A Nuclear Magnetic Resonance Relaxation Time Study of the Manganese(II)–Inosine 5′-Triphosphate Complex in Solution†

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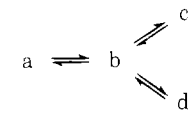
ABSTRACT: The nature of binding between manganese (Mn(II)) ions and inosine 5′-triphosphate (ITP) was studied using ^{13}C, ^{1}H, and ^{31}P nuclear magnetic resonance (NMR) techniques. The longitudinal relaxation data for protons H-8 and H-1′ indicate that the complex present in solution involves one manganese ion interacting with one ITP molecule. The transverse relaxation time temperature dependence of the hypoxanthine ring carbon nuclei indicates that the manganese ions interact at two distinct sites on the inosine ring, namely at C=O and N-7. The equations for a four-site exchange process are derived. The lifetimes of the metal ion bound to the phosphate groups on ITP are 6.7×10^{-8} and 5×10^{-8} sec when bound to the C=O and the N-7 groups, respectively, at 27°.

In a continuing study of metal ion–nucleotide interactions, the manganous ion (Mn(II)) complex with inosine 5′-triphosphate (ITP)[1] (Figure 1) has been studied. Metal ion–nucleotide complexes have been studied by many researchers since Szent-Gyorgyi (Szent-Gyorgyi, 1957) proposed that the Mg(II) ion complex with adenosine 5′-triphosphate (ATP) was biologically active due to a backbonded Mg–ATP complex. Nuclear magnetic resonance (NMR) spectroscopy has shown that the divalent metal ions Cu(II), Ni(II), Co(II), Zn(II), and Mn(II) interact with the phosphate groups of ATP as well as with the adenine ring (Cohn and Hughes, 1962; Sternlicht et al., 1965a,b, 1968; Glassman et al., 1971, 1973b, 1974a,b; Kuntz et al., 1972, 1975; Brown et al., 1973; Wee et al., 1974; Feldman and Wee, 1974; Lam et al., 1974; Naumann et al., 1974). Reviews on metal ion–nucleoside and metal ion–nucleotide stability constants, and including other data on these complexes, have been written (Phillips, 1966; Izatt et al., 1971; Weser, 1968; Tu and Heller, 1974; Frey and Stuehr, 1974; Eichhorn, 1973; Kotowycz and Lemieux, 1973).

Since 1957, several methods of studying metal ion–nucleotide interactions have been proposed. The results of the ultraviolet study of metal ion–nucleoside interactions (Schneider et al., 1964) have been questioned (Glassman et al., 1973b). The bis Mn–ATP complex, in which the metal ion is bound to the triphosphate chain of one molecule and to the base of a neighboring molecule, proposed by Sternlicht and coworkers (Sternlicht et al., 1968), has been shown to be based on an inconsistent analysis (Wee et al., 1974; Frey and Stuehr, 1974; Lam et al., 1974). A "trapped" water molecule between the metal ion and the N-7 position of the base has been shown for the Ni–ATP system (Glassman et al., 1971) and for the Co–ATP system (Kuntz et al., 1972). Direct coordination is observed between Mn(II) ions and the N-7 nitrogen in Mn–ATP solutions (Lam et al., 1974). It is felt that neither the $Mn(ATP)_2$ species (Sternlicht et al., 1968) nor the complex proposed by Wee et al., (1974) in which the metal ion is bound simultaneously to the phosphate groups of one ATP molecule and to two adenine rings (either directly or through a water molecule bridge) is present in solution (Zetter et al., 1973; Glassman et al., 1973b; Lam et al., 1974). The present data indicate that these species are also not present in the biologically important Mn–ITP complex. In the Mn–ITP complex the Mn(II) ions can interact with the inosine base moiety not only at the N-7 nitrogen but also with the carbonyl oxygen at C-6 (Kotowycz and Suzuki, 1973). Knowledge of the Mn–ITP complex in solution will aid in the understanding of, for example, the enzyme system succinyl coenzyme A synthetase from pig heart which requires a metal ion and either GTP or ITP as a substrate (Brownie and Bridger, 1972; Bridger, 1974).

The ^{13}C NMR transverse relaxation time data on the Mn–ITP system is analyzed as a four-site exchange system:



(no a ⇌ c; a ⇌ d, or d ⇌ c)

The theory for this exchange process is presented in the Appendix.

Experimental Procedure

Natural abundance ^{13}C and ^{31}P NMR transverse relaxation time measurements were obtained in the Fourier transform mode with proton noise decoupling on a Bruker HFX-90 NMR spectrometer (22.6 and 36.4 MHz, respectively) interfaced with a Nicolet computer. The deuterium resonance of the solvent D_2O was used as the lock signal. All measurements were carried out under controlled temperatures using the Bruker temperature control unit. Sam-

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[1] Abbreviations used are: T_{2p}, nuclear transverse relaxation time due to the paramagnetic ion; T_1, nuclear spin-lattice (longitudinal) relaxation time; T_{1p}, nuclear spin-lattice relaxation time due to the paramagnetic ion; T_{1e} electron spin-lattice relaxation time; ITP, inosine 5′-triphosphate.

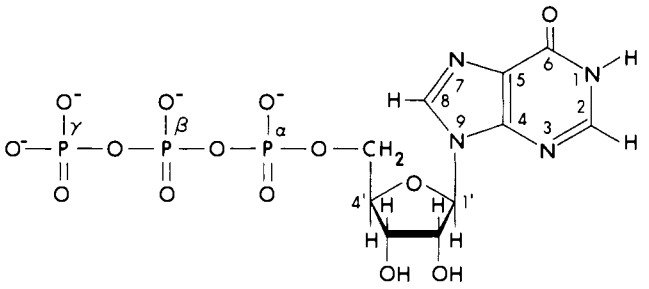


FIGURE 1: Structure of inosine 5′-triphosphate.

