ($\Delta H_P$), Line Widths ($\Delta\nu_{2M}$), and Metal-Proton Distances ($R$) for the NiATP (1:1) Complex at pD 3, 5.5, and 8.5

| | $\Delta H_P$, Hz | $\Delta\nu_{2M}$, Hz | $R$, Å |
|---|---|---|---|
| $H_2$ | $-32 \pm 3^a$ | 68 ± 3 | 5.0 |
| | $-25 \pm 3^b$ | 51 ± 5 | 5.2 |
| | $-20 \pm 3^c$ | 50 ± 5 | 5.2 |
| $H_8$ | $-70 \pm 7^a$ | 149 ± 10 | 4.2 |
| | $-35 \pm 3^b$ | 110 ± 10 | 4.5 |
| | $-37 \pm 3^c$ | 120 ± 10 | 4.4 |
| $H_1'$ | $-56 \pm 5^a$ | 92 ± 3 | 4.7 |
| | $-23 \pm 3^b$ | 74 ± 6 | 4.9 |
| | $-26 \pm 3^c$ | 78 ± 6 | 4.9 |

[a] pD 3. [b] pD 5.5. [c] pD 8.5.

**Table VI.** Calculated Bound State Line Widths in the 1:1 and 1:2 $Ni^{2+}$-ATP Complexes at pD 3

| | $\Delta\nu_{2M}^{(1)}$, Hz | $\Delta\nu_{2M}^{(2)}$, Hz |
|---|---|---|
| $H_2$ | 66 ± 2 | 68 ± 3 |
| $H_1'$ | 93 ± 2 | 85 ± 3 |

identified with the two $C_5'$ protons. In the anti-gauche-gauche conformation of ATP in divalent metal complexes,[33,40-42] $H_8$ is the nearest proton to the metal ion followed by $H_5'$ and $H_5''$. The metal-proton distances obtained above for $H_8$, $H_2$, and $H_1'$ are compatible with this conformation. Also, $H_x$, whose metal-free shift could be determined from its paramagnetic shifts (Figure 3), may be identified with the $H_4'$ proton.[43] The two remaining $H_y$ protons are then the ribose $H_2'$ and $H_3'$. This is quite reasonable since these two protons are approximately equidistant from the metal ion in the CoATP complex.

Additional study of the cobaltous ATP system has been carried out at pD 5.8 and 8.5. In this pD range the proton signals for higher metal concentrations (i.e., $[Co]_0 \sim [ATP]_0$) were obscured by the water signal. Hence the bound state parameters ($\Delta H_P$ and $\Delta\nu_{2M}$) had to be calculated by least-squares fitting of the low metal concentration shifts (Figure 8) with eq 10 and the line width data with eq 28 and 30. For the pD 5.8 calculations we used the formation constants calculated above, since the charge state of the ring is not expected to affect the binding constants,[44] as indeed is confirmed by comparison of our $K_1$ value with that obtained by pH titration (Table I). For the pD 8.5 calculations, the formation constants reported in ref 10 and 32 were used. The results are summarized in Table IV.

**$Ni^{2+}$-ATP.** The experimental proton field shifts at pD 3 and 5.5 and the paramagnetic line broadening at pD 3 are shown in Figures 9, 10, and 11, respectively. Examining the three figures, a similar concentration-dependence character is observed which may be interpreted as indication of the prevalence of fast exchange conditions. First, in the whole experimental range a single distinct signal is observed for each resonance, which is shifted from the free to the bound state position. And second, an attempt to fit the line widths with the general relation[45]

$$T_2^{-1} = f_A/T_{2A} + f_M/T_{2M} + f_A f_M \tau_M \Delta\omega_M^2$$

where $T_{2A}$ is the relaxation time of nuclei in the uncoordinated ligand, and $f_A = 1 - f_M = (1 + P_M)^{-1}$, revealed that the last term is too small to have a significant contribution. The $H_2$ and $H_1'$ data were analyzed by the nonlinear approximation method for the calculation of the formation constants. The results are given in Table I. The bound state (1:1 complex) parameters are given in Table V. It appears that in the NiATP complex $T_{2M}^{-2} \gg \Delta\omega_M^2$, hence the relevant relation for the line-broadening analysis is $\Delta\nu_{2P} = P_M T_{2M}^{-1}$.[36] Let $\Delta\nu_{2M}^{(1)}$ and $\Delta\nu_{2M}^{(2)}$ be the line widths in the bound state in the 1:1 and 1:2 complexes, respectively, then we have:

$$\Delta\nu_{2P} = ([ML] \cdot \Delta\nu_{2M}^{(1)} + 2[ML_2] \cdot \Delta\nu_{2M}^{(2)})/L_0 \quad (33)$$

The $H_2$ and $H_1'$ line width data were analyzed with eq 33 to yield the values of $\Delta\nu_{2M}^{(1)}$ and $\Delta\nu_{2M}^{(2)}$ (Table VI).

The water coordination in the nickelous-ATP 1:1 and 1:2 complexes has been studied by fitting the line widths (Figure 12) with eq 32 (the water signal shifts were too small to enable significant study). The results are given in Table III, indicating a more pronounced shortening of the electronic relaxation time in the nickelous relative to the cobaltous ATP complex.

We should point out that our results for the proton saturation shifts in the NiATP complex are lower than those obtained by Glassman et al.[9] and Sternlicht et al.[4] from temperature studies. It is our opinion however that temperature studies should be very carefully interpreted and that simple temperature-dependence assumption may be erroneous. To demon-

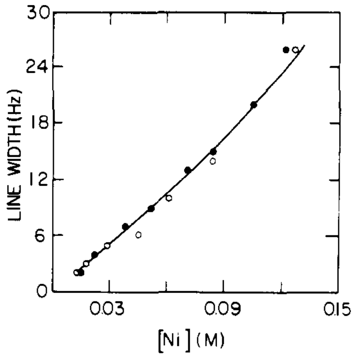


**Figure 12.** The paramagnetic line broadening of the water coordinated in the $Ni^{2+}$-ATP system at pD 3 (●) and pD 5.5 (○).

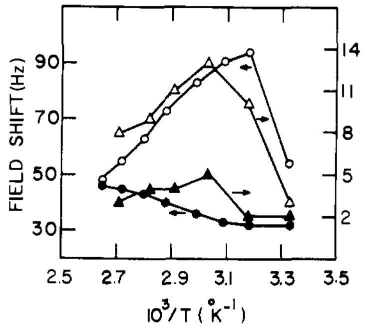


**Figure 13.** Temperature dependence of the $H_2$ (▲) and $H_1'$ (△) field shifts in 1:18 and in 1.1:1 (●, ○ respectively) $Ni^{2+}$-ATP systems ($[ATP]_0$ = 0.082 M, pD 3).

strate this we studied the protons field shifts temperature dependence of 1:18 and 1.1:1 $Ni^{2+}$:ATP solutions (Figure 13). (The paramagnetic shifts were measured relative to metal-free ATP shifts in the appropriate temperatures.) The temperature dependence of the shifts in the $Ni^{2+}$ diluted solution may be interpreted[4] as governed by a chemical exchange process: as the temperature is raised the exchange becomes more rapid and the shifts of the bulk signals increase. In the limit of fast exchange a $1/T$ dependence is expected.[46] Clearly, the decrease of the shift (Figure 13) is much faster than predicted, in a similar "anomalous" way as found in the CoATP complex.[4] Moreover, in the study of the 1.1:1 solution the bound signals were observed directly, and these are expected to shift with $1/T$ dependence in the whole temperature range. The results contradict this expectation completely. Taking into account the possible degrees of freedom of the ATP molecule[40,47] and the complexation and self-stacking temperature dependence we conclude that the temperature study of ATP complexes reflects mainly conformational and complexational changes rather than exchange controlled temperature behavior.

Additional study of the nickelous-ATP complex at pD 8.5 yielded bound state parameters similar, within the error range, to those obtained at pD 5.5 (Table V).

