

forms cannot constitute significant amounts of the conformational population. The high energy all-chair ground state of CSC-PHP makes the possibility that boat forms contribute to the conformational population more probable, but again the agreement between calculated and experimental spectra indicates such contributions are probably not significant.

**(D) Carbon-13 Magnetic Resonance as a Tool in Conformation Analysis and Compound Identification.** It must be concluded from the results presented here that carbon-13 spectroscopy is a powerful and relatively simple tool for identification of uncharacterized paraffin hydrocarbon isomers and for analyzing the conformational properties of molecular systems. Although much remains to be done, an accumulating library of chemical shift and parametric values and relevant ex-

perience will provide an increasingly useful technique in the field of conformational and configurational analysis and characterization of complex molecules.<sup>41</sup>

**Acknowledgment.** This research was supported by the National Institutes of Health under Grant No. GM-08521. D. K. D. was the recipient of a National Science Foundation traineeship during a part of the time in which this work was accomplished.

(41) It has come to our attention that between the time at which this work was completed (D. K. Dalling, Ph.D. Thesis, University of Utah, 1969) and that at which it was accepted for publication, other workers (N. L. Allinger, B. J. Gordon, I. J. Tyminski, and M. T. Wuesthoff, *J. Org. Chem.*, **36**, 739 (1971)) have characterized the four PHP isomers treated in this work by synthetic organic methods. Their results are in agreement with those presented herein, and upon comparing the requisite labor involved the advantages of using cmr spectroscopic techniques as compared with traditional chemical methods are readily apparent.

## <sup>13</sup>C Relaxation Studies on the Manganese(II)-Adenosine 5'-Triphosphate Complex in Solution

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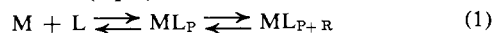
**Abstract:** The nature of binding between manganese ions and adenosine 5'-triphosphate (ATP) was studied using natural abundance Fourier transform <sup>13</sup>C nmr techniques. Relaxation times for the <sup>13</sup>C nuclei in the presence of Mn(II) ions were determined together with their temperature dependence. The adenine base carbons show specific effects while the ribose carbons remain unaffected. The transverse relaxation time data indicate a consecutive binding mechanism, namely that the metal ion binds first to the phosphate groups of ATP and then, in the second step, the metal interacts with the N(7) nitrogen. The equations for this exchange process are derived. The rate constant for the interaction of the metal ion with the adenine base is  $2.7 \times 10^7 \text{ sec}^{-1}$  at 27°. The distances between the metal ion and the carbon and proton nuclei of the adenine base of ATP were evaluated on the basis of the longitudinal relaxation time studies. The results indicate that the metal binds directly to the N(7) nitrogen.

The interaction between metal ions with nucleotides has been studied extensively for several years.<sup>2,3</sup> The search has been for the elusive backbond phosphate-metal ion-ring interaction that was originally proposed by Szent-Györgyi in 1957.<sup>4</sup> This hypothesis was to explain why in many enzymatic reactions which require adenosine 5'-triphosphate (ATP) the actual enzyme substrate was the complex Mg-ATP.

Nmr has shown that the divalent metal ions Zn(II), Cu(II), Ni(II), Co(II), and Mn(II) all interact with the adenine ring of ATP<sup>5-7</sup> as well as with the phosphate groups. The nature of this ring interaction has only been established in the case of Ni(II) and Co(II). With these two metal ions it has been demonstrated<sup>8,9</sup> that

there is a bridging water molecule between the metal ion and the adenine ring and that this "trapped" water molecule probably forms a hydrogen bond with the N(7) nitrogen of the base. The metal ion Mn(II) substitutes for Mg(II) *in vitro* in most enzymatic reactions that require Mg-ATP as a substrate. The nature of the paramagnetic ion-Mn-ring interaction of ATP is therefore important as the study may shed light on the diamagnetic metal ion Mg-ATP complex.

Recently, natural abundance <sup>13</sup>C nmr transverse relaxation times due to the presence of paramagnetic metal ions have been measured<sup>10</sup> and these measurements indicate the potential of using the <sup>13</sup>C nmr probe for studying nucleotide-metal ion systems. In this paper we wish to report our studies on <sup>13</sup>C longitudinal and transverse relaxation time measurements on the Mn-ATP system. The results yield information about the nature of the metal ion binding site on the adenine ring. In addition, the most consistent interpretation of our data indicates that the following consecutive binding mechanism (eq 1) occurs in solution, where L



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

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(8) T. A. Glassman, C. Cooper, L. W. Harrison, and T. J. Swift, *Biochemistry*, **10**, 843 (1971).

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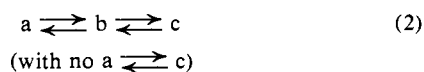
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is the ligand, namely ATP, M is the Mn(II) ion,  $ML_P$  is the Mn-ATP complex with the metal ion bound only to the three phosphate groups, and  $ML_{P+R}$  is the Mn-ATP complex in which the metal ion is bound simultaneously to the three phosphate groups and to the adenine ring. This mechanism has been observed for the Ni-ATP system.<sup>11</sup>

### Theory

The three-site chemical exchange has been treated by McConnell.<sup>12</sup> Swift and Connick<sup>13</sup> applied the modified Bloch equations to a three-component system (a, b, c) for the case in which the concentration of species a is much greater than the concentration of species b or c. In addition, site a undergoes exchange with either site b or site c with no exchange occurring between site b or site c. Swift and Connick<sup>13</sup> then applied their equations to a study of paramagnetic metal ions in an aqueous solution.

