The interactions between ATP, monovalent cations, and divalent cations on rabbit muscle pyruvate kinase have been examined using $^7\text{Li}$, $^{31}\text{P}$, and $^1\text{H}$ nuclear magnetic resonance. Water proton nuclear relaxation studies are consistent with the binding of Li$^+$ to the K$^+$ site on pyruvate kinase with an affinity of 120 mM in the absence of substrates and 16 mM in the presence of P-enolpyruvate. 

Titration with pyruvate demonstrate that pyruvate binds to the enzyme with an affinity of 0.85 mM in the presence of Li$^+$ and 0.4 mM in the presence of K$^+$.

The presence or absence of pyruvate at the active site of pyruvate kinase is increased upon titration with the metal-nucleotide analogue, Cr(H$_2$O)$_6$ATP. Mn$^{2+}$ EPR spectra were used to determine the distribution of the enzyme between the so-called isotropic and anisotropic conformations of the enzyme (Ash, D. E., Kayne, P., and Reed, G. H. Arch. Biochem. Biophys. (1978) 190, 571–577). Li-Cr distances of 5.6 and 11.0 Å were calculated for the anisotropic and isotropic forms, respectively, in the absence or presence of pyruvate. When the divalent cation site on the enzyme was saturated with Mg$^{2+}$, these distances increased to 6.7 and 9.5 Å, respectively, regardless of the presence or absence of pyruvate.

$^{31}\text{P}$ nuclear relaxation studies with the diamagnetic metal-nucleotide analogue, Co(NH$_3$)$_5$ATP, indicated that addition of Mn$^{2+}$ ion to the divalent cation site on the enzyme increased the longitudinal relaxation rates of all three phosphorus nuclei of the analogue. The $^{31}\text{P}$ data indicate that the presence of pyruvate at the active site effects a decrease in the Mn$^2+$-P distances, bringing Mn$^{2+}$ and Co(NH$_3$)$_5$ATP closer together at the active site. The data also permit an evaluation of the role of the metal coordinated to the $\beta$-P and $\gamma$-P of ATP at the active site.

Due to the large amount of active site structural information which has already been obtained for the rabbit muscle enzyme, pyruvate kinase, it is an ideal model system for the development of new spectroscopic techniques or for the exploration of new spectroscopic probes. Thus, in our laboratory, a long-standing interest in monovalent cation active transport across the mammalian plasma membrane led us to examine $^7\text{Li}$ NMR as a probe of monovalent cation sites, first on pyruvate kinase (1) and later with kidney (Na$^+$ + K$^+$)-ATPase (2) and then with sarcoplasmic reticulum Ca$^{2+}$-ATPase (3). Similarly, we have used the complexes of Cr(III) and Co(III) with ATP to characterize the interactions of divalent metals and ATP at the active sites of the ATPases (3, 4). We have relied at times on the wealth of information which exists on the interactions of these probes with pyruvate kinase and other related phosphoryl transfer systems. It was in the interest of a further characterization of $^7\text{Li}$ and the Cr(III) and Co(III) complexes of ATP as spectroscopic probes, and in order to address several ambiguities in the active site structure of pyruvate kinase, that we undertook the experiments described in this paper.

Pyruvate kinase is a tetrameric enzyme of $M_r = 237,000$, which catalyzes the reversible phosphorylation of adenosine diphosphate (ADP) from phosphoenolpyruvate (P-enolpyruvate) to form adenosine triphosphate (ATP). This enzyme requires both monovalent (K$^+$) and divalent (Mg$^{2+}$) cations for activity and recent studies indicate that the enzyme binds, and may require for activity, two divalent cations at the active site (5). Spectroscopic and kinetic studies have yielded a model of the active site in which 17 distances between relevant substrate nuclei and/or metal ions can be reconciled. The data are consistent with molecular contact between the $\gamma$-phosphoryl phosphorus of ATP and the carbonyl oxygen of pyruvate, consistent with direct phosphoryl transfer. The enzyme-bound divalent cation appears to form second sphere complexes with the phosphoryl groups of P-enolpyruvate and ATP and may activate the transferred phosphoryl group indirectly through a water ligand.

On the other hand, as pointed out by Mildvan et al. (6), several ambiguities remain. One of the these concerns the activating monovalent cation, normally K$^+$.

Studies with $^{205}\text{Tl}^+$ (7) and in our lab with $^7\text{Li}^+$ (1) have demonstrated that the binding of P-enolpyruvate causes a substantial decrease in the separation of the monovalent cation site and the high affinity catalytic site for Mn$^{2+}$ on the enzyme. Comparison of the $^{205}\text{Tl}^+$ and $^7\text{Li}^+$ data led us to suggest that the low activity of the enzyme with Li$^+$ (2% of that with Tl$^+$ or K$^+$) might be a consequence of a "poor fit" for Li$^+$ at the active site, with a larger M$^+\cdot$Mn$^{2+}$ separation in the case of Li$^+$ than with Tl$^+$ (1, 7). This model was disputed by Ash et al. (8), but Raushel and Villafranca (9, 10) later showed that the activating abilities of the alkali cations were indeed inversely related to the M$^+\cdot$Mn$^{2+}$ separations measured for these ions at the active site of pyruvate kinase, as we had originally suggested. Still, the relation of the monovalent cation site to the other moieties...
pyruvate and Li⁺/pyruvate were prepared similarly, except that these were neutralized to pH 7.5 with Tris base, or pH 6.6 with 1 M LiOH, respectively.