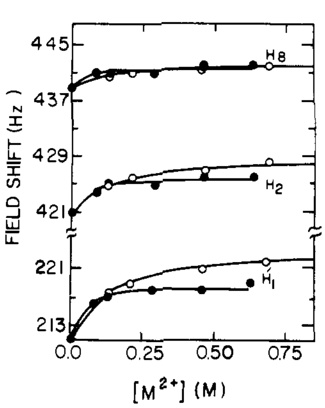


**Figure 14.** Measured proton field shifts in the $M^{2+}$-ATP system, $M^{2+}$ = $Mg^{2+}$ (●) or $Ca^{2+}$ (○). The shifts (to low field) are given relative to *p*-dioxane ($[ATP]_0$ = 0.082 M).

**Table VII.** Proton Field Shifts in the $Mg^{2+}$- and $Ca^{2+}$-ATP (1:1) Complexes at pD 3

| $M^{2+}$ | $H_2$ | $H_8$ | $H_1'$ |
|---|---|---|---|
| $Mg^{2+}$ | $-4.2 \pm 0.2$ | $-2.7 \pm 0.2$ | $-7.4 \pm 0.2$ |
| $Ca^{2+}$ | $-7.6 \pm 0.1$ | $-3.4 \pm 0.1$ | $-12.7 \pm 0.5$ |

**$Mg^{2+}$-ATP and $Ca^{2+}$-ATP.** Measurements of proton field shifts in the magnesium and calcium ATP complexes at pD 5.5 and 8.5 revealed no significant change (within the experimental error) relative to the metal-free ATP solution, in accordance with previous sudies.[6,48] At pD 3 however significant shifts were observed (Figure 14). The data were analyzed assuming the formation mainly of 1:1 complex in the metal's concentrations range investigated and using the binding constants $10^{2.0}$ and $10^{1.1}$ for the MgATP and CaATP complexes as determined by fluorescence study at pH 2.8.[49] The results are given in Table VII. The broadening of the lines in either complex was found to be negligible.

**$Co^{2+}$-ATP-Adenosine.** The complexation of adenosine (A) with $Co^{2+}$ in the absence and the presence of ATP has been studied. The aim was to examine the possibility of the formation of a ternary $Co^{2+}$ATP-A complex, i.e., to demonstrate the ability of the CoATP monocomplex to bind a second molecule via ring binding alone. The measurements were performed at pD 2.5. (Adenosine has a p*K* ~ 3.6 for ring protonation.[20,24]) The effect of the cobaltous ions (0.040 M) on the $H_8$, $H_2$, and $H_1'$ resonance of A (0.038 M) is shown in Figure 15b. The very small effect ($H_2$ and $H_1'$ were almost unchanged and $H_8$ only slightly broadened and downfield shifted) is expected, considering the low formation constant between A and divalent metal ions.[2] Moreover the solvent (HDO) signal was shifted downfield to an extent compatible with the formation of a regular hexacoordinated cobaltous aqueous complex. The adenosine binding is thus an outer-sphere coordination. When ATP (0.009 M) was added to the $Co^{2+}$-A solution, a considerable effect on the A resonance (with $H_8$ undergoing the largest) was induced (Figure 15c). Clearly A which binds very weakly to the $Co^{2+}$ ion binds more strongly to the CoATP complex. The dependence of the field shifts of the $H_8$, $H_2$, and $H_1'$ resonances of A, as well as that of the solvent (HDO) on the concentration of ATP added to the 1:1 $Co^{2+}$:A solution, has been studied. The results are shown in Figure 16. As the ATP concentration was raised more A was bound and water molecules were expelled from the cobaltous coordination sphere. This resulted in shifting the water signal back upfield, and saturation was achieved at about half the original shift, for 1:1 $Co^{2+}$:ATP concentrations, indicating that three water molecules were taken out from the aqueous complex as the CoATP complex was formed. However, as the ATP concentration exceeded that of $Co^{2+}$ (and A), the competition between the free ATP and the adenosine bound in the ternary complex resulted in the latter being removed from the complex, as is inferred from Figure 16. It should be noted, however, that an ATP concentration-dependent irregularity of the adenosine $H_8$ and $H_2$ shifts is observed. The $H_8$ shift is being saturated much faster than that of $H_2$ and "decays" much faster for [ATP] > [A]. This phenomenon is not yet