In the present work, we wish to report in more detail than Swift and Connick<sup>13</sup> presented the equations for the exchange process



as these equations are of interest to biological systems. Species a is unbound ATP, species b is the complex with the metal ion bound only to the three phosphates of ATP, and species c is the complex with the metal ion bound to the three phosphates and simultaneously to the adenine ring. We have also started with the modified Bloch equations<sup>12,13</sup> for the three site exchange process given by eq 2. The resulting equation for the absorption mode is

$$v = \frac{-\omega_1 M_0^a (k_a + k_x)}{(k_a + k_x)^2 + (\Delta\omega_a + \Delta\omega_p)^2} \quad (3)$$

where  $\Delta\omega_a$ ,  $\Delta\omega_b$ , and  $\Delta\omega_c$  are the differences between the resonance frequency for sites a, b, and c and the actual frequency.  $T_{2j}$  is the usual transverse relaxation

$$k_x = \frac{-k_b(k_c^2 + \Delta\omega_c^2)k_{ab}k_{ba} + k_c k_{bc} k_{cb} k_{ab} k_{ba}}{[(k_b^2 + \Delta\omega_b^2)(k_c^2 + \Delta\omega_c^2) + 2(\Delta\omega_b \Delta\omega_c - k_b k_c)k_{bc} k_{cb} + k_{cb}^2 k_{bc}^2]}$$

$$\Delta\omega_p = \frac{\Delta\omega_b(k_c^2 + \Delta\omega_c^2)k_{ba}k_{ab} + \Delta\omega_c k_{bc} k_{cb} k_{ab} k_{ba}}{[(k_b^2 + \Delta\omega_b^2)(k_c^2 + \Delta\omega_c^2) + 2(\Delta\omega_b \Delta\omega_c - k_b k_c)k_{bc} k_{cb} + k_{cb}^2 k_{bc}^2]}$$

$$k_a = 1/T_{2a} + k_{ab}$$

$$k_b = 1/T_{2b} + k_{ba} + k_{bc}$$

$$k_c = 1/T_{2c} + k_{cb}$$

time in the  $j$  environment and  $k_{ij}$  is the exchange rate of the nucleus from site  $i$  to site  $j$ .

In eq 3, the term  $\Delta\omega_p$  describes the resonance shift and is useful for systems which satisfy eq 2 and display a significant chemical shift, e.g., Ni(II) complexes. However,  $(k_a + k_x)$  is the half-width at half-height, since eq 3 is a normal Lorentzian curve for which  $1/T_2$  is defined as

$$T_2^{-1} = k_a + k_x \quad (4)$$

(11) C. M. Frey and J. E. Stuehr, "Metal Ions in Biological Systems," Vol. I, H. Sigel, Ed., Marcel Dekker, New York, N. Y., 1973.

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

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

In analogy with Swift and Connick,<sup>13</sup> this equation for the line width may be rewritten as

$$T_2^{-1} = T_{2a}^{-1} + k_{ab} + k_x \quad (5)$$

$$T_2^{-1} = T_{2a}^{-1} + T_{2p}^{-1}$$

where  $T_{2p}$  now contains all the relaxation effects due to the presence of the paramagnetic ions.

Since this study deals only with the interaction of Mn(II) with ATP, the temperature effects that are predicted by eq 3 were investigated using the following limiting cases.

#### (I) Chemical controlled region

$$1/T_{2c}^2 \gg k_{cb}^2, k_{ba}^2 \gg \Delta\omega_c^2, \Delta\omega_b^2, T_{2b}^{-2} \quad (6)$$

$$T_{2p}^{-1} = k_{ab} \frac{k_{bc}}{k_{ba} + k_{bc}}$$

with  $k_{bc} \gg k_{ba}$

$$T_{2p}^{-1} = k_{ab} = \tau_{ab}^{-1} = \frac{[b]}{[a]} \tau_{ba}^{-1} \quad (7)$$

$\tau_{ab}$  is the lifetime for the exchange between free ATP and ATP with the metal ion bound to the phosphates.

#### (II) Fast exchange region

$$k_{cb}^2 \gg T_{2c}^{-2} \gg \Delta\omega_c^2 \quad (8)$$

$$k_{ba}^2 \gg T_{2b}^{-2} \gg \Delta\omega_b^2$$

$$T_{2p}^{-1} = \frac{k_{ab} k_{ba}}{k_{ba} k_{cb}} T_{2c}^{-1} = \frac{[b][c]}{[a][b]} T_{2c}^{-1} \quad (9)$$

$$T_{2p}^{-1} = \frac{[c]}{[a]} T_{2c}^{-1}$$

In the chemical controlled region, case I, we see that nmr is sensitive to the exchange rate of the entire molecule rather than to the exchange rate of species b to species c. In case II, nmr is sensitive to the transverse relaxation of species c.