**NMR Methods**—The **Li⁺** NMR experiments were performed using a JEOL PS-100P Fourier transform spectrometer. Measurements were made at probe temperatures of 23 ± 1 °C. All experiments were performed with Li⁺-loaded NMK tubes. The carbon probe, a 5-kHz crystal filter, and lowering the magnetic field from 23.5 to 15.2 kG, the 'Li⁺' signal resonated at 25.15 MHz. Lowering the field 12 h before the experiment produced a stable field. Unfortunately, lowering the field prohibits the normal use of the deuterium lock; hence, experiments were done unlocked. Usually only one transient was required for good signal to noise ratios. Spin-lattice relaxation rates (1/T1) were measured using an 180°-90° pulse sequence where the 90° pulse was approximately 20 μs. Null times were measured giving a T2 = null time/ln2. Samples having an initial volume of 1.0 ml contained 90 mM LiCl, 80 mM Pipes buffer, and 100-200 μM enzyme sites.

The **31P** NMR spectra and relaxation rates are determined by pulsed Fourier transform methods at two frequencies, 40 MHz and 145 MHz. Low field spectra are obtained at 2.3 tesla on a Jeol PS-100P Fourier transform spectrometer operating at 40.5 MHz, equipped with a field frequency lock on an internal deuteron reference. The spectra are obtained in the time domain mode, and a signal averaging is performed with a 980A Texas Instruments computer dedicated to the spectrometer. Broad-band decoupling of the proton resonances (~15 W) is employed. The 90° pulse width is 12–15 μs. The spectra are processed with an exponential filter to improve the signal to noise ratio. High field spectra are obtained at 8.5 tesla with a Nicolet Magnetics Corp. NT-360/Oxford spectrometer equipped with the 1290/290B data system. An internal 'H lock and uninterrupted incoherent 'H decoupling at low levels (~1 W) are employed. The 90° pulse width at 145.75 MHz is 28–32 μs. The spectra are recorded using quadrature phase detection with the carrier frequency in the middle of the spectrum. Standard Nicolet software (NMCFT V#10221) is used to correct and process the free induction decays. The 1D spectra are collected as 8K FIDs which are zero-filled to yield 16K FT spectra. T1, T2, and T2* are measured. The sweep width of the spectra is ±13856.04 Hz, and exponential multiplication is employed on the T1, data sets to optimize sensitivity, using a time constant matched to the effective decay constant T2*.

Measurements are made at probe temperatures of 23 ± 1 °C. Typical sample volumes for the NMR experiments are 1.0 ml (40.3 MHz) and 2.4 ml (145.7 MHz) containing approximately 20% D2O for the purpose of providing a field frequency lock. Sample tubes (Wilmad 513-7PP), 10-mm outside diameter, were used with a vortex plug. The chemical shifts are expressed with reference to 85% H3PO4 as an external standard; positive values are downfield from the standard.

For the one-pulse spectra, the rf pulse angle θ, for the optimum signal to noise ratio, is calculated according to the expression:

$$\cos \theta = e^{-\gamma B_{1} T_{R}}$$

where τ and T1 represent the repetition time and spin–lattice relaxation time, respectively (15). The longitudinal relaxation rates (1/T1) are measured using the inversion-recovery pulse sequence (180°-τ-90°-T1).

The proton relaxation rate experiments were performed using a variable frequency, pulsed NMR spectrometer. The magnet for this instrument is a Varian 4012A electromagnet with a 2100A power supply which have been modified for solid state operation. The rf components and pulse programmer have been designed and built by Siemens. The frequency synthesizer is a PRD 7838 synthesizer. Measurements are made at a probe temperature of 23 ± 1 °C. The experiment is carried out in 4-mm tube (0.1 ml) at 24 MHz. The relaxation rates are measured using the inversion-recovery sequence. Null times are measured giving a T1, equal to the null time divided by ln2.

**NMR Samples**—Just prior to performing the experiments, a sample of CoATP is thawed and passed through a column (0.4 × 7 cm) of Chelex. The solution is adjusted to pH 7.5 with 1 M Tris-Cl (chelaxed), pH 7.4, and diluted to yield a solution containing 100 mM Tris-Cl, pH 7.5. The NMR samples contain the following components: 17–21

---

1. The abbreviations used are: TMA, tetramethylammonium; Pipes, 1,4-piperazinediethanesulfonic acid; FID, free induction decay; Hopes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
nm CoATP, 80 mM Tris-Cl buffer, pH 7.5, 20% D2O, and 0-25 μM MnCl2. Those samples which have enzyme contain 40 μM pyruvate kinase (in enzyme sites) and 10 mM KCl. For those experiments containing pyruvate, TMA/pyruvate is added to yield a final concentration of 17.6 mM.

Theoretical Basis for Calculations—The calculations in this paper are based on previously described theory (16-25) and will only be summarized here. The paramagnetic contribution to the longitudinal relaxation rate, 1/T1p, of a nucleus is given by:

\[
\frac{1}{T_1p} = \frac{1}{T_1} - \frac{1}{T_{1(o)}} = \frac{pq}{\tau_m + T_{1M}}
\]

where 1/T1 is the observed relaxation rate in the presence of the paramagnet; 1/T1(o) is the relaxation rate in the absence of the paramagnet; p is the ratio of the concentration of paramagnetic ion to the concentration of the observed nucleus; q is the number of nuclei in the coordination sphere of the paramagnetic ion; and pq (pq = f) is the mole fraction of the nuclei in the coordination sphere; 1/τm is the ligand exchange rate between the bound and unbound form; and 1/T1M is the relaxation rate in the coordination sphere of the paramagnetic ion. If 1/τm is fast compared to 1/T1M (i.e. conditions of fast exchange), the paramagnetic contribution to the relaxation rate, 1/T1p, is given by the Solomon-Bloembergen equation:

\[
\frac{1}{T_1p} = \frac{C}{B} \frac{r}{\tau_s (1 + \omega^2 r^2 + \omega^2 s^2)} + \frac{4r_0}{1 + 4\omega^2 s^2}
\]

where B is a constant containing the electronic spins s and the zero-field splitting of Mn²⁺ and τs is a time constant for transient symmetry distortions of the Mn²⁺ complex. This correlation time has two limiting cases. For case 1

\[\omega_s^2 r^2 > 1\]