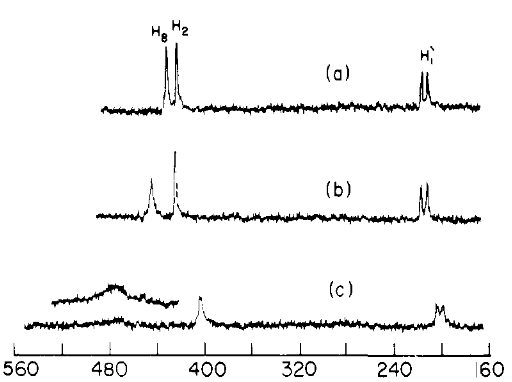


**Figure 15.** Proton magnetic resonance spectra of the $H_8$, $H_2$, and $H_1'$ resonances of A (0.038 M), pD 2.5, in (a) metal free solution, (b) with 0.040 M $Co^{2+}$, (c) with 0.040 M $Co^{2+}$ and 0.009 M ATP. The shifts are measured downfield from a *p*-dioxane internal standard.

completely clear to us and a more thorough examination is needed.

In order to estimate the influence of the ring-ring interacton on their resonance shifts in the $Co^{2+}$-ATP-A complex the above study was repeated, but $Mg^{2+}$ was used instead of $Co^{2+}$. The results shown in Figure 16 reveal that negligible shifts are induced by one ring current on the second ring protons. We also consider this to be the case in the bis $M^{2+}$-ATP complex.

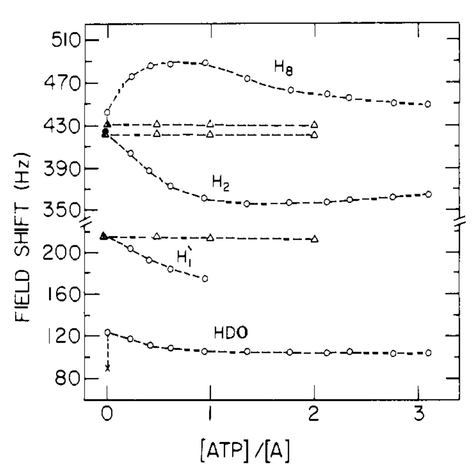


**Figure 16.** The field shifts of the HDO and the adenosine $H_8$, $H_2$, and $H_1'$ resonances as functions of the relative concentration of added ATP to 1:1 $Co^{2+}$:A and 3:1 $Mg^{2+}$:A ([A] = 0.038 M) solutions. The shifts are measured downfield from a *p*-dioxane internal standard: (x) A alone; (○) A + $Co^{2+}$ + ATP; (△) A + $Mg^{2+}$ + ATP.

## Discussion

**(A) The Formation Constants.** Comparing the formation constant obtained for the metal complexes of $ATP^{3-}$ and $^{+}DATP^{3-}$ (Table I), we find that the ring protonation does not significantly affect the metals binding to ATP as reflected in the formation constants. This implies that the main metal binding site is the phosphate chain, and indeed the secondary phosphate hydrogen ionization results in considerable changes in the binding constants.[21] It is interesting to note that in the $Co^{2+}$-ATP system $K_2 \lesssim K_1$, while in the $Ni^{2+}$-ATP system $K_2 \ll K_1$. In view of the bis complex model proposed below, this may be attributed to a lower tendency of $Ni^{2+}$ to form outer-sphere complexes. As the ligand exchange mechanism in metal complexes is expected to be mainly of the $S_N1$ type and to a lesser extent $S_N2$, it seems to us that information on the relative importance of the $S_N2$ process in the $Co^{2+}$- and $Ni^{2+}$-ATP complexes (i.e., from thermodynamic study) may contribute to the understanding of the bis complex. In a recent potentiometic titration study, Frey and Stuehr[50] reported that they found no evidence for a $Ni(ATP)_2$ complex. In view of the small value obtained for $K_2$ and the low concentrations (2-5 $\times 10^{-3}$ M) they used, their results are now explicable.