### Results

The <sup>13</sup>C nmr line assignments were based on earlier work on nucleotides<sup>14,15</sup> and nucleosides.<sup>16</sup> The ribose carbon resonances were assigned on the basis of the work of Mantsch and Smith.<sup>15</sup> To evaluate the effect on the relaxation rates arising from the metal, the differences between the pure ATP <sup>13</sup>C signals and those for Mn-ATP solutions were calculated using

$$T_{ip}^{-1} = T_{i,metal}^{-1} - T_{i,blank}^{-1} \quad i = 1, 2 \quad (10)$$

where  $T_{1p}$  and  $T_{2p}$  refer to the spin-lattice and transverse relaxation times, respectively, due to the presence of the metal ion. On progressive addition of the manganese ions, the <sup>13</sup>C  $T_{1p}$  and  $T_{2p}$  values for the ribose carbon nuclei were not affected. However, the adenine base <sup>13</sup>C resonances showed specific effects. The results are shown in Figures 1 and 2. The  $T_{1p}^{-1}$  and  $T_{2p}^{-1}$  values for C(5) and C(8) were affected most strongly on the addition of Mn(II) ions. Average results are reported as  $T_{1p}^{-1}$  and  $T_{2p}^{-1}$  in Table I.

(14) D. E. Dorman and J. D. Roberts, *Proc. Nat. Acad. Sci. U. S.*, **65**, 19 (1970).

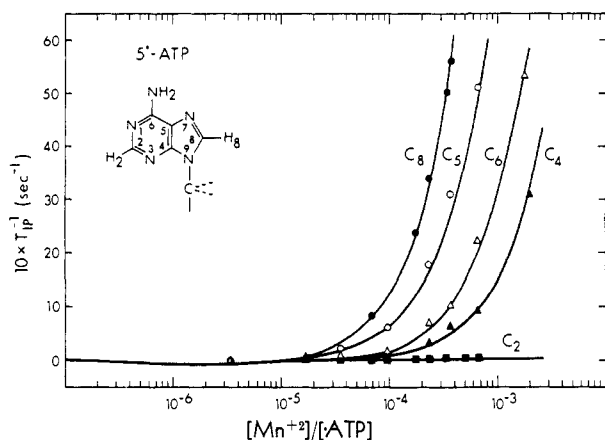
(15) H. H. Mantsch and I. C. P. Smith, *Biochem. Biophys. Res. Commun.*, **46**, 808 (1972).

(16) A. J. Jones, M. W. Winkley, D. M. Grant, and R. K. Robins, *Proc. Nat. Acad. Sci. U. S.*, **65**, 27 (1970).

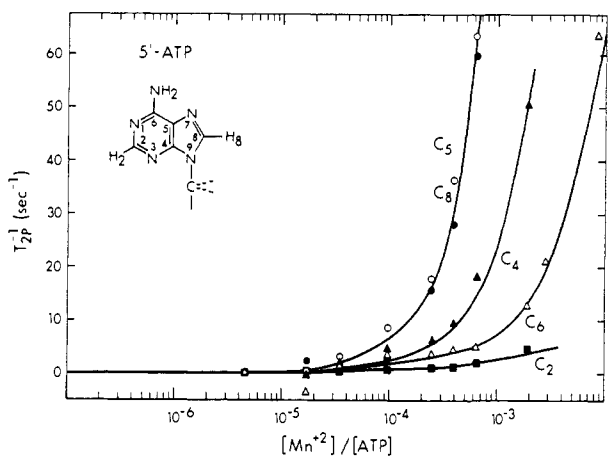
**Table I.** Summary of  $^{13}\text{C}$   $T_{1p}^{-1}$  and  $T_{2p}^{-1}$  Values for Mn(II)-ATP Solutions<sup>a</sup>

	C(6)	C(2)	C(4)	C(8)	C(5)
$T_1$ (pure ATP), sec	$6.33 \pm 0.25$	$0.17 \pm 0.01$	$5.90 \pm 0.25$	$0.13 \pm 0.01$	$6.54 \pm 0.25$
$T_{1p}^{-1},^b \text{ sec}^{-1}$	$1.1 \pm 0.15$		$0.59 \pm 0.07$	$5.0 \pm 0.2$	$2.8 \pm 0.3$
$T_{2p}^{-1},^b \text{ sec}^{-1}$	$4.1 \pm 0.8$	$\leq 0.03$	$10.7 \pm 0.5$	$28.6 \pm 2.4$	$33.4 \pm 2.6$

<sup>a</sup> Experiments were carried out in  $\text{D}_2\text{O}$  at a pD of 7.0 at  $30^\circ$ .  $[\text{ATP}] = 0.30 \text{ M}$ ;  $[\text{Mn}]/[\text{ATP}] = 3.7 \times 10^{-4}$ . <sup>b</sup> Average values from five measurements normalized to the  $[\text{Mn}]/[\text{ATP}]$  ratio of  $3.7 \times 10^{-4}$ .



**Figure 1.** The effect of Mn(II) on the  $^{13}\text{C}$  longitudinal relaxation rates of the adenine base nuclei of ATP. The measurements were carried out in  $\text{D}_2\text{O}$  at a pD of 7.0 at  $30^\circ$ . The ATP concentration is 0.30 M.