In this case, Equation 4 reduces to  

\[
\frac{1}{T_1p(\omega_s^2)} = \frac{1}{4r_0} \left(1 + 4\omega^2 s^2 (\omega_2 \omega_1)^2 \right)^2 (1 + 4\omega^2 s^2) \tau_s^2
\]

In this case, T1p displays maximum frequency dependence, and τs = T1p at ωs. For case 2

\[\omega_s^2 r^2 \ll 1\]

As can be seen by consideration of Equation 4, T1p = 1/(5Br2) for this case and is independent of frequency. For the ratio of 1/(T1p) at two frequencies, (ω1 and ω2) given by

\[
\frac{1}{T_1p(\omega_1)} = \frac{1}{T_1p(\omega_2)} = \frac{1}{T_1p(\omega)} \left(1 + 4\omega^2 s^2 \tau_s^2 \right) (1 + 4\omega^2 s^2)
\]

Thus when T1p dominates τs, Equations 5 and 6 can be used together with measurements of 1/(T1p(ω)) at two frequencies to estimate τs. Ternary Mn²⁺-Enzyme-Co(NH₂)₂ATP Complex—Under the experimental conditions employed here, Co(NH₂)₂ATP is present in both binary and ternary Mn²⁺ complexes. Thus, the observed paramagnetic relaxation rate is a weighted average of the rates due to each complex of CoATP:

\[
\frac{1}{T_1p} = \frac{[SM]}{[S]} \left(\frac{1}{T_1p(\omega)}\right)_{\text{binary}} + \frac{[MES]}{[S]} \left(\frac{1}{T_1p(\omega)}\right)_{\text{ternary}}
\]

where [SM], [MES], and [S] denote the concentrations of the binary complex, ternary complex, and total Co(NH₂)₂ATP, respectively. 1/(T1p(ω)binary) and 1/(T1p(ω)ternary) are the relaxation rates of CoATP in the binary and ternary complexes. [SM] and [MES] are calculated from their respective dissociation constants (5).

RESULTS

Binding Constants for K⁺, Li⁺, and Pyruvate to Pyruvate Kinase—The interactions of Li⁺ with pyruvate kinase and the binding of pyruvate in the presence of Li⁺ were examined by following the rate of water proton longitudinal relaxation in solutions containing the enzyme, Mn²⁺, Li⁺, and pyruvate. Fast exchange of water protons at the single high affinity Mn²⁺ site on the enzyme provides an enhancement of the relaxation of bulk water in these solutions, as has been shown in previous studies (15). As reported previously (21), addition of K⁺ to solutions containing pyruvate kinase effects a decrease in 1/τ1, the enhancement of water relaxation. As shown in Fig. 1, a similar effect is observed with Li⁺. The affinity of K⁺ estimated from kinetic studies is 14 mM in the presence of Mn²⁺ (22) while the binding of Li⁺ obtained here (120 mM) is considerably weaker (Table I). This value compares with a value of 11 mM estimated from kinetic studies (1). In the presence of P-enolpyruvate, the affinities for both K⁺ and Li⁺ are increased dramatically. The value of K₅ for K⁺ compares well with that determined by Nowak and Mildvan (21), while the value for Li⁺ is in agreement with the estimate from kinetic studies (1). The weak binding of Li⁺ and the low enhancement at high levels of Li⁺ raise the question of displacement of Mn²⁺ from the enzyme by Li⁺. That this is not the case was shown by us previously (1).

In order to measure the affinity of the pyruvate kinase-Mn²⁺-M⁺ (M⁺ = K⁺ or Li⁺) complex for pyruvate, the appropriate solutions of enzyme and ions were titrated with pyruvate as shown in Fig. 2. The affinity for pyruvate is only slightly weaker in the presence of Li⁺ than in solutions containing K⁺ as shown in Table I.

Li⁺ Nuclear Relaxation Studies in Solutions of Pyruvate Kinase and Cr(H₂O)₆ATP—Measurements were made of Li⁺ longitudinal relaxation rates, 1/T₁, in solutions of pyruvate kinase as a function of added Cr(H₂O)₆ATP in order to determine the location of the monovalent cation site with respect to the bound paramagnetic Cr(III) ion. Titration in the presence and absence of Mg²⁺ and pyruvate. Since pyruvate kinase is known to exist in two conformations, and since the distribution between these two states is known to be temperature dependent (23), all 'Li⁺ NMR studies were performed both at 5 and 23 °C. In the absence of enzyme, the relaxation rates of Li⁺ solutions ranged from 0.067 s⁻¹ at 23 °C to 0.11 s⁻¹ at 5 °C. Addition of Mg²⁺ or pyruvate in the absence or presence of enzyme caused no significant change in the relaxation rate. Addition of enzyme caused a diamagnetic increase in the relaxation rate, which was typical of the effects of dissolved macromolecules.