**(B) The Effect of Ring Protonation on the Metal-Ring Interaction.** Evidence that ATP (and other nucleotides) has a folded structure has been presented.[19,40,41,47] This folding makes possible the formation of metal complexes in which the metal binds simultaneously to the phosphate moiety and to the adenine ring. However, the lack of variation of divalent metal complex formation constants with variation of the ring structure,[51] as well as the thermodynamic values obtained for secondary phosphate hydrogen ionization in ATP and other nucleotides,[52] indicates that in the pH range 6-9, i.e., in the unprotonated ring state, the ring chain interaction is very small. On the other hand, it has been suggested[49,53-55] that when the ring becomes protonated there is electrostatic interaction between the positive ring and the negative chain. Our results provide additional evidence for interaction between the phosphate chain and the protonated ring, which subsequently enhance the metal-ring interaction. Inspecting the bound state data of the $Co^{2+}$- and $Ni^{2+}$-ATP complexes at pD 3 and 5.5 (Tables II, IV and V), it is evident that the ring protonation results in bringing the adenine ring nearerto the metal ion (actually the ribose ring is also affected), i.e., an "over-folding" of the ATP molecule is effected. This is revealed in broader line widths and more pronounced hyperfine interaction shifts. Comparing the changes in the proton field shifts it may be concluded that the $1/R^3$ dependent pseudocontact interaction is increased relative to the contact interaction. As to the diamagnetic metal complexes of ATP, the chain-ring interaction causes enhancement of the metal-ring interaction to an extent which is enough to be reflected in the proton field shifts. It seems reasonable to assume that the diamagnetic metal ions do interact with the adenine ring of ATP in a similar way to the paramagnetic ions. However, since the diamagnetic effect is very short ranged, the metal-ring interaction in the unprotonated ring state may be too small to be reflected in the proton shift. It should be noted that most of those who reject the assumption of metal-ring interaction in diamagnetic metal-ATP complexes[6,15,48] performed their measurements in the unprotonated ring state. Recently Glassman et al.[56] predicted by using the generalized perturbation theory that the $Mg^{2+}$ and $Ca^{2+}$ ions may interact with the $N_1$ or $N_3$ ring nitrogens of ATP (in contrast to the paramagnetic ions). Our results (Table VII) are compatible with this prediction, as the $H_8$ signal is seen to be less affected by the ATP complexation with the diamagnetic ions. Note that $H_8$ and $H_2$ are shifted to low field, i.e., in the same direction as caused by the ring protonation. This supports the assumption of the diamagnetic ions binding to the ring (either directly or via a hydrogen bond to a coordinated water), since the binding of a positive charge to a nitrogenous ring site may result in redistribution of the electronic charge in the adenine ring, similar to ring protonation, hence causing low-field shifts of the proton signals. As $H_1'$ is the most affected, it may imply that the diamagnetic ions interact with the $N_3$ of the ATP ring.

**(C) The Effect of Secondary Phosphate Hydrogen Ionization on the Metal-Ring Interaction.** The measurements in the pD range 5.5-8.5 indicate no significant change in the metal-ring interaction as reflected in the proton-bound state parameters. Thus, within the limits of the experimental error it can be said that the secondary phosphate ionization does not affect the metal binding to the adenine ring. This is compatible with the small chain-ring interaction in this pD range as discussed above. However, evidence for lowering the p*K* of the secondary phosphate ionization due to the presence of a metal ion[44,57] provides an additional way of interpreting the lack of variation

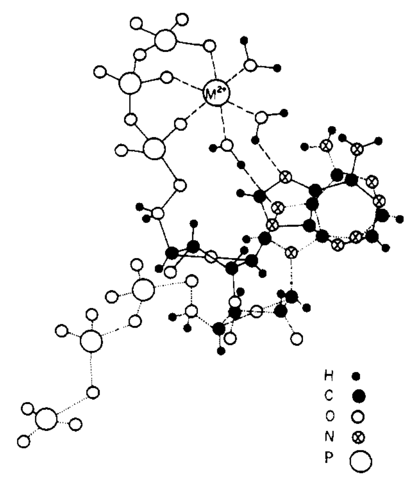


Figure 17. The proposed structure of the bis $M^{2+}$-ATP complex. The molecule presented by the dotted line lies beneath the other one.

of the NMR data (including $^{31}P$ results) in the concerned pD range.