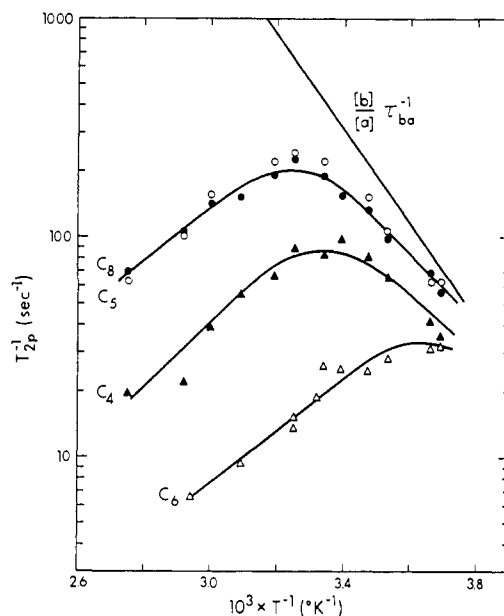


**Figure 2.** The effect of Mn(II) on the  $^{13}\text{C}$  transverse relaxation rates of the adenine base nuclei of ATP. The measurements were carried out in  $\text{D}_2\text{O}$  at a pD of 7.0 at  $30^\circ$ . The ATP concentration is 0.30 M.

Transverse relaxation rates were further studied as a function of temperature from  $-5$  to  $90^\circ$  over which the ATP was stable. The temperature profile of  $T_{2p}^{-1}$  is shown in Figure 3. Carbons 5, 8, 4, and 6 exhibited similar behavior, converging to the same slope at low temperature. Carbon 2 and the ribose carbon widths remained unchanged over this temperature interval and concentration.

## Discussion

The changes in  $T_{1p}$  and  $T_{2p}$  of the adenine base carbon nuclei indicate that manganese ions interact with the adenine ring whereas the sugar carbons are unchanged and hence are not likely involved in the binding with



**Figure 3.** The temperature dependence of  $T_{2p}^{-1}$  of the  $^{13}\text{C}$  nuclei of ATP that are affected by the presence of the Mn(II) ions.  $T_{2p}^{-1}$  values are normalized to a  $[\text{Mn}]/[\text{ATP}]$  ratio of  $2.5 \times 10^{-3}$ .  $[\text{ATP}] = 0.30 \text{ M}$ .

the manganese ions. This agrees with previous studies.<sup>5,7,8</sup> In 1968, Sternlicht, *et al.*,<sup>17</sup> proposed a model for the complex formed between metal ions and ATP, namely  $\text{M}(\text{ATP})_2$ . However, this complex has not been observed for Mn(II),<sup>18</sup> Ni(II),<sup>19</sup> or Mg(II).<sup>8</sup> complexes with ATP. In addition, Frey and Stuehr<sup>11</sup> have shown that the mechanism of metal-ATP binding as proposed<sup>17</sup> is not self-consistent. The temperature jump kinetic data obtained by Sternlicht, *et al.*,<sup>17</sup> for the interaction of Mn(II) with ATP can be fit<sup>20</sup> by the presence in solution of  $\text{Mn}(\text{ATP})$  and  $\text{Mn}_2(\text{ATP})$  species as was done for the Mg(II)-ATP system. Under our conditions, it is expected that the metal ion will exist predominantly in the  $\text{Mn}(\text{ATP})$  form.

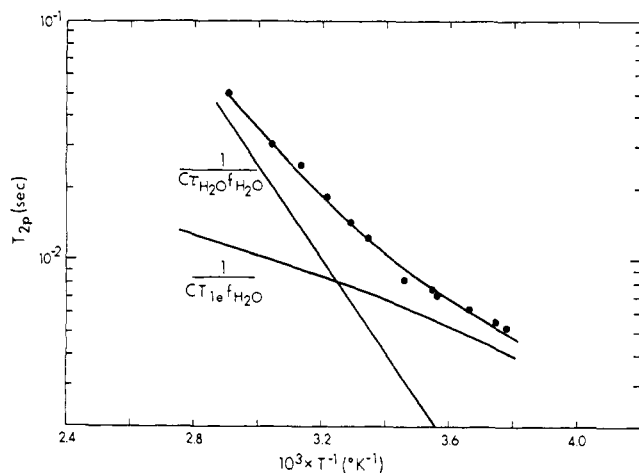
Above  $30^\circ$  (Figure 3), the transverse relaxation of species c,  $T_{2c}^{-1}$ , is dominating the relaxation of carbons 6 and 4. In this region  $T_{2p}^{-1} = f'(T_{2c})^{-1}$ , where  $f' = [c]/[a]$ . Therefore it is valid to compare  $T_{2p}$  with  $T_{1p}$ , as above  $30^\circ$   $T_{1p}$  must be controlled by  $T_{1c}$ . The ratios  $T_{1p}:T_{2p}$  are 4 and 20 for carbons 6 and 4, respectively (Table I). This implies that  $T_{1p}$  and  $T_{2p}$  are governed by different relaxation mechanisms. Hence, under our

(17) H. Sternlicht, D. E. Jones, and K. Kustin, *J. Amer. Chem. Soc.*, **90**, 7110 (1968).

(18) M. S. Zetter, H. W. Dodgen, and J. P. Hunt, *Biochemistry*, **12**, 778 (1973).