In all cases, addition of Cr(H₂O)₆ATP to solutions of enzyme resulted in an increase in the longitudinal relaxation rate of 'Li⁺ until the enzyme was saturated with Cr(H₂O)₆ATP, consistent with close approach of Li⁺ and Cr(III) at the active site. A typical titration with Cr(H₂O)₆ATP is shown in Fig. 3, and a summary of the titrations in the absence and presence of pyruvate and Mg²⁺ is presented in Table II. As can be seen, the presence of pyruvate increases the values of 1/T₁, while Mg²⁺ effects a decrease in 1/T₁, both in the presence and absence of pyruvate.

Mn²⁺ EPR Studies of the Distribution of Pyruvate Kinase between Isotropic and Anisotropic Conformations—Pyruvate kinase undergoes conformational changes under several conditions.

14062 NMR Studies of Substrate and Ion Sites on Pyruvate Kinase
NMR Studies of Substrate and Ion Sites on Pyruvate Kinase

TABLE I

<table>
<thead>
<tr>
<th>Complex</th>
<th>Dissociation constant of [ligand]</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Mn-[K+]</td>
<td>140</td>
</tr>
<tr>
<td>E-Mn-[Li+]</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>E-Mn-[K+]·(P-enolpyruvate)</td>
<td>0.8 ± 0.06</td>
</tr>
<tr>
<td>E-Mn-[Li+]·(P-enolpyruvate)</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>E-Mn·K+[pyruvate]</td>
<td>0.4 ± 0.09</td>
</tr>
<tr>
<td>E-Mn·Li+[pyruvate]</td>
<td>0.65 ± 0.10</td>
</tr>
</tbody>
</table>

* Dissociation constant of [ligand] from indicated complex.
* Determined without divalent cations by difference spectroscopy.
* Determined with Mn²⁺ present by kinetic methods (22).
* Also determined in Ref. 21.
* Also determined in Ref. 8.

Table 1. Binding of potassium, lithium, and pyruvate to pyruvate kinase.

The solutions contained: A, 140 mm Tris, pH 7.5; B, 140 mm Tris, pH 7.5; C, 50 mm Tris, pH 5.0.

Determined by difference spectroscopy.

The percentage of the anisotropic component in the spectrum falls in the same order as the activating abilities of the ions: K⁺ > Na⁺ > Li⁺. Ash et al. (8) used the Mn(II) EPR spectra of pyruvate kinase-Mn²⁺(P-enolpyruvate)-Li complexes to determine the percentage of anisotropic or "K-like" species in the presence of Li⁺. In order to determine the distribution of the enzyme between the anisotropic and isotropic conformations and in order to calculate Li-Cr distances in these complexes, we have examined the Mn(II) EPR spectra of enzyme-Mn²⁺ complexes in the absence and presence of pyruvate and Cr(H₂O)₆ATP, a diamagnetic analogue of Cr(H₂O)₆ATP. Fig. 4 shows the Mn(II) EPR spectra of pyruvate kinase complexes with pyruvate and either K⁺ or Li⁺. The contributions to these spectra from the anisotropic or K⁺-like form and the isotropic form of the enzyme were calculated from these spectra using spectral subtraction and integration software described previously (24). The anisotropic conformation accounted for 92% of the Mn(II) spectral intensity at 23 °C and only 45% at 5 °C.

Calculation of Li-Cr Distances in Complexes of Enzyme, Mg²⁺, and Pyruvate—The existence of two conformational states with, as Ash et al. (8) have shown, two different orientations for Li⁺ at the active site means that the observed relaxation rates for Li⁺ will contain a contribution from each species. Expressed in terms of the observed 1/T₁Li:

\[ 1/T_{\text{obs}} = p_1/T_{\text{M(1)}} + p_2/T_{\text{M(2)}} \]

where p₁ and p₂ are the fractional populations of the two species (i.e., p₁ + p₂ = 1 and 1/T_{M(1)} and 1/T_{M(2)} are the characteristic relaxation rates of the two bound forms. Since the populations of the two forms are altered by varying the temperature, we can use the observed relaxation rates to calculate the values of 1/T₁M(1) and 1/T₁M(2) for the enzyme-Li-Cr(H₂O)₆ATP-pyruvate complex with and without Mg²⁺. Appropriate values of 1/T₁M for the enzyme complexes with Li⁺ and Cr(H₂O)₆ATP are shown in Table II.

The calculation of Li-Cr distances in these complexes re-
plexes, Gupta et al. (5) have described interactions between Mn2+ and Co(NH3)ATP on pyruvate kinase. In particular, they have shown that Mn2+-Co(NH3)4ATP is prone to small amounts of spontaneous breakdown, in which Co(III) is reduced to paramagnetic Co(II) over periods similar to those required for nuclear relaxation measurements with paramagnetic probes (26). It is possible that the higher values of 1/T1m (and consequently smaller values for Mn-P distances) obtained in the previous study (25) are a result of reduction of Co(III) with time in those experiments.

In the studies presented below, all solutions of Co(NH3)4ATP were bubbled gently with O2 just prior to NMR measurements, and each relaxation time measured here was obtained on a freshly prepared sample of Co(NH3)4ATP. As we have shown, these procedures effectively prevent the reduction of Co(III) to Co(II) (27), although more extensive bubbling eventually causes broadening of the 31P resonances.