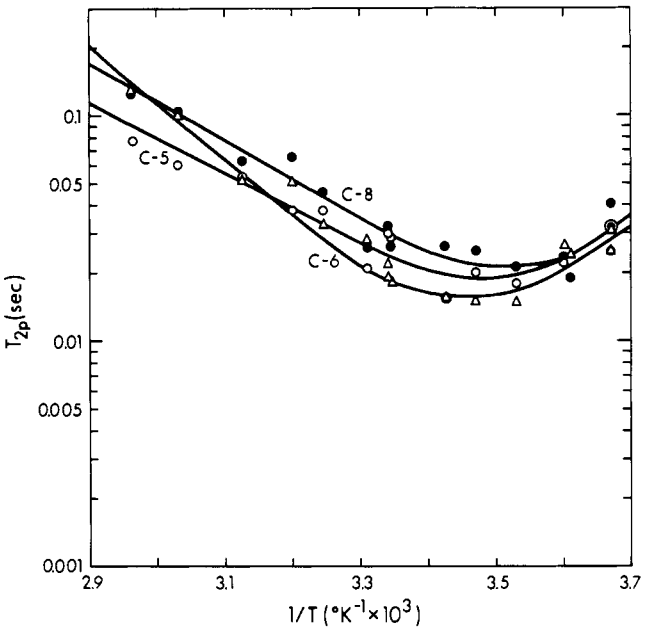


FIGURE 2: The temperature dependence of T_{2p} for carbons C-5, C-6, and C-8 of ITP in the presence of Mn(II) ions. T_{2p} values are normalized to a [Mn]/[ITP] ratio of 3.66×10^{-3}, [ITP] = 0.3 *M*; pD = 6.5 ± 0.2.

ples were in the probe for at least 15 min before the measurements were begun.

Transverse relaxation times due to the paramagnetic ion (T_{2p}) were measured from the line widths at half-height from the Fourier transformed spectra and calculated using

$$T_{2p}^{-1} = \pi[\Delta\nu_{1/2,\text{obsd}} - \Delta\nu_{1/2,o}] = \pi\Delta\nu_{1/2,p} \quad (1)$$

where $\Delta\nu_{1/2,\text{obsd}}$ is the observed line width at half-height of the ligand resonance for the metal ion solution, $\Delta\nu_{1/2,o}$ is the line width at half-height for the same resonance without metal ions, and $\Delta\nu_{1/2,p}$ is the effective paramagnetic line width. In the ^{31}P measurements, corrections were made for the $J(^{31}P - {}^{31}P)$ splitting of 19.5 Hz. Depending on the required resolution, the free induction decay (FID) signals were stored in either 8K or 4K data points corresponding to a frequency range of 3000 Hz. The transformed spectrum then yields 4K or 2K data points, respectively.

The spin-lattice relaxation times (T_1) of natural abundance proton decoupled ^{13}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. Spin-lattice relaxation times were evaluated from proton decoupled

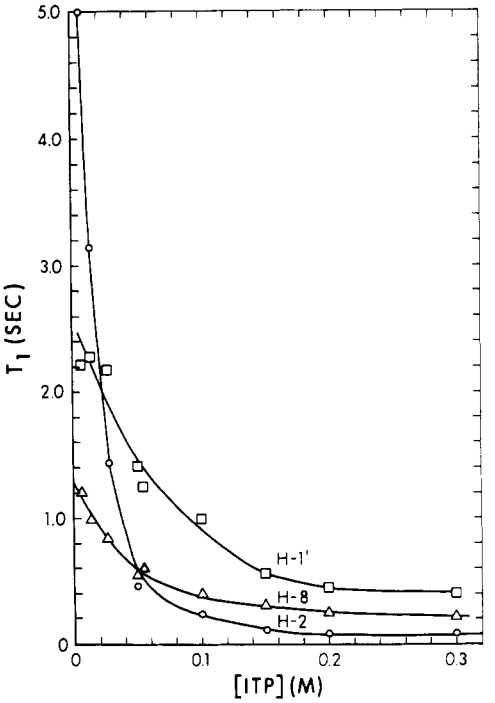


FIGURE 3: The concentration dependence of the longitudinal relaxation times of protons H-2, H-8, and H-1′ of ITP. pD = 6.5 ± 0.2, T = 33°. EDTA was added to the solutions for these studies as discussed in the text.

partially relaxed Fourier transform spectra obtained using the triple pulse sequence (Freeman and Hill, 1971). For each spectrum of 3000 Hz, 1500 FID signals were stored in 2K data points. Spin-lattice relaxation times due to the paramagnetic ion (T_{1p}) were evaluated using

$$T_{1p}^{-1} = T_{1,\text{obsd}}^{-1} - T_{1,0}^{-1} \quad (2)$$

where $T_{1,\text{obsd}}$ is the observed spin-lattice relaxation time of the ^{13}C nucleus for the metal ion solution and $T_{1,0}$ is the spin-lattice relaxation time for the same resonance without metal ions. The spin-lattice relaxation times of protons were also measured on the Varian HA-100-15 NMR spectrometer (100 MHz) using the deuterium resonance as the lock signal and the triple pulse sequence. Each spectrum was stored in 2K data points with a bandwidth of 1000 Hz. Samples with different concentrations of Mn(II) ions were used for the relaxation time experiments and the data normalized. The viscosity measurements were performed in a constant temperature bath at 33°.

ITP (trisodium salt) of highest grade was obtained from Sigma Chemical Company. Samples were purified by passing a solution through a Dowex 50 or Chelex 100 cation exchange resin. ITP solutions were then prepared in D_2O and were adjusted to a pD of 6.5 (Glasoe and Long, 1960). The ITP concentrations were determined by uv spectrometry (ϵ_{max} $1.22 \times 10^{+4}$, pD 6). Stock solutions of Mn(II) ions were prepared from Fisher certified reagent $MnCl_2 \cdot 4H_2O$ crystals.