(19) C. M. Frey and J. E. Stuehr, *J. Amer. Chem. Soc.*, **94**, 8898 (1972).

(20) J. E. Stuehr, Department of Chemistry, Case Western Reserve University, private communication.



**Figure 4.** The temperature dependence of the water proton transverse relaxation times for the Mn-ATP system. The curve is the calculated sum of the two straight lines evaluated using eq 15. The ATP concentration is 0.27 M,  $f_{\text{H}_2\text{O}} = 1.1 \times 10^{-4}$  where  $f_{\text{H}_2\text{O}} = n[\text{Mn}]/[\text{H}_2\text{O}]$ . The assumed complex is  $\text{MnATP}(\text{H}_2\text{O})_3$  so that  $n = 3$ . To evaluate  $C$ , a value of  $A/h = 4.6 \times 10^5$  Hz was used.

conditions (25.15 MHz), the Solomon-Bloembergen equations for  $T_{1M}$  and  $T_{2M}$  become<sup>21,22</sup>

$$(f'T_{1p})^{-1} = T_{1e}^{-1} = \frac{2}{5} [S(S+1)g^2\beta^2\gamma_1^2/r^6]\tau_c \quad (11)$$

$$(f'T_{2p})^{-1} = T_{2e}^{-1} = \frac{1}{3} \frac{S(S+1)}{\hbar^2} A^2\tau_e \quad (12)$$

where  $\tau_c$  and  $\tau_e$  are the correlation times for the dipolar and scalar interactions, respectively. These equations indicate that  $(f'T_{2p})^{-1}$  is controlled by scalar relaxation, while  $(f'T_{1p})^{-1}$  by dipolar relaxation.

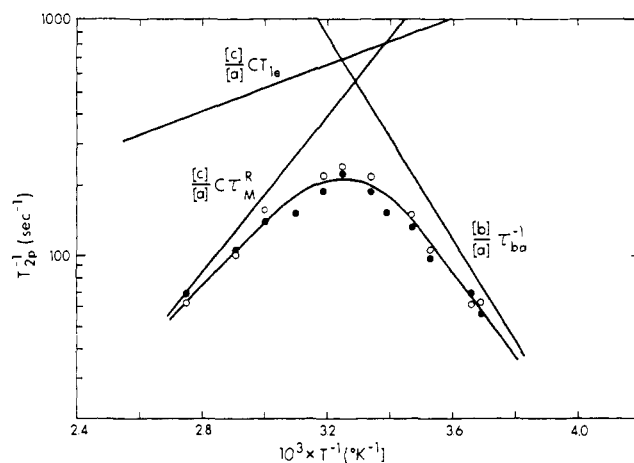
The temperature dependence of  $T_{2p}^{-1}$  (Figure 3) can be used to study the ring-metal ion interaction since in the rapid exchange region, the transverse relaxation of the  $^{13}\text{C}$  nuclei is governed by the scalar relaxation mechanism. This property is in contrast to the  $^{31}\text{P}$   $T_{2p}^{-1}$  data which are in the chemical controlled region<sup>6</sup> and the  $^1\text{H}$   $T_{2p}^{-1}$  data which are dominated by the dipolar relaxation.<sup>7</sup> The analysis of the temperature profile curves of carbons 8, 5, 4, and 6 (Figure 3) requires a knowledge of  $T_{2e}$ ,  $\tau_{ba}$ , and the distribution of ATP in environments b and c. As discussed in the theory section, in the chemical controlled region (low-temperature region), chemical exchange between species a and b will dominate  $T_{2p}^{-1}$  (eq 7). The exchange rate  $\tau_{ba}^{-1}$  is the same as  $\tau_m^{-1}$  obtained from phosphorus exchange studies by Sternlicht, *et al.*<sup>6</sup> ( $2.3 \times 10^5$  sec<sup>-1</sup> at 27°). In the fast exchange region (high-temperature region),  $T_{2p}^{-1}$  will be governed by  $T_{2e}^{-1}$  (eq 9) and therefore will be dominated by the scalar mechanism (eq 12). Equation 12 may be rewritten as<sup>21,22</sup>

$$T_{2e}^{-1} = C\tau_e = C(T_{1e}^{-1} + \tau_{cb}^{-1})^{-1} \quad (13)$$

where  $C = [S(S+1)/3](A/\hbar)^2$ ,  $T_{1e}$  is the longitudinal electronic relaxation time, and  $\tau_{cb}^{-1}$  is the exchange rate of the metal ion-ring interaction. From hereon we will refer to  $\tau_{cb}$  as  $\tau_m^R$  so as to distinguish it from  $\tau_m$  ( $=\tau_{ba}$ ), the metal ion-phosphate exchange time.

(21) I. Solomon, *Phys. Rev.*, **99**, 559 (1955).

(22) I. Solomon and N. Bloembergen, *J. Chem. Phys.*, **25**, 261 (1956).



**Figure 5.** The analysis of the temperature dependence of  $T_{2p}^{-1}$  for the carbon nuclei 5 and 8. The limiting values to the curve are represented with the straight lines as discussed in the text.