As shown in Fig. 5, titration of solutions of CoATP results in increases in 1/T1 of the 31P resonances of the nucleotide in proportion to the concentration of added Mn2+. In order to determine a lower limit of 1/T1 for the Li-Cr(III) interactions described here. Taking into account the frequency dependence of water proton relaxation rates in various enzyme-Cr(H2O)4ATP complexes, Gupta et al. (5) determined values of T1 of 1.2-2.3 × 10^{-10} s. Moreover, EPR spectra of free Cr(H2O)4ATP show a line at g = 2.0 with a peak to peak line width of 650 G, setting a lower limit of 1 × 10^{-10} s on the electron spin relaxation time, T1, of Cr(H2O)4ATP. Since the dominant contribution to T1 for H2O-Cr(III) interactions on pyruvate kinase is almost certain to be T1, the same correlation time is likely to pertain for the Li-Cr(III) interactions described here. Taking the values for 1/T1m and the value for T1 shown in Table II, we obtain the distances for the various Li-enzyme complexes shown in Table II.

31P Nuclear Relaxation Studies of the Mn2+-Co(NH3)4ATP Complex—In order to examine the interactions between Mn2+ and Co(NH3)4ATP on pyruvate kinase, we first considered the binary Mn2+-Co(NH3)4ATP complex. 31P NMR studies of this complex have been described (25), but we have obtained results which differ significantly from the previous study. As we have recently shown, it is possible that the higher values of 1/T1m (and consequently smaller values for Mn-P distances) obtained in the previous study (25) are a result of reduction of Co(III) with time in those experiments.

In the studies presented below, all solutions of Co(NH3)4ATP were bubbled gently with O2 just prior to NMR measurements, and each relaxation time measured here was obtained on a freshly prepared sample of Co(NH3)4ATP. As we have shown, these procedures effectively prevent the reduction of Co(III) to Co(II) (27), although more extensive O2 bubbling eventually causes broadening of the 31P resonances.

As shown in Fig. 5, titration of solutions of CoATP results in increases in 1/T1 of the 31P resonances of the nucleotide in proportion to the concentration of added Mn2+. In order to...
NMR Studies of Substrate and Ion Sites on Pyruvate Kinase

Fig. 4. Electron paramagnetic resonance spectra at 23 °C of pyruvate kinase complexes with K⁺ and Li⁺. Solutions contained 3 mM pyruvate kinase, 50 mM TMA/Hepes, pH 7.5, 100 mM TMA/Cl, 0.9 mM MnCl₂, and A, 60 mM KCl; B, 60 mM KCl, 21 mM pyruvate; and C, 50 mM Li⁺, 21 mM pyruvate.

Fig. 5. The effect of Mn(II) on the longitudinal relaxation rate of the phosphorus nuclei of Co(NH₄)₂ATP at 40 MHz (c) and 145 MHz (b, w, d, w, w). The solutions contained 20 mM Co(NH₄)₂ATP and 80 mM Tris, pH 7.5. Titrations at 145 MHz are shown for the α-P (β-isomer (w), and A-isomer (w)), β-P (Δ-isomer (w)), and γ-P (w) resonances.

Examine the frequency dependence of the Mn²⁺-induced relaxation of the phosphorus nuclei of Co(NH₄)₂ATP, titrations were performed both at 40 and 145 MHz. Since the Pₐ resonances of the Δ and A isomers of CoATP overlap at both these frequencies, it is not possible to compare relaxation parameters for the Δ and A diastereomers. On the other hand, the resonances for the Pₐ and P₇ nuclei of the two isomers are sufficiently separated at 145 MHz to extract relaxation parameters for each of the Δ and A isomers. The assignments of the resonances shown in Fig. 6 were made (26) on the basis of two-dimensional chemical shift correlation NMR measurements and analysis of reaction mixtures containing CoATP, glucose, and hexokinase, an enzyme known to be specific for the Δ diastereomer of metal-ATP complexes. As can be seen in Table III, the Pₐ of the A isomer of CoATP shows a substantially larger paramagnetic interaction with Mn²⁺ than is the case of the Δ isomer.

Estimation of the Correlation Time, τc, for the Binary Mn²⁺-Co(NH₄)₂ATP Complex—The most direct method for determining the correlation time, τc, for the Mn²⁺-CoATP interaction is by measuring the frequency dependence of 1/T₁p. According to the Solomon-Bloembergen theory, significant dependence of 1/T₁p on frequency should be detectable if r is in the range of 1/ω₀. The ratio of 1/T₁M for Mn²⁺ at 40.48 MHz and 145.75 MHz (Table III) indicates that 1/T₁M for the Mn²⁺-CoATP complex is frequency dependent. The correlation time of 8.22 × 10⁻⁸ s is calculated using Equation 6 and assuming no frequency dependence of τc, itself. This value of τc can be thought of as an upper limit, due to the circumstances of the experiment. The longer time required for T₁ measurements at 40 MHz leaves open the possibility of a greater systematic error at 40 MHz due to reduction of Co(III) to Co(II). Also we have compensated for pulse imper-
fections which can lead to artificially short \( T_1 \) values at 145 MHz on our NMR spectrometer. On the other hand, such compensation is not possible on the JEOL PFT-100 spectrometer. Both these potential problems would lead to underestimates of \( T_1 \) values at 40 MHz, and the net effect would be an overestimate of \( \tau_r \).

**Determination of the Distances in the Binary Mn\(^{2+}\)-Co(NH\(_3\)_2)ATP Complex.** The bound-state longitudinal relaxation rates, \( 1/T_{1p} \), are calculated using a value of 15 mM for the dissociation constant of the Mn\(^{2+}\)-CoATP complex (28).