Results

T_{2p} values for the ^{13}C nuclei are plotted vs. the reciprocal of the absolute temperature in Figure 2. The slope for C-8 and C-5 in the high temperature region is different from the slope of C-6. The maximum line width occurs at approximately 20°. In Figure 3, the longitudinal relaxation times

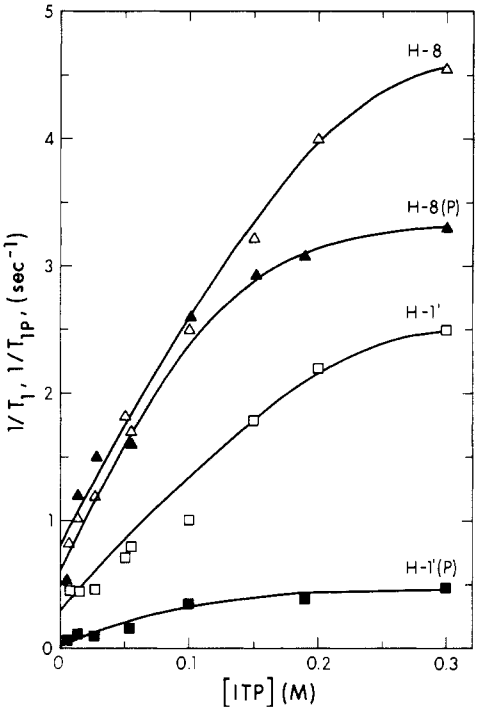


FIGURE 4: Plot of T_1^{-1} and T_{1p}^{-1} as a function of the ITP concentration for protons H-8 and H-1′. The T_1^{-1} data are from Figure 3. The [Mn]/[ITP] ratio is normalized to 1×10^{-4}. $T = 33°$. The error limits are ±10%. The symbols are: (△) T_1 = H-8; (□) T_1 = H-1′; (▲) T_{1p} = H-8; (■) T_{1p} = H-1′.

Table I: ^{13}C Longitudinal Relaxation Times (sec) as a Function of the ITP Concentration.[a]

[ITP] (M)	T_1 C-8	T_1 C-2
0.3	0.25 ± 0.01	0.24 ± 0.01
0.1	0.45 ± 0.03	0.50 ± 0.03
0.075	0.51 ± 0.03	0.56 ± 0.03
0.05	0.68 ± 0.07	0.68 ± 0.07

[a] Temperature, 42°, pD = 6.5 ± 0.2.

T_1 (33°) for protons H-8, H-2, and H-1′ are plotted vs. the ITP concentration. The change in the T_1 of H-8 and H-1′ between 0.3 and 0.055 *M* ITP is 2.7 and 3.1, respectively, whereas between 0.3 and 6×10^{-3} *M*, the change is approximately 5.8 for both protons. EDTA was added to the solutions for these studies to eliminate the effects of trace metal ions on the proton T_1 measurements ([EDTA]/[ITP] $\simeq 10^{-3}$). The importance of following this procedure has recently been emphasized (Wasylishen and Cohen, 1974). The T_{1p}^{-1} values for protons H-8 and H-1′ at 33° are plotted in Figure 4 as a function of the ITP concentration, and are compared with the H-8 and H-1′ proton T_1^{-1} values. The T_{1p}^{-1} values for these protons change by about a factor of 6.5 when 0.3 and 6×10^{-3} *M* ITP solutions are compared.

The longitudinal relaxation times of C-2 and C-8 at 42° are listed in Table I for several concentrations of ITP. The change in T_1 of C-8 and C-2 between 0.3 and 0.05 *M* is approximately a factor of 2.8. T_1 measurements at lower concentrations must wait for enriched ^{13}C samples. Measurements on the 0.05 *M* solution took 6 hr. The values of T_{1p} and T_{2p} for carbons C-6, C-2, C-4, C-8, and C-5 are listed in Table II at 42° for [ITP] = 0.3 *M*, [Mn]/[ITP] = 1.73 $\times 10^{-3}$, and pD = 6.6 ± 0.2. T_{1p} and T_{2p} differ by about a factor of four. Therefore, there is spin leakage into the hypoxanthine ring and, hence, direct coordination between the ring and the Mn(II) ion. Finally, in Figure 5, the T_{2p} temperature dependence of carbon C-8 is analyzed. The estimated uncertainty in the measurements is given in Tables I and II.

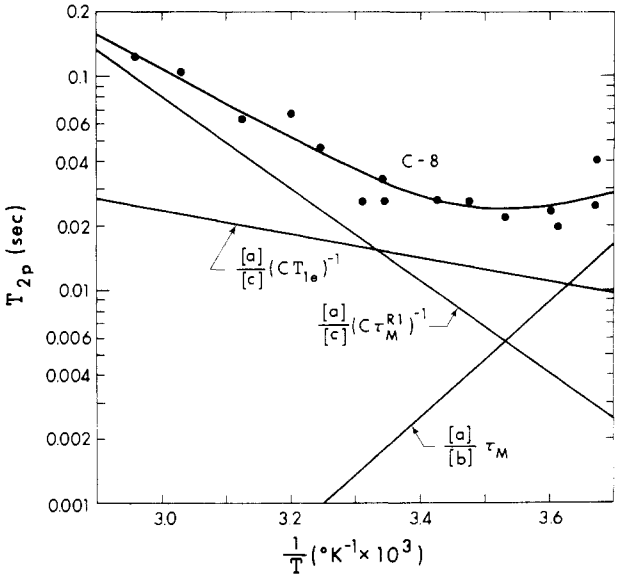


FIGURE 5: The analysis of the temperature dependence of T_{2p} for carbon C-8. The limiting values to the curve are represented with straight lines. The correction for the dipolar term has not been made in this analysis as discussed in the text.