To determine if the  $T_{1e}$  temperature dependence alone could account for the slope of the curves of the  $T_{2p}^{-1}$  values in the high-temperature region (Figure 3), the theory of Rubenstein, *et al.*,<sup>23</sup> was applied. The temperature dependence of  $T_{1e}^{-1}$  is predicted by

$$T_{1e}^{-1} = \frac{2}{50} \Delta^2 [4S(S+1) - 3] \times \left[ \frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2} \right] \quad (14)$$

where

$$\tau_v = \tau_v^0 \exp(V_v/RT)$$

$\Delta$  is the zero field splitting,  $\tau_v$  is the correlation time for the impact of water molecules of the solvent upon the complex, and the other terms have the usual meaning. To verify the applicability of eq 14 to the Mn-ATP-aquo complex, the values of  $T_{2p}$  for the water protons as a function of temperature were determined for a Mn-ATP solution and were analyzed using

$$(T_{2p}f_{\text{H}_2\text{O}})^{-1} = C(\tau_{\text{H}_2\text{O}}^{-1} + T_{1e}^{-1})^{-1} \quad (15)$$

When values for  $\Delta$  equal to 195 G,<sup>24</sup>  $\tau_v$  equal to  $8.5 \times 10^{-12}$  sec at 27°,<sup>24</sup> and  $V_v$  equal to 2.6 kcal were used, eq 14 and 15 did fit the data for  $T_{2p}$  of the water protons (Figure 4). ( $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  parameters are listed in the Appendix.)

Knowing the temperature dependence of  $T_{1e}$ , it is seen that the  $T_{1e}$  temperature dependence does not account for the steep slope of carbons 5, 8, 4, and 6 (Figure 5). In addition, the effect of  $\tau_m^R$  on the temperature dependence of  $T_{2p}^{-1}$  (eq 13) must be considered. Actually a wide range of values of  $A/h$  and  $\tau_m^R$  will fit the temperature profile for carbons 5 and 8. However, from eq 13 and 14 we can calculate the  $\tau_m^R$  as at the point of intersection of the two straight lines,  $T_{1e} = \tau_m^R$  (Figure 5,  $T \approx 22^\circ$ ). In addition, similar lines describing  $T_{1e}$  and  $\tau_m^R$  for all the adenine ring carbon nuclei must cross at the same temperature, *i.e.*, all the adenine carbon nuclei display the same exchange rate  $\tau_m^R$ . Therefore combining these requirements to-

(23) M. Rubenstein, A. Baram, and Z. Luz, *Mol. Phys.*, **20**, 67 (1971).

(24) G. H. Reed, J. S. Leigh, and J. E. Pearson, *J. Chem. Phys.*, **55**, 3311 (1971).

gether with eq 13 and 14 leads to a reasonably unique set of values for  $A/h$  and  $\tau_m^R$ . These values are given in Tables II and III together with  $\Delta H^{\ddagger}_R$  and  $\Delta S^{\ddagger}_R$

**Table II.** Scalar Coupling Constants for the Adenine Base Carbon Nuclei of ATP<sup>a</sup>

	C(8)	C(5)	C(4)	C(6)
$A/h$ , Hz	$4.9 \times 10^5$	$4.9 \times 10^5$	$2.8 \times 10^5$	$1.2 \times 10^5$

<sup>a</sup> The solution composition is: [ATP] = 0.3 M; [Mn] =  $7.6 \times 10^{-4}$  M; [b] = 0.8[Mn]; [c] = 0.2[Mn].

**Table III.** Activation Parameters and Rate Constant for the Process<sup>a</sup>  $c \rightleftharpoons b$

$\Delta H_R^{\ddagger}$ , kcal	$\Delta S_R^{\ddagger}$ , cal/deg	$1/\tau_m^R$ , sec <sup>-1</sup>
7.1	-1.2	$2.7 \times 10^6$

<sup>a</sup> Evaluated at 27°. The solution composition is [ATP] = 0.3 M; [Mn] =  $7.6 \times 10^{-4}$  M; [b] = 0.8[Mn]; [c] = 0.2[Mn].

values. The  $A/h$  values observed for the interaction of Mn(II) with the <sup>13</sup>C nuclei are of the same order of magnitude as those observed between Mn(II) with <sup>17</sup>O of H<sub>2</sub>O,<sup>13</sup> <sup>1</sup>H of H<sub>2</sub>O,<sup>13,19</sup> <sup>31</sup>P in ATP,<sup>6</sup> and <sup>13</sup>C in pyruvate.<sup>23</sup>

In Figure 5, the temperature profile of  $T_{2p}^{-1}$  for carbons 5 and 8 is shown with the limiting values of  $([b]/[a]) \cdot \tau_m^{-1}$ ,  $C([c]/[a])T_{1e}$ , and  $C([c]/[a])\tau_m^R$ . It is found that the best-fit values to the curve are obtained with  $A/h = 4.9 \times 10^5$  Hz,  $1/\tau_m^R = 2.7 \times 10^7$  sec<sup>-1</sup> at 27°,  $[b]/[a] = 0.8[Mn]/[ATP]$ , and  $[c]/[a] = f' = 0.2[Mn]/[ATP] = 0.2f$ , where  $f = [Mn]/[ATP]$ . Hence not all of the manganese ions interact with the adenine ring as was originally believed<sup>7</sup> but only 20% of the ions are complexed to the ring.