**(D) The Paramagnetic M(ATP)$_2$ Complex.** The fundamental assumption made as the basis for the analysis of our experimental data was that the two ATP molecules in the 1:2metal ligand complex are magnetically equivalent as far as the metaling interaction is concerned. Actually, due to the fast chemical exchange in the metal-ATP system, any differences between these two molecules are averaged out. Thus, the above assumption leads to the requirement that the average metal-ring interaction in the bis complex would be similar to that in the mono complex. The results for both the $Co^{2+}$- and $Ni^{2+}$-ATP complexes demonstrate the validity of this assumption. The measurements have been carried out over wide metal concentration ranges, with the appropriate concentration of the ATP complexes varying from the limit where M(ATP)$_2$ predominates to where MATP predominates. The ability to fit the whole concentration range of the results with a single set of parameters clearly indicates that within the experimental error there is no significant difference between the metal-ring interaction in the 1:1 and 1:2 complexes. Had there been a significant distinction between the two, such as continuous fitting could not have been possible.

The possible bis complexes having the metal ion equivalently bound to the two adenine rings (it is to be remembered that the binding is via a water molecule bridge) are: (a) simultaneously binding to the phosphate chain and the adenine ring of both ATP molecules, (b) binding to the phosphate chain of one ATP molecule and to the adenine ring of the other,[12] and (c) binding to the phosphate chain and adenine ring of one ATP molecule and to the ring of the other one. The first model is rejected on the basis of the study of the coordinated water in the $M^{2+}$-ATP system. If the metal were bound to the two phosphate chains in the bis complex, then the number of coordinated water molecules should have been reduced considerably relative to the mono complex. Our results (Table III) clearly contradict this. Also, $^{31}P$ NMR competition study of metal complexation with ATP and AMP[12] negates the formation of such a bis complex. The second model, from the point of view of the metal-ring binding, is equivalent to the 1:1 complex and could not be simultaneously fitted to both our low- and high-metal-concentration results. Thus the only model for the bis $M^{2+}$-ATP complex consistent with all the experimental results is the following. The metal ion binds directly (inner-sphere coordination) to the three ATP phosphates and to three water molecules. The adenine ring is outer-sphere coordinated via a hydrogen bond to one of the coordinated water molecules and the second ATP molecule is similarly outer-sphere coordinated by ring binding solely. The study of the adenosine binding to $Co^{2+}$ confirms that the MATP complex can bind a second ATP (or A) molecule only via ring binding. The expulsion of A molecules from the ternary MATP-A complex by excess ATP molecules proves unequivocally that a bis M(ATP)$_2$ complex is formed. It also indicates that ATP is a stronger competitor than A for outer-sphere cordination; hence the phosphate chain plays some role in stabilizing the outer-sphere coordinated molecule apparently through a bridging interaction with the ribose of the other ATP molecule. The bis complex is however mainly stabilized by the base stacking of the two ATP rings. Recent study of the intramolecular interactions of adenosine 5′-monophosphate[58] showed that self-association of two nucleotide molecules occurs through vertical stacking involving almost 100% base overlap, while the phosphate moieties are well apart. On the basis of these and our results a proposed structure of the bis $M^{2+}$-ATP complex is given in Figure 17.

**Table VIII.** Bound State Field Shifts ($\Delta H_P$), Line Widths ($\Delta\nu_{2M}$), and Metal-Proton Distances ($R$) in the Cobaltous and Nickelous Aqueous Complexes at 27 °C

| $M^{2+}$ | $\Delta H_P(H_2O)$, Hz | $\Delta\nu_{2M}(H_2O)$, Hz | $R$, Å[a] |
|---|---|---|---|
| $Co^{2+}$ | $-6270 \pm 30$ | $692 \pm 20$ | 2.8 |
| $Ni^{2+}$ | $-642 \pm 10$ | $2025 \pm 30$ | 2.8 |

[a] Calculated taking the metal-oxygen distance as 2.1 Å, 0.96 Å for the oxygen-proton distance, and 104°24′ for the HOH angle.