Table II: Summary of ^{13}C T_{1p} and T_{2p} Values (sec) for Mn–ITP Solutions.[a]

	C-6	C-2	C-4	C-8	C-5
T_{1p}	0.51 ± 0.05		1.50 ± 0.25	0.32 ± 0.05	0.36 ± 0.04
T_{2p}	0.10 ± 0.02		0.42 ± 0.08	0.13 ± 0.02	0.09 ± 0.02

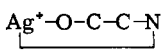
[a] Experiments were carried out in D_2O at a pD of 6.6 ± 0.2 at 42°. [ITP] = 0.30 *M*; [Mn]/[ITP] = 1.73×10^{-3}.

Discussion

From Figure 2 it can be seen that the slope for carbon C-6 in the high temperature region is different from the slope for carbons C-5 and C-8. This implies that there are at least two sites for metal ion–ring interactions (Figure 1) on the hypoxanthine ring, namely the N-7 nitrogen and the carbonyl oxygen at C-6 (C=O). Although the individual points in Figure 2 may have a range as large as 30%, the existence of the crossover is not in question because the measurements at each value of $1/T$ were made simultaneously on the same solution.

The Mn(II) ion cannot bind simultaneously to the N-7 nitrogen and to the C=O oxygen. This can be demonstrated by taking the hypoxanthine ring distances and angles from monosodium inosine 5′-monophosphate (Rao and Sundaralingam, 1969; Sletten, 1971; Kraut and Jensen, 1963). By assuming that the carbonyl oxygen lone pairs occur at 115° with respect to the double bond, and that the lone pair of N-7 bisects the C–N–C angle, projections from the two lone pairs of electrons (C=O and N-7) intersect at 37°. Simultaneous binding of Mn(II) to both the carbonyl

oxygen and N-7 would therefore be extremely strained. A similar conclusion was reached in the X-ray studies of 9-methylhypoxanthinecopper(II) crystal (Sletten, 1971), as well as of the inosine 5′-monophosphate-nickel(II) crystal (Clark and Orbell, 1974). However, Ag^+ ions appear to form a five-membered chelate of the type



(Bamberg and Hemmerich, 1961; Hala, 1965; Tu and Reinosa, 1966). There is no further evidence (Izatt et al., 1971) to support this type of complex occurring with the first-row transition metal ions such as Mn(II), Ni(II), or Cu(II). Further experiments, such as X-ray studies on this system, can prove the validity of our approach, namely, that the Mn(II) ions interact with the C=O oxygen and the N-7 positions of the hypoxanthine base independently.

To show that the metal ion interactions which influence the ^{13}C transverse relaxation times (Figure 2, metal ion binding to N-7 and C=O) are due to the Mn(ITP) species, and not to the $Mn(ITP)_2$ species in which the metal ion binds simultaneously to two hypoxanthine rings, the proton longitudinal relaxation times were studied. From Figure 3, the T_1 values for protons H-8 and H-1′ increase by a factor of 2.7 and 3.1, respectively, as the ITP concentration decreases from 0.3 to 0.055 *M*. The increase in T_1 is 5.6 for both protons when the ITP concentration decreases from 0.3 to 6×10^{-3} *M*. The H-8 and H-1′ relaxation is dominated mainly by the sugar protons (Gueron et al., 1973). These data are now compared with the T_1 relaxation times for the carbon nuclei with directly bonded protons, namely C-2 and C-8. The relaxation of carbons with attached protons in dipolar in nature (Solomon, 1955; Doddrell et al., 1972; Lyerla and Grant, 1972). Therefore, changes in the spin-lattice relaxation times of these carbon nuclei yield accurate information on changes in the tumbling correlation time. The longitudinal relaxation times of C-2 and C-8 increase as the ITP concentration decreases from 0.3 to 0.05 *M* (Table I). These carbon nuclei relax approximately 2.8 times more slowly at the lower concentration. This is very similar to the change seen by H-8 and H-1′. Therefore, carbons C-2 and C-8 and protons H-8 and H-1′ are "seeing" the change in the tumbling correlation time as the ITP concentration decreases. Observation of the proton spin-lattice relaxation times at lower concentrations of ITP should then yield accurate information with regard to the tumbling correlation time. The increase in the H-8 and H-1′ T_1 relaxation times as the ITP concentration decreases from 0.055 to 0.006 *M* indicates that there is still a significant amount of self-association present in 0.055 *M* ITP solutions. Therefore, the T_1 values for C-8 and C-2 at 0.006 *M* should be approximately 1.2 sec.

Since the paper by Bloembergen et al. (1948), researchers have attempted to correlate changes in the spin-lattice relaxation time with changes in the viscosity of the solutions. However, it is known that this need not be the case (Pople, 1967; Dwek and Richards, 1967; Jardetzky, 1964; Reeves and Yue, 1970; Armitage et al., 1974). The ratio of the viscosities between the 0.3 *M* and 0.006 *M* ITP solutions and between 0.3 *M* ITP solutions and distilled water are 1.7 and 2.1, respectively. These results are similar to the ATP viscosity results (Wee et al., 1974) from which Wee et al. (1974) argued that the dipole-dipole interaction is controlled by the viscosity of the solution. The present authors feel that the effect of varying the nucleotide concentration on the tumbling correlation time can best be approached by measuring the proton longitudinal relaxation times of metal-free ITP solutions (Figure 3). That is, changes in the tumbling correlation time of molecules that self-associate cannot be studied by measuring the macroscopic viscosity.