Sternlicht and coworkers<sup>7</sup> found that the exchange rate of Mn(II) with the adenine ring was the same as the exchange rate of the Mn(II) with the phosphates and interpreted this result as implying that the adenine ring-metal ion bond was broken simultaneously as the phosphate-metal ion bond. However, we have demonstrated that this equality does not necessarily mean that the bond with the adenine ring is made and broken at the same rate as the bond with the phosphate groups. But, as suggested by Frey and Stuehr,<sup>11</sup> what was seen by Sternlicht, *et al.*,<sup>7</sup> was a rate-determining interaction of the metal ion with the phosphate oxygens followed by a rapid interaction with a ring position. The lifetimes obtained from proton resonances simply reflect the slower process.

The  $T_{1p}$  measurements can be used to estimate the conformation of the Mn-ATP complex and yield further insight into the binding of Mn(II) to the adenine ring. The determination of the distances between the metal ion and the various carbon nuclei requires a knowledge of  $f'$  and  $\tau_c$  (eq 11). The lifetime  $\tau_c$  is the "effective" rotational correlation time which may either be (1) that of the phosphorus ligand,<sup>6</sup>  $\tau_c \sim 1 \times 10^{-9}$  sec, or (2) that of the water ligand in the Mn-ATP solution,  $\tau_c \sim 3 \times 10^{-10}$  sec.<sup>6</sup> Calculated distances using both  $\tau_c$  values and  $f'$  are presented in Table IV along with the recalculated proton-metal distances from the data of Sternlicht, *et al.*<sup>7</sup>

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When  $\tau_c = 3 \times 10^{-10}$  sec, the correlation time of the water ligands, the predicted distances are very short, *e.g.*, the manganese-C(8) distance is 2.5 Å. Moreover, we believe that  $\tau_c$  corresponding to the water proton correlation time is too fast for both proton and carbon nuclei of ATP as rotation about the Mn-water oxygen bond is expected to exist.<sup>26</sup> With the metal ion binding to the ring as well as to the phosphate groups,  $\tau_c$  of the carbon and proton nuclei is expected to be similar to that of the phosphorus nuclei. In the earlier <sup>1</sup>H nmr study,<sup>7</sup>  $\tau_c$  of the water ligand correlation time was believed to be correct for the distance calculations instead of  $\tau_c$  of the phosphate groups because the latter calculations gave distances that were too long to be acceptable.<sup>7</sup> Now comparing the distances found knowing that only 20% of the manganese is ring bound and that  $\tau_c$  is the phosphate correlation time (Table IV, row II) with the distances from the molecular Mn-ATP model (Table IV, row III),<sup>27</sup> it is found that calculated distances based upon nmr data are most consistent with Mn(II) binding directly to the adenine ring at N(7).

In summary, nmr theory has been extended to the three site exchange  $a \rightleftharpoons b \rightleftharpoons c$  where  $[a] \gg [b] > [c]$ . The theory has been successfully applied to the Mn-ATP system where species a, b, and c are free ATP, manganese-phosphate bound ATP, and phosphate-manganese-ring bound ATP, respectively. This study has shown that approximately 20% of the manganese ions are ring coordinated. Furthermore, longitudinal relaxation studies have demonstrated that the correlation times of the phosphate, proton, and carbon nuclei are similar in value to those one might expect with the metal ion-ring interaction being present. The results of the longitudinal relaxation time study indicate that the manganese ion binds directly to N(7) of the adenine ring.

## Experimental Section

Natural abundance proton decoupled <sup>13</sup>C nmr spectra were obtained in the Fourier transform mode on a Varian HA-100-15 nmr spectrometer (25.15 MHz) interfaced with a Digilab FTS/NMR-3 data system, the Nova 1200 computer, and the pulse unit (FTS/NMR 400-2). The deuterium resonance of the solvent was used as the lock signal. All measurements were carried out under controlled temperatures using the Varian temperature control unit and were calibrated with a thermocouple before and after each run. Samples were put in the probe at least 30 min before the measurements were begun.

Transverse relaxation times were measured from the line widths at half-height from the Fourier transformed spectra. For each spectrum, 2000 free induction decay (FID) signals were stored in 16K (16384) data points over a corresponding frequency domain of 4000 Hz. The Digilab system yields 16K data points in the transformed real spectrum by zero filling and carrying out a 32K transform. (~0.25 Hz per data point over 4000 Hz). The pulse widths and noise-decoupler offset values were optimized by maximizing the signal intensities at a reduced power of the noise decoupler.

Spin-lattice relaxation times were evaluated from proton decoupled partially relaxed Fourier transform spectra obtained using the  $(-180^\circ - \tau - 90^\circ - T -)$ <sub>n</sub> pulse sequence.<sup>28-30</sup> For each spectrum, 500 FID signals were accumulated. Least-squares

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**Table IV.** Metal-Nuclei Distances (Å) for the Mn(II)-ATP Complex

$\tau_c$ , sec	$f' = [c]/[a]$	C-6	C-4	C-5	C-8	H-8	H-2	H-1
$3 \times 10^{-10}$	$0.2f^a$	$3.2 \pm 0.2$	$3.6 \pm 0.2$	$2.8 \pm 0.2$	$2.5 \pm 0.2$	$2.7^b$	$3.5^b$	$4.5^b$
$1 \times 10^{-9}$	$0.2f^a$	$4.0 \pm 0.2$	$4.4 \pm 0.2$	$3.4 \pm 0.2$	$3.1 \pm 0.2$	$3.4^b$	$4.4^b$	$5.7^b$
Model study		$4.0^c$	$4.6^c$	$3.4^c$	$3.2^c$	$3.3^c$	$6.9^c$	
						$3.5^d$	$6.8^d$	$6.5^d$