We have assumed in these calculations that free and bound CoATP are in fast exchange (i.e., \( r_m \ll T_1m \)). This is justified by a measurement of the paramagnetic contribution to the \( T_2 \) relaxation rate, \( 1/T_{2p} \), of \( 0.02 \times 10^{-4} \) s\(^{-1} \), which greatly exceeds \( 1/T_{1p} \) (354 s\(^{-1} \)) and which sets a lower limit on the exchange rate. Since resonances for the \( \alpha\)-P and \( \beta\)-P of CoATP are poorly resolved and strongly coupled at 40 MHz, individual relaxation rates could not be calculated at that frequency. Hence, the correlation time calculated for the \( \gamma\)-P is used for all the phosphorus resonances in the distance calculation.

The \( \Delta \) and \( \Delta \) diastereomers of CoATP have overlapping \( \gamma\)-P resonances, and the distance calculation at the \( \gamma\)-P is thus necessarily an average. The \( \Delta \) and \( \Delta \) isomers are sufficiently well resolved for the \( \alpha\)-P and \( \beta\)-P resonances on the other hand that separate distances can be calculated for these nuclei. As shown in Table III, the Mn\(^{2+}\)-\( \beta\)-P distances are the same for the two diastereomers while the Mn\(^{2+}\)-\( \alpha\)-P distances are clearly different. The distances calculated here are too large to permit inner sphere and probably even second sphere coordination of Mn\(^{2+}\) and the phosphates of CoATP. While this is somewhat surprising, it is interesting to compare our results with the \(^{13}\)C and \(^{15}\)N nuclear relaxation measurements of Levy and Dechter (29), who found a second metal site on AMP. Based on several Mn\(^{2+}\)-carbon and Mn\(^{2+}\)-nitrogen distances, they proposed inner sphere coordination of Mn\(^{2+}\) by the N-7 nitrogen of the adenine ring. Likewise, it has been found by Martin and Mariam (30) that simultaneous coordination of N-7 and \( \alpha\)-P of AMP does not occur.

**31P Nuclear Relaxation Studies of Co(NH\(_3\)_2)ATP in Ternary and Quaternary Complexes with Pyruvate Kinase.**—To determine the interaction of Mn\(^{2+}\) and the phosphorus nuclei of CoATP at the active site of pyruvate kinase, a series of \(^{31}\)P nuclear relaxation measurements similar to those with the binary complex were made. As for the studies with the binary complex, the NMR samples for the enzyme experiments were also bubbled with \( O_2 \) prior to NMR measurements. The presence of this small amount of added \( O_2 \) does not affect the activity of pyruvate kinase. The enzyme exhibits similar activities in the presence and absence of added \( O_2 \). The addition of Mn\(^{2+}\) to solutions containing pyruvate kinase and CoATP resulted in a gradual increase in the relaxation rate, \( 1/T_{1p} \), for all three phosphorus nuclei of CoATP (Fig. 7). In these experiments, it was not possible to saturate the enzyme with Mn\(^{2+}\); since at levels of 10-25 \( \mu \)M Mn\(^{2+}\), the \(^{31}\)P line widths had already begun to broaden to the point where line widths began to exceed the separation between lines so that individual resonances were too broad to resolve.

The presence of pyruvate kinase clearly enhances the relaxation rates of all three phosphorus nuclei of \( \beta\)-, \( \gamma\)-bidentate CoATP, demonstrating the formation of a ternary enzyme-Mn-CoATP complex. Similar enhancements of relaxation rates are also observed in solutions containing pyruvate (data not shown, but see Table IV). Although pyruvate kinase activity is specific for the \( \Delta \) diastereomer of the metal-ATP complex (31), increases in the relaxation rates of the \( \Delta \) diastereomer are also observed. CoATP does not bind tightly to pyruvate kinase and under the conditions of these experiments, it is present in both the binary and ternary (enzyme) complexes with Mn\(^{2+}\). The measured relaxation rates reflect a weighted average of the relaxation rates for these two complexes. Using the appropriate dissociation constants, one can calculate the contribution of the ternary complex to the observed paramagnetic relaxation rates. A dissociation constant of 15 \( \mu \)M is used for the binary Mn\(^{2+}\)-CoATP complex. Since \( \beta\)-, \( \gamma\)-bidentate CoATP and \( \beta\)-, \( \gamma\)-bidentate Cr(H\(_2\)O\(_3\))ATP can be assumed to display similar properties of binding with pyruvate kinase, the binding constants previously determined for Cr(H\(_2\)O\(_3\))ATP (6) are used here in the calculations for the CoATP complexes. The values of \( T_{1m} \) obtained from these calculations for the ternary enzyme-Mn-CoATP and quater-
nary enzyme-Mn-CoATP-pyruvate complex are given in Table IV.

**Estimation of Correlation Times for the Ternary and Quaternary Enzyme Complexes**—In order to provide estimates for the correlation times for the $^{31}$P-Mn$^{2+}$ interactions in the ternary and quaternary enzyme complexes, we have measured the $^{31}$P nuclear relaxation rates at two frequencies, 40.48 and 145.75 MHz (Table IV). In both cases, a significant inverse frequency dependence is observed. Equations 6 and 5, which assume no frequency dependence and maximal dependence of $\tau_r$ respectively, were used to calculate the two extreme values of $\tau_r$. The values obtained in this way are shown in Table IV, and can be compared with values previously obtained or estimated in other studies of pyruvate kinase-Mn-complexes (9-11, 32, 33).