From Figure 4 it can be seen that in the Mn-ITP solutions protons H-8 and H-1′ relax about 6.5 times faster at 0.3 *M* ITP than at 6×10^{-3} *M* ITP. This is similar to the change found by Wee et al. (1974) for the Mn-ATP system. In the Mn-ATP complex, H-8 relaxes 7.2 times faster at 0.3 *M* ATP than at 1×10^{-3} *M* ATP (Wee et al., 1974). To within the precision of the measurements (±10%) the ratios for protons H-8 and H-1′ for Mn-ITP solutions are comparable to the ratios for these protons for metal-free ITP (Figure 4). That is, changes in the relaxation times of C-2, C-8, H-8, and H-1′ accurately predict the changes in the tumbling correlation time and, therefore, the changes in T_{1p}^{-1} as a function of the concentration of ITP. An ML_2 species with the metal ion bound to one triphosphate chain and simultaneously to two hypoxanthine rings need not be proposed to account for the concentration dependence of the proton T_{1p}^{-1} values in the Mn-ITP system as was done by Wee et al. (1974) in the Mn-ATP system on the basis of the viscosity measurements. Stability constant measurements also indicate that the $Mn(ATP)_2$ complex could not be measured in solution (Mohan and Rechnitz, 1974).

The Mn-ITP system is therefore analyzed by considering that a single ligand ring is bound to one metal ion. Unfortunately the proton relaxation time study does not rule out the possibility of some $Mn(ITP)_2$ species existing in which the metal ion binds to the phosphates of one ITP molecule and to the ring of another ITP molecule at high concentrations of ITP. It is felt that such an event occurring on a one-to-one basis would be extremely fortuitous. A transverse relaxation time study of enriched ^{13}C samples at low concentrations of nucleotide (0.01 *M*) would definitely answer the question as to the presence of an $Mn(ITP)_2$ species, as would a study of 9-(β-D-glucopyranosyl)inosine 6′-triphosphate. A study of 9-(β-D-glucopyranosyl)adenine 6′-triphosphate with Ni(II) showed that the $Ni(ATP)_2$ complex was not present (Glassman et al., 1973b). Since self-association is present in the nucleotide solutions (Ts'o, 1970), our analysis of the data based on the assumption of interactions in a 1:1 complex may be an oversimplification of the system. However, the Mn-ITP and Mn-ATP data (Lam et al., 1974), when analyzed in terms of a 1:1 complex, are self-consistent.

The data in Figure 3 warrant further discussion. The much larger change in T_1 for H-2, relative to H-8 and H-1′, is probably due to hydrogen bonding between neighboring sugar and hypoxanthine rings. In this way, as the ITP concentration decreases, proton H-2 is "seeing" not only a change in the tumbling correlation time, but is also affected by the increase in distance to the sugar protons which causes the relaxation time of H-2 to increase. Figure 3 also demonstrates that stability constant information can be determined from spin-lattice relaxation data if one could measure the proton T_1 values at infinite dilution. Thus stability constants can be determined between interacting diamagnetic molecules or ions even when a chemical shift is not measurable. The equations are similar in form to the equations used in determining a stability constant from chemical shift data (Wang and Li, 1966, 1968; Shimokawa et al., 1973).

In the analysis of the ^{13}C transverse relaxation time (T_{2p}) data (Figure 2), following the previous discussion, the

concentration of any $Mn(ITP)_2$ species is assumed to be negligible with respect to the various Mn(ITP) species. Due to the rapid exchange rates that are observed and the similarity of the present system to the Mn–ATP system, the ^{13}C T_{2p} data were analyzed in terms of the following exchange mechanism:

$$\begin{array}{ccccc} & \overset{a}{} & & \overset{b}{} & \overset{c}{} \\ M + L & \rightleftharpoons & ML_P & \rightleftharpoons & ML_{P+R1} \\ & & \Updownarrow & & \\ & & ML_{P+R2} & & \\ & & d & & \end{array}$$

Species a is the free ligand ITP, species b is the metal ion–phosphate bound ligand (ML_P), species c is ML_{P+R1}, in which the metal ion is bound simultaneously to the phosphates and the N-7 nitrogen, and species d is ML_{P+R2}, where the metal ion is bound simultaneously to the phosphate groups and to the carbonyl oxygen at C-6. It is assumed that there is no exchange between sites a and c, a and d, and c and d.

In the slow exchange region NMR "sees" only the a $\rightleftharpoons$ b exchange (Kuntz et al., 1975) (Appendix). That is, τ_{ab} is measured experimentally using ^{31}P NMR, and from this term, τ_{ba} is calculated using the equation $\tau_{ba}^{-1} = \tau_{ab}^{-1}[a]/[b]$. In the fast exchange region, the concentration of species b is estimated from the ^{13}C T_{1p} data by assuming a range of probable binding distances (e.g., N-7 to metal ion and the C=O oxygen to metal ion distance of 2.0–2.5 Å) and calculating the corresponding amount of ring binding using the Solomon equation (Solomon, 1955)

$$T_{1p}^{-1} = f' \frac{2}{5} S(S + 1)\gamma_I^2\beta^2 g^2(\tau_c/r^6) \qquad (3)$$

where $f' = [c]/[a]$ or $[d]/[a]$, and the other terms have their usual meaning. The ^{13}C T_{1p} values are listed in Table II at 42°. The ITP correlation time, τ_c, at 42° was calculated using the pure ATP relaxation times for C-8 and C-2 (0.13 sec at 30°; $\tau_c = 1 \times 10^{-9}$ sec) (Sternlicht et al., 1965a; Lam et al., 1974) and the pure ITP relaxation times for C-8 and C-2 (0.24 sec). Hence τ_c (42°) = (13/24) × $10^{-9} \simeq 5 \times 10^{-10}$ sec. The assumption in this calculation is that the change in the tumbling rate of the free molecule will be the same as for the complex.