<sup>a</sup> The value  $f$  is the ratio of the total manganese concentration to the ATP concentration. <sup>b</sup> Proton-metal ion distances recalculated from the data of Sternlicht, *et al.*,<sup>7</sup> using  $f' = 0.2f$ . <sup>c</sup> The distances from the Mn(II) ion to the carbon and proton nuclei were calculated from the molecular model by fixing the metal  $2.3 \text{ \AA}$ <sup>22</sup> from N(7) in the same plane as the adenine base with the Mn-N(7)-C(5) bond angle equal to  $135^\circ$ . The adenine base bond lengths and bond angles were taken from X-ray studies (J. Kraut and L. H. Jensen, *Acta Crystallogr.*, **16**, 79 (1963)). The error limits are estimated to be  $\pm 0.3 \text{ \AA}$ . <sup>d</sup> Distances from the proposed model of the Mn-ATP complex as given by Sundaramlingam.<sup>27</sup> The error limits are estimated to be  $\pm 0.3 \text{ \AA}$ .

analyses were carried out on the data from 14 different  $\tau$  settings, which yielded spin-lattice relaxation time values with standard deviations less than 8%. For small  $T_1$  values ( $\leq 0.2$  sec), each spectrum was obtained from 5000 FID signals and each experiment was repeated twice. The pulse widths were 100 and 50  $\mu$ sec for the 180 and 90° pulses, respectively. The <sup>13</sup>C pulse frequency was offset in a way such that the signals to be studied were within 1500 Hz from the carrier.<sup>31</sup> Therefore, for ATP, the carbon signals in the adenine base region and the sugar region were measured separately.

ATP (disodium salt) of highest grade was obtained from Sigma Chemical Co. Samples were purified by passing a 0.5 M ATP (pH  $\sim$ 7) solution through a Dowex 50 exchange resin. ATP solutions were then prepared in D<sub>2</sub>O and were adjusted to a pD of 7.0.<sup>32</sup> Stock solutions of Mn(II) were prepared from Fisher certified reagent MnCl<sub>2</sub>·4H<sub>2</sub>O crystals. The solvent (D<sub>2</sub>O) was from Columbia Organic Chemical Co.

The ATP concentrations were determined by uv spectrometry ( $\epsilon_{\max} 1.572 \times 10^4$  at 260 m $\mu$  at pH 7). The metal solution was added with calibrated H. E. Pedersen micropipets. The pH of the solution was checked after the last addition of paramagnetic ions and remained constant at pD  $7.0 \pm 0.3$ . Samples with different concentrations of Mn(II) ions were used for the relaxation time experiments and the normalized data are given in Table I and Figures 3 and 5.

The H<sub>2</sub>O proton line broadening experiments were carried out on a 0.27 M ATP solution containing  $2.0 \times 10^{-3}$  M Mn(II) ions on a Varian HA-100-15 nmr spectrometer at 100.0 MHz in the continuous wave mode. The temperature was calibrated using a thermocouple.

**Acknowledgments.** The authors express their appreciation to Dr. J. E. Stuehr for a preprint of his

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manuscript and to the National Research Council of Canada for financial assistance.

## Appendix

The activation parameters and the rate constant for the exchange of water molecules with the Mn-ATP-aquo complex are listed in Table V. The proton coupling constant  $A/h$  is  $4.6 \times 10^5$  Hz.

**Table V.** Activation Parameters and Rate Constant for Water Molecule Exchange<sup>a</sup>

$\Delta H^\ddagger$ , kcal	$\Delta S^\ddagger$ , cal/deg	$\tau_{\text{H}_2\text{O}}^{-1}$ , sec <sup>-1</sup>
8.4	2.3	$1.4 \times 10^7$

<sup>a</sup> The calculations were carried out at 27° for a solution with  $[\text{ATP}] = 0.27 \text{ M}$  and  $f_{\text{H}_2\text{O}} = 1.1 \times 10^{-4}$ .  $f_{\text{H}_2\text{O}} = n[\text{Mn}]/[\text{H}_2\text{O}]$ , where  $n$  is the number of water molecules in the assumed complex MnATP(H<sub>2</sub>O)<sub>3</sub>. The proton frequency is 100.0 MHz.

The activation parameters (Table V) differ slightly from the values obtained by Zetter, *et al.*<sup>18</sup> Their value of  $\tau_v$  at 30° ( $1.1 \times 10^{-12}$  sec) is too short since substituting it into eq 14 at K-band frequencies, a decrease in  $T_{1e}^{-1}$  with increasing temperature is predicted. However, this is contrary to the epr studies of Reed, *et al.*,<sup>24</sup> who observe an increase in the line width of the epr signal with an increase in temperature. Therefore, since Zetter, *et al.*,<sup>18</sup> assumed that  $T_{1e}^{-1}$  was independent of temperature, their values of  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  are in slight disagreement with the present work.