**Determinations of Distances in the Ternary and Quaternary Enzyme Complexes**—The distances between enzyme-bound Mn$^{2+}$ and the $^{31}$P nuclei of bound CoATP can be calculated from Equation 2 and the values for $1/T_L$ (i.e. $1/T_{1M}$) obtained here, provided that the observed relaxation rates are not dominated by the exchange term in Equation 1. That this is not the case is shown in two ways: 1) values for $1/T_L$ are substantially greater than for $1/T_{1M}$ as shown in Table IV, the spin-spin relaxation rate for the $\gamma$-P of CoATP of 3.8 $\times$ $10^8$ s$^{-1}$ is 2 orders of magnitude greater than the longitudinal relaxation rate of 368 s$^{-1}$. 2) The longitudinal relaxation rates show a significant frequency dependence. As shown above, this is observed in the present case. Thus the assumption of the fast exchange condition would seem to be valid in the present case.

The distances calculated for the ternary and quaternary enzyme complexes are shown in Table IV. The distances are consistent with a pyruvate-induced conformational change at the active site of pyruvate kinase. While such a conformational change has been observed previously, the effect of this transition on the conformation of ATP at the active site has not previously been determined.

**DISCUSSION**

The NMR studies described here are consistent with the many previous active site structural studies on pyruvate kinase. These data suggest that Li$^+$ at the monovalent cation site is 5.8 Å from Cr(III) at the ATP site, both in the presence and the absence of pyruvate. Addition of Mg$^{2+}$ increases this distance slightly, perhaps due to electrostatic repulsion. Previous studies of enzyme-Mg-pyruvate-Cr(H$_2$O)$_6$ATP complexes indicate that the carboxyl and carbonyl carbons of pyruvate are 6.1 Å from Cr(III) at the active site. This distance is midway between the Li-Cr distances in the absence and presence of Mg$^{2+}$. The Li-Cr distance in the presence of Mg$^{2+}$ fits well into the model previously proposed (6) for the active site of pyruvate kinase, a portion of which involving Li$^+$ is shown in Fig. 8. Binding of pyruvate induces a conformational change which decreases the Li-Mn distance from 8.3 Å to 5.8 Å (1). It has been suggested that the function of the monovalent cation in the pyruvate kinase mechanism is to assist in the orientation of the carboxyl group of pyruvate during the phosphorl transfer reaction. This model is supported by comparison of Mn-pyruvate distances on the one hand, and Mn distances to T$_1^*$ (7), monomethyl ammonium ion (34), and Li$^+$ (1, 8-10). However, additional support for the binding of monovalent ions near the carboxyl carbon of bound pyruvate is provided by the present study.

The present studies also clarify a discrepancy between the Li$^+$-Mn$^{2+}$ distance calculated in the absence of pyruvate or P-enolpyruvate (1) and the analogous T$_1^*$-Mn$^{2+}$ separation reported previously (7). The Li$^+$-Mn$^{2+}$ distance we calculated previously of 11.0 Å was obtained assuming that the enzyme was saturated with Li$^+$ under the conditions of the NMR experiment, 50 mM Li$^+$ and 2.1 mM pyruvate kinase sites. Our value for the affinity of Li$^+$ for the enzyme in the absence of other substrates is 120 mM, a value substantially higher than the value we had obtained from kinetic studies of 11 mM. Applying the new $K_D$ for Li$^+$ to a recalculation of the distance yields a value of 8.3 Å for the Li$^+$-Mn$^{2+}$ separation. This value is essentially the same as the value measured from T$_1^*$ NMR, 8.2 Å (7).

The EPR studies of the "isotropic" and "anisotropic" forms of the enzyme-Mn$^{2+}$-Li$^+$-pyruvate complex are similar to those reported for the enzyme-Mn$^{2+}$-Li$^+$-(P-enolpyruvate) complex. In both cases, the so-called anisotropic or K$^+$-like form of the enzyme predominates at 23 °C, while the fraction existing in the isotropic form is increased as temperature is decreased. The data presented here indicate that pyruvate produces slightly more of the K$^+$-like conformation than does P-enolpyruvate (92% compared to 81% (8)).

The Mn$^{2+}$-P distances obtained in the present study for the binary Mn$^{2+}$-CoATP complex are larger than those previously measured (25). This would appear to be a consequence of reduction of Co(III) to paramagnetic Co(II), as we have described elsewhere (26). When we carried out the $^{31}$P NMR measurements with no precautionary treatments of our CoATP solutions, we obtained results essentially similar to the previous report. On the other hand, pretreatment of our Co(NH$_3$)$_2$ATP solutions either by O$_2$ bubbling or by addition of NH$_3$ to prevent and/or reverse Co(III) reduction, we obtain the results shown here. The results indicate that the Mn$^{2+}$ interaction with CoATP does not involve inner sphere or even outer sphere coordination of the phosphates of ATP. This may be due to electrostatic repulsion of Mn$^{2+}$ and Co$^{3+}$, and may provide an explanation for the weak association between Mn$^{2+}$ and CoATP ($K_D \geq 15$ mM (28)). A more likely coordination site for a second metal in this type of complex has been suggested by Levy and Dechter (29), who have determined from $^{23}$C and $^{23}$N relaxation studies that the second metal in a MnATP complex is coordinated to the N-7 nitrogen of the adenine ring on ATP. Proton NMR studies of the Mn$^{2+}$-CoATP could possibly clarify this point.