The analysis of the T_{1p} ^{13}C data for carbons C-4, C-5, C-6, and C-8 indicates that the amount of ring binding is self-consistent when the metal ion is located between 2.3 and 2.5 Å from the N-7 nitrogen and the C-6 carbonyl oxygen. The total ring binding is 8–12%, with approximately one-eleventh of the ring bound metal ion coordinated to C=O, and the remainder bound to N-7. The smaller amount of ring binding for the Mn–ITP system relative to the Mn–ATP system (Lam et al., 1974) is not surprising in light of the calculations of Glassman et al. (1973a). These workers (Glassman et al., 1973a) have applied generalized perturbation theory (Klopman, 1968) and have calculated the "ability" of metal ions to interact with various purine ring systems. The calculations demonstrate that there is a significant difference in the N-7 lone-pair electrons between inosine and adenosine.

From Table II it can be seen that T_{2p} values are smaller than the corresponding T_{1p} values. In NMR relaxation terms, this indicates that there is a significant amount of unpaired spin density that is transferred from the metal ion to the hypoxanthine ring (Swift, 1973), and hence the metal ion is directly bonded to the ring (Dwek, 1973; Swift, 1973; Espersen et al., 1974). In terms of the Solomon and Bloembergen equations (Solomon, 1955; Solomon and Bloembergen, 1956), these data indicate that for the Mn–ITP complex T_{1p} is dipolar in nature (eq 3), while the hyperfine term (eq 5 and 6) controls T_{2p}. As the high temperature portions of the curves for C-5 and C-8 are parallel (Figure 2), spin-leakage to the C=O carbon does not enter the remainder of the purine ring. In addition, in the Mn–ATP system, T_{2p} for C-6 is approximately 2.5% of the T_{2p} values for C-5 and C-8 (Lam et al., 1974). Hence the spin-leakage to C-6 from the metal ion binding at N-7 is expected to be small, as in the Mn–ATP system. The large effect that is seen on the T_{2p} C-6 values is due to Mn(II) ion binding at the carbonyl oxygen. Hence the T_{2p} data for C-5 and C-8 (due to binding at N-7) and the T_{2p} data (Figure 2) for C-6 (due to binding at C=O) can be analyzed as a function of the temperature.

The curve fitting analysis for carbons C-8, C-5, and C-6 was carried out as discussed previously (Lam et al., 1974). A correction for the dipolar term (Table II) could not be made in the following calculation due to the experimental difficulties in performing a natural abundance ^{13}C T_{1p} temperature study. The temperature-dependent curves (Figure 2) are fitted using the slow exchange region (low temperature) equation (Appendix)

$$T_{2p}^{-1} = ([b]/[a])\tau_{ba}^{-1} \qquad (4)$$

and the fast exchange or high temperature region equation

$$T_{2p}^{-1} = \frac{[c]}{[a]} T_{2c}^{-1} + \frac{[d]}{[a]} T_{2d}^{-1} \qquad (5)$$

$$1/T_{2j} = \frac{S(S + 1)}{3}(A/h)^2(T_{1e}^{-1} + \tau_{jb}^{-1})^{-1} = C(T_{1e}^{-1} + \tau_{jb}^{-1})^{-1} \qquad (6)$$

T_{1e} is the longitudinal electronic relaxation time, and τ_{jb}^{-1} is the exchange rate between species c or d with species b. We will refer to τ_{cb} as τ_M^{R1} and τ_{db} as τ_M^{R2} to distinguish it from $\tau_M(\tau_M = \tau_{ba})$, the metal ion–phosphate exchange rate. The metal ion–phosphate exchange rate was measured by ^{31}P FT NMR and found to be in the slow exchange region as is the Mn–ATP system (Sternlicht et al., 1965a; Kuntz et al., 1975). A plot of T_{2p}^{-1} vs. T^{-1} is a straight line and $\Delta H^{\ddagger}$ for this process is 10.9 ± 0.5 kcal/mol. The value of τ_M^{-1} at 27° is 2.3×10^5 sec^{-1} which may be calculated by solving for $\tau_M^{-1} = \tau_{ab}^{-1}[a]/[b]$ where [b] = 0.89[Mn], and [a] = 0.3 *M* ITP.

The temperature dependence of the electron spin-lattice relaxation rate is predicted using the following equation (Rubenstein et al., 1971):

$$T_{1e}^{-1} = \frac{2}{50}\Delta^2[4S(S + 1) - 3] \times \left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \qquad (7)$$

where $\tau_v = \tau_v^0 \exp V_v/RT$, Δ is the zero field splitting, τ_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. The Δ and τ_v values for Mn–adenosine triphosphate and Mn–uridine triphosphate (Reed et al., 1971) were used for the Mn–ITP complex. The temperature dependence of T_{1e} does not account for the steep slope of carbons 5, 8, and 6 (Figure 2). Therefore, τ_M^{R1} and τ_M^{R2} are required to fit the high temperature region of Figure 2. Ac-

Table III: Scalar Coupling Constants for the Inosine Base Carbon Nuclei of ITP.[a]

	C-8	C-5	C-6
A/h (Hz)	3.7×10^5	4.0×10^5	1.2×10^6

[a] The solution composition is [ITP] = 0.3 M; [Mn] = 1.1×10^{-3} M; [b] = 0.89[Mn]; [c] = 0.10[Mn]; [d] = 0.01[Mn].

Table IV: Activation Parameters and Lifetimes of the Metal Ion Involved in the Ring Interaction.[a]

τ_M^{R1}	$\Delta H^{\ddagger}_{R1}$	τ_M^{R2}	$\Delta H^{\ddagger}_{R2}$
5×10^{-8} sec	9.4 kcal/mol	6.7×10^{-8} sec	14 kcal/mol

[a] Evaluated at 27°. The solution composition is [ITP] = 0.3 M; [Mn] = 1.1×10^{-3} M; [b] = 0.89[Mn]; [c] = 0.10[Mn]; [d] = 0.01[Mn].

tually a range of A/h values and τ_M^{R1} will fit the temperature profiles of carbons 5 and 8. However, we can calculate τ_M^{R1}, since, at the point of intersection of the two straight lines, $T_{1e} = \tau_M^{R1}$ (Figure 5, $T \approx 27°$). Obviously these two straight lines must cross at the same temperature for both carbons 5 and 8. This limits the values of A/h and τ_M^{R1} to a reasonably unique set. As this cannot be done for C-6, the values for A/h and τ_M^{R2} may not be as reliable as those for C-5 and C-8. These values, and the corresponding $\Delta H^{\ddagger}$ values are listed in Tables III and IV.