## Conclusions

It is confirmed that metal ions bind ATP predominantly through metal chain interaction and that ring protonation does not affect this interaction. Secondary phosphate ionization, though it has considerable effect on the binding constants, has no significant effect on the chain or metal interactions with the unprotonated ring. The adenine ring protonation causes "over-folding" of the ATP molecule; hence it causes the metal ion and the ring to come closer. This is reflected in enhanced hyperfine interactions experienced by the ring (and ribose) protons. In the $M^{2+}$-ATP system with an excess of nucleotide concentration, a bis complex is formed in which the phosphate chain (and three water molecules) of one ATP is inner-sphere coordinated, and the adenine rings of both ATP molecules are outer-sphere coordinated through a water bridge.

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## Appendix

The bound state parameters of water coordinated in the $Co^{2+}$ and $Ni^{2+}$ aqueous complexes were needed for the scaling part of the $M^{2+}$-ATP results. Field shift and line width measurements have thus been carried out at a temperature of 27 °C, using metal ion concentrations in the range $10^{-2}$-$5 \times 10^{-1}$ M. The derived bound-state parameters are given in Table VIII.

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# Studies of Proteins in Solution by Natural-Abundance Carbon-13 Nuclear Magnetic Resonance. Spectral Resolution and Relaxation Behavior at High Magnetic Field Strengths

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**Abstract:** Natural-abundance $^{13}$C nuclear magnetic resonance spectra of hen egg-white lysozyme at 63.4 kG are compared with spectra at 14.2 kG. The increase in resolution when going from the low to the high field is much greater for aliphatic and methine aromatic carbon resonances than for carbonyl and nonprotonated aromatic carbon resonances. This result is consistent with experimental and calculated spin–lattice relaxation times, which demonstrate the dominance of chemical shift anisotropy as a relaxation mechanism for nonprotonated unsaturated carbons of a native protein at magnetic field strengths much above 40 kG. For the aliphatic and methine aromatic carbons, the $^{13}$C–$^{1}$H dipolar relaxation mechanism should be dominant at all magnetic field strengths now available for NMR.

Most $^{13}$C nuclear magnetic resonance studies of proteins have been carried out at low magnetic field strengths, such as 14.2[1-3] or 23.5 kG.[4,5] With the increased availability of high-field high-resolution Fourier transform NMR spectrometers,[6,7] it becomes important to evaluate the effect of high magnetic field strengths on the information content of $^{13}$C NMR spectra of proteins. It is not necessarily true that resolution and sensitivity will automatically increase with magnetic field strength. It has been suggested[8] that, for some carbons of native biopolymers, *sensitivity* at high magnetic field strengths (such as 50–90 kG) may be adversely affected by long spin–lattice relaxation times ($T_1$). This suggestion was based on the assumption of purely $^{13}$C–$^{1}$H dipolar relaxation for all carbons. It is now well documented that, in general, this relaxation mechanism is dominant for $^{13}$C resonances of proteins at 14.2 kG, even when dealing with nonprotonated carbons.[2] However, there is evidence that at 63.4 kG chemical shift anisotropy (CSA) contributes strongly to the relaxation of nonprotonated unsaturated carbons of organic molecules in solution.[7] If, as expected, CSA is also the dominant relaxation mechanism for some carbons of a protein (at high field), then the $T_1$ values of these carbons may be significantly shorter than predicted on the basis of the $^{13}$C–$^{1}$H dipolar mechanism. However, the dominance of the CSA relaxation mechanism will adversely affect the use of high magnetic field strengths for improving spectral *resolution* (see below).

In this report we present natural-abundance $^{13}$C Fourier transform NMR spectra and spin–lattice relaxation data for

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