The $^{31}$P nuclear relaxation studies of the ternary and quaternary pyruvate kinase complexes provides the opportunity for several comparisons of active site structure. For example,
it has not previously been possible to examine the quaternary complex of enzyme, Mn\(^{2+}\), ATP, and pyruvate, since this complex is a functional one, and breaks down to products much faster than the time required for NMR measurements. In the present case, using CoATP, which is not a functional substrate for pyruvate kinase, we have been able to examine the effect of pyruvate on the conformation of the ternary enzyme-Mn\(^{2+}\)-CoATP complex. As seen in Table IV, pyruvate has been used ATP and oxalate. Since neither of these complexes is a functional one, it is difficult to say which of these represents the presence of a second metal (coordinated to one-metal complex (using ATP instead of CoATP) has been clearly defined. It would be interesting in this context to use the value of \(\tau_e\) to be used for distance calculations. The data of Sloan and Mildvan (11) were obtained at 40.5 MHz, while our studies were performed at 40.5 and 145.75 MHz. The value of \(\tau_e\) we obtain from the frequency dependence of \(1/T_{1P}\) of phosphorus, assuming no frequency dependence of \(\tau_e\), of 2.3 \(\times\) \(10^{-9}\) s is a reasonable one to use for comparison of the two data sets. Recalculation of the data for the enzyme-Mn\(^{2+}\)-ATP complex using this value for \(\tau_e\) gives Mn-P distances of 6.4, 6.3, and 6.1 Å for the \(\alpha\), \(\beta\), and \(\gamma\)-phosphorus nuclei of ATP. These values are well within the limits of error for our values in the pyruvate kinase-Mn\(^{2+}\)-CoATP-pyruvate complex. If any conclusion can be drawn from this comparison, it would be that any changes in the conformation of ATP due to Co(III) coordination are masked by the error limits in the calculations, at least a part of which is the uncertainty regarding the appropriate value of \(\tau_e\). Regardless of the choice of a correlation time, there would appear to be a small effect of Co(III) on the conformation of ATP which we may infer by considering each set of data independently. If only relative distances are considered, the errors in the distances in Table IV may be considered to be quite small. If the error in \(1/T_{1P}\) is \(\pm 10\%\), then the relative errors in the calculated distances, due to the sixth root dependence of Equation 2, are \(\pm 1.6\%\). The distances we calculate in Table IV are thus consistent with a bent conformation for the triphosphate moiety of CoATP. The data for the enzyme-Mn-ATP complex in the absence of the second metal are consistent either with an extended conformation of the triphosphate chain or with a rotation of the chain so that the plane defined by the bent chain is perpendicular to the Mn-P vectors. In either case, it is clear that the coordination of Co(III) effects a significant change in the conformation of the triphosphate chain of bound ATP. This conclusion is drawn from consideration of relative distances only, and thus is independent of one’s choice of a correlation time.

\[f(\tau_e)\text{, on the other hand, } \tau_e \text{ itself can be frequency dependent, and thus the estimation of } \tau_e \text{ by measurement of the frequency dependence of } 1/T_{1P} \text{ is best performed in the frequency range in which the value of } \tau_e \text{ is to be used for distance calculations. The data of Sloan and Mildvan (11) were obtained at 40.5 MHz, while our studies were performed at 40.5 and 145.75 MHz. The value of } \tau_e \text{ we obtain from the frequency dependence of } 1/T_{1P} \text{ of phosphorus, assuming no frequency dependence of } \tau_e \text{, of } 2.3 \times 10^{-9} \text{ s is a reasonable one to use for comparison of the two data sets. Recalculation of the data for the enzyme-Mn}^{2+}-\text{ATP complex using this value for } \tau_e \text{ gives Mn-P distances of 6.4, 6.3, and 6.1 Å for the } \alpha\text{-, } \beta\text{-, and } \gamma\text{-phosphorus nuclei of ATP. These values are well within the limits of error for our values in the pyruvate kinase-Mn}^{2+}\text{-CoATP-pyruvate complex. If any conclusion can be drawn from this comparison, it would be that any changes in the conformation of ATP due to Co(III) coordination are masked by the error limits in the calculations, at least a part of which is the uncertainty regarding the appropriate value of } \tau_e\text{. Regardless of the choice of a correlation time, there would appear to be a small effect of Co(III) on the conformation of ATP which we may infer by considering each set of data independently. If only relative distances are considered, the errors in the distances in Table IV may be considered to be quite small. If the error in } 1/T_{1P} \text{ is } \pm 10\%\text{, then the relative errors in the calculated distances, due to the sixth root dependence of Equation 2, are } \pm 1.6\%\text{. The distances we calculate in Table IV are thus consistent with a bent conformation for the triphosphate moiety of CoATP. The data for the enzyme-Mn-ATP complex in the absence of the second metal are consistent either with an extended conformation of the triphosphate chain or with a rotation of the chain so that the plane defined by the bent chain is perpendicular to the Mn-P vectors. In either case, it is clear that the coordination of Co(III) effects a significant change in the conformation of the triphosphate chain of bound ATP. This conclusion is drawn from consideration of relative distances only, and thus is independent of one’s choice of a correlation time.}

\[\text{REFERENCES}\]


\[\text{TABLE V}\]

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
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<tr>
<td>Sloan and Mildvan</td>
<td>Frequency dependence for H of ATP</td>
<td>5 (\times) (10^{-10})</td>
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<td>James et al. (32)</td>
<td>Frequency dependence of water protons</td>
<td>7.26 (\times) (10^{-10})</td>
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<td>Raushel and Villafranca (9, 10)</td>
<td>Comparison of (4^2Li) and (4^2Li)</td>
<td>37 (\times) (10^{-10})</td>
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<tr>
<td>Reuben and Cohn (33)</td>
<td>Frequency dependence of water protons</td>
<td>94 (\times) (10^{-10})</td>
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NMR Studies of Substrate and Ion Sites on Pyruvate Kinase