In summary, the theory for the four-site exchange system has been developed and has been successfully applied to the Mn-ITP system. Species a, b, c, and d are free ITP, manganese-phosphate bound ITP, phosphate-manganese-N-7 ring bound ITP and phosphate-manganese-C=O ring bound ITP. This study has shown that approximately 11% of the bound Mn(II) ions are ring coordinated. The transverse relaxation time study indicates that there is direct metal ion-ring binding at nitrogen N-7 and at the carbonyl oxygen.

Appendix

The four-site chemical exchange is treated in an identical manner to the three-site exchange system (McConnell, 1958; Swift and Connick, 1962; Lam et al., 1974)

$$-AG_a + \frac{G_b}{\tau_{ba}} + \frac{G_c}{\tau_{ca}} + \frac{G_d}{\tau_{da}} = i\omega_1 M_0^a$$

$$\frac{G_a}{\tau_{ab}} - BG_b + \frac{G_c}{\tau_{cb}} + \frac{G_d}{\tau_{db}} = i\omega_1 M_0^b$$

$$\frac{G_a}{\tau_{ac}} + \frac{G_b}{\tau_{bc}} - CG_c + \frac{G_d}{\tau_{dc}} = i\omega_1 M_0^c$$

$$\frac{G_a}{\tau_{ad}} + \frac{G_b}{\tau_{bd}} + \frac{G_c}{\tau_{cd}} - DG_d = i\omega_1 M_0^d$$

where

$$A = \frac{1}{T_{2a}} + \frac{1}{\tau_{ab}} + \frac{1}{\tau_{ac}} + \frac{1}{\tau_{ad}} - i\Delta\omega_a$$

T_{2j}^{-1} is the transverse relaxation rate of site j; τ_{ij}^{-1} is the exchange rate from site i to site j. Setting $\tau_{ac}^{-1} = \tau_{ca}^{-1} = \tau_{ad}^{-1} = \tau_{da}^{-1} = \tau_{cd}^{-1} = \tau_{dc}^{-1} = 0$ and solving for the line width in the usual manner, the relationship for $1/T_2$ becomes

$$T_2^{-1} = k_a + k_x = T_{2a}^{-1} + \tau_{ab}^{-1} + k_x$$

where

$$k_x = \frac{k_{ab}k_{ba}(-k_b k_c^2 k_d^2 + k_c k_d^2 k_{cb} k_{bc} + k_d k_c^2 k_{db} k_{bd})}{\{k_b^2 k_c^2 k_d^2 + k_d^2 k_{cb}^2 k_{bc}^2 + k_c^2 k_{bd}^2 k_{db}^2 + 2k_d k_c k_{bc} k_{cb} k_{db} k_{bd} - 2k_b k_c k_d^2 k_{cb} k_{bc} - 2k_b k_c^2 k_d k_{db} k_{bd}\}}$$

$$k_a = T_{2a}^{-1} + \tau_{ab}^{-1}$$

$$k_b = T_{2b}^{-1} + \tau_{ba}^{-1} + \tau_{bc}^{-1} + \tau_{bd}^{-1}$$

$$k_c = T_{2c}^{-1} + \tau_{cb}^{-1}$$

$$k_d = T_{2d}^{-1} + \tau_{db}^{-1}$$

$$k_{ij} = \tau_{ij}^{-1}$$

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

The effect of the metal ion on the line width, T_{2p}^{-1}, is $\tau_{ab}^{-1} + k_x$. The following limiting cases are of importance in the analysis of the Mn-ITP system. Case I: slow exchange

$$\frac{1}{T_{2p}} = \tau_{ab}^{-1} = \frac{[b]}{[a]}\tau_{ba}^{-1}$$

Case II: fast exchange

$$\tau_{cb}^{-1} \gg T_{2c}^{-1}$$

$$\tau_{db}^{-1} \gg T_{2d}^{-1}$$

$$\tau_{cb}^{-1}, \tau_{db}^{-1} \gg \tau_{ba}^{-1}$$

Hence

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

References

Armitage, I. M., Huber, H., Live, D. H., Pearson, H., and Roberts, J. D. (1974), *J. Magn. Reson. 15,* 142.

Bamberg, P., and Hemmerich, P. (1961), *Helv. Chim. Acta 44,* 1001.

Bloembergen, N., Purcell, E. M., and Pound, R. V. (1948), *Phys. Rev. 73,* 679.

Bridger, W. A. (1974), *Enzymes, 3rd Ed. 10,* 581.

Brown, F. F., Campbell, I. D., Henson, R., Hirst, C. W. J., and Richards, R. E. (1973), *Eur. J. Biochem. 38,* 54.

Brownie, E. R., and Bridger, W. A. (1972), *Can. J. Biochem. 50,* 719.

Clark, G. R., and Orbell, J. D. (1974), *J. Chem. Soc., Chem. Commun.* 139.

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

Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56,* 3683.

Dwek, R. A. (1973), Nuclear Magnetic Resonance (N.M.R.) in Biochemistry, Oxford, Clarendon Press, p 174.

Dwek, R. A., and Richards, R. E. (1967), *Discuss. Faraday Soc. 43,* 196.

Eichhorn, G. L. (1973), in Inorganic Biochemistry, Eichhorn, G. L., Ed., New York, N.Y., Elsevier, p 1191.

Espersen, W. G., Hutton, W. C., Chow, S. T., and Martin R. B. (1974), *J. Am. Chem. Soc. 96,* 8111.

Feldman, I., and Wee, V. (1974), *Biochemistry 13,* 1836.

Freeman, R., and Hill, H. D. W. (1971), *J. Chem. Phys. 54,* 3367.

Frey, C. M., and Stuehr, J. E. (1974), in Metal Ions in Biological Systems, Sigel, H., Ed., New York, N.Y., Marcel Dekker, p 51.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64,* 188.

Glassman, T. A., Cooper, C., Harrison, L. W., and Swift, T. J. (1971), *Biochemistry 10,* 843.
Glassman, T. A., Cooper, C., Kuntz, G. P. P., and Swift, T. J. (1974a), *FEBS Lett. 39,* 73.
Glassman, T. A., Cooper, C., Kuntz, G. P. P., and Swift, T. J. (1974b), *FEBS Lett. 45,* 184.
Glassman, T. A., Klopman, G., and Cooper, C. (1973a), *Biochemistry 12,* 5013.
Glassman, T. A., Suchy, J., and Cooper, C. (1973b), *Biochemistry 12,* 2430.
Gueron, M., Chachaty, C., and Son, T.-D. (1973), *Ann. N.Y. Acad. Sci. 222,* 307.
Hala, J. (1965), *J. Inorg. Nucl. Chem. 27,* 2659.
Izatt, R. M., Christensen, J. J., and Rytting, J. H. (1971), *Chem. Rev. 71,* 439.
Jardetzky, O. (1964), *Adv. Chem. Phys. 7,* 499.
Kotowycz, G., and Lemieux, R. U. (1973), *Chem. Rev. 73,* 669.
Kotowycz, G., and Suzuki, O. (1973), *Biochemistry 12,* 3434.
Klopman, G. (1968), *J. Am. Chem. Soc. 90,* 223.
Kraut, J., and Jensen, L. H. (1963), *Acta Crystallogr. 16,* 79.
Kuntz, G. P. P., Glassman, T. A., Cooper, C., and Swift, T. J. (1972), *Biochemistry 11,* 538.
Kuntz, G. P. P., Lam, Y.-F., and Kotowycz, G. (1975), *Can. J. Chem. 53,* 926.
Lam, Y.-F., Kuntz, G. P. P., and Kotowycz, G. (1974), *J. Am. Chem. Soc. 96,* 1834.
Lyerla, Jr., J. R., and Grant, D. M. (1972), in Magnetic Resonance, Series One, Vol. 4, McDowell, C. A., Ed., Baltimore, Md., University Park Press, p 155.
McConnell, H. M. (1958), *J. Chem. Phys. 28,* 430.
Mohan, M. S., and Rechnitz, G. A. (1974), *Arch. Biochem. Biophys. 162,* 194.
Naumann, C. F., Prijs, B., and Sigel, H., (1974), *Eur. J. Biochem. 41,* 209.
Phillips, R. (1966), *Chem. Rev. 66,* 501.
Pople, J. A. (1967), *Discuss. Faraday Soc. 43,* 192.
Rao, S. T., and Sundaralingam, M. (1969), *J. Am. Chem. Soc. 91,* 1210.
Reed, G. H., Leigh, J. S., and Pearson, J. E. (1971), *J. Chem. Phys. 55,* 3311.
Reeves, L. W., and Yue, C. P. (1970), *Can. J. Chem. 48,* 3307.
Rubinstein, M., Baram, A., and Luz, Z. (1971), *Mol. Phys. 20,* 67.
Schneider, P. W., Brintzinger, H., and Erlenmeyer, H. (1964), *Helv. Chim. Acta 47,* 992.
Shimokawa, S., Fukui, H., Sohma, J., and Hotta, K. (1973), *J. Am. Chem. Soc. 95,* 1777.
Sletten, E. (1971), *Chem. Commun.,* 558.
Solomon, I. (1955), *Phys. Rev. 99,* 559.
Solomon, I., and Bloembergen, N. (1956), *J. Chem. Phys. 25,* 261.
Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965a), *J. Chem. Phys. 43,* 3123.
Sternlicht, H., Shulman, R. G., and Anderson, E. W. (1965b), *J. Chem. Phys. 43,* 3133.
Sternlicht, H., Jones, D. E., and Kustin, K. (1968), *J. Am. Chem. Soc. 90,* 7110.
Swift, T. J. (1973), in NMR of Paramagnetic Molecules, Principles and Applications, La Mar, G. N., Horrocks, Jr., W. DeW., and Holm, R. H., Ed., New York, N.Y., Academic Press, p 53.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37,* 307.
Szent-Gyorgyi, A., (1957), Bioenergetics, New York, N.Y., Academic Press, p 64.
Ts'o, P. O. P. (1970), Fine Structure of Proteins and Nucleic Acids, New York, N.Y., Marcel Dekker, p 49.
Tu, A. T., and Heller, M. J. (1974), in Metal Ions in Biological Systems, Sigel, H., Ed., New York, N.Y., Marcel Dekker, p 1.
Tu, A. T., and Reinosa, J. A. (1966), *Biochemistry 5,* 3375.
Wang, S. M., and Li, N. C. (1966), *J. Am. Chem. Soc. 88,* 4592.
Wang, S. M., and Li, N. C. (1968), *J. Am. Chem. Soc. 90,* 5069.
Wasylishen, R. E., and Cohen, J. S. (1974), *Nature (London) 249,* 847.
Wee, V., Feldman, I., Rose, P., and Gross, S. (1974), *J. Am. Chem. Soc. 96,* 103.
Weser, U. (1968), *Struct. Bonding (Berlin) 5,* 41.
Zetter, M. S., Dodgen, H. W., and Hunt, J. P. (1973), *Biochemistry 12,* 778.