The monovalent cation site of pyruvate kinase and other enzymes: NMR investigations[1,2]

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ABSTRACT

Several monovalent cations have ideal physical properties for investigation of their interactions with biomacromolecules by NMR techniques. $^{7}Li^{+}$, $^{14}NH_4^{+}$, and $^{133}Cs^{+}$ have high natural abundance, and enriched samples of $^{6}Li^{+}$ and $^{15}NH_4^{+}$ are easily obtained. All of these nuclei share the common property of having narrow line widths, thereby providing high sensitivity for NMR experiments. Numerous $^{23}Na^{+}$ NMR studies have been reported but $^{39}K^{+}$, $^{85}Rb^{+}$, and $^{87}Rb^{+}$ have not been studied extensively because these nuclei have low sensitivity and broad line widths. Recently NMR studies of monovalent cations have been extended to explore the monovalent cation sites of enzymes activated by such cations. With pyruvate kinase we determined that the extent of activation elicited by monovalent cations correlated well with the distance between the monovalent and divalent cations (structural site) sites. Those ions that were the best activators were closest to the M^{2+} site. In the course of this investigation we developed a general method to determine correlation times for dipolar relaxation between monovalent cation sites and paramagnetic centers on enzymes, and we have extended this work to other enzymes that are activated by monovalent cations.—**Villafranca, J. J.; Raushel, F. M.** The monovalent cation site of pyruvate kinase and other enzymes: NMR investigations. *Federation Proc.* 2961–2965; 1982.

Several enzymes display a requirement for the addition of a monovalent cation for maximal activity. Suelter (16) in 1970 described several classes of enzymes that were activated by monovalent cations. He concluded that a common theme existed among them, i.e., reactions in which a keto-enol tautomerization was a likely event all involved activation by monovalent cations. The role of the monovalent cation in these reactions was supposedly to stabilize the enolate anion for further reaction through this species. Although this is an attractive idea it suffers largely from lack of experimental proof to verify enzyme-bound enolate-cation species.

Within this class of enzymes activated by monovalent cations are several that catalyze phosphoryl transfer reactions. Perhaps the best studied is pyruvate kinase whose reaction is

$$\text{ATP} + \text{pyruvate} \overset{M^{2+},\,M^{+}}{\rightleftharpoons} \text{ADP} + \text{phosphoenolpyruvate} \quad (1)$$

The enzyme is a tetramer and the role of the divalent cation is firmly established as coordinating to the ATP and enzyme during catalysis (3). The role of the monovalent cation is less clear. On substitution of Mn^{2+} for the physiologically important Mg^{2+} in this reaction, a paramagnetic probe is introduced at the active site of the enzyme and several structural experiments suggest themselves. NMR studies of the paramagnetic influence of Mn^{2+} on relaxation rates of several nuclei of substrates allow distances to be determined among these nuclei (8). This can also be applied to measure the distance between the monovalent and divalent cation sites, and this application was studied in our laboratory. In brief, discussion of the NMR properties of various monovalent cations follows along with our experimental data on two enzyme systems.

PROPERTIES OF MONOVALENT CATIONS

Table 1 lists monovalent cations of the alkali metal series. All are observable by NMR spectroscopy and all have a nuclear spin greater than ½. These nuclei therefore possess a quadrupole moment Q, which affects their relaxation properties and hence their use in high-resolution NMR studies with enzymes. The table gives the quadrupole moment of each nucleus, its natural abundance, and NMR sensitivity relative to the natural abundance of ^{13}C (1.1%) to provide a benchmark for comparison. The U.S. government removes the ^{6}Li from most commercially available lithium salts so the only practical way to obtain ^{6}Li is from a government laboratory such as Oak Ridge. An important point to notice in this table is that, with the possible exception of ^{39}K (assuming enriched ^{6}Li is used by the investigator), all nuclei are easily observed in standard multinuclear NMR spectrometers (at reasonable concentrations of 1–100 mM).

Table 1 also presents the line widths obtained for dilute samples of each of

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TABLE 1. Properties of monovalent cations[a]

Nucleus	Spin	Q	Natural abundance	NMR sensitivity, relative to ^{13}C	Line width, Hz, 25 C
^{6}Li	1	0.00046	7.42[b]	3.58	0.002
^{7}Li	$^{3}/_{2}$	−0.042	92.58[b]	1540	0.01
^{23}Na	$^{3}/_{2}$	0.11	100	525	4.5
^{39}K	$^{3}/_{2}$	0.055	93.08	2.69	4.8
^{85}Rb	$^{5}/_{2}$	0.247	72.15	43	130
^{87}Rb	$^{3}/_{2}$	0.12	27.85	277	140
^{133}Cs	$^{7}/_{2}$	−0.003	100	269	0.02

[a] Data from ref 7. [b] U.S. government removes the ^{6}Li.

these ions. An equation describing the factors that contribute to the line width is

$$\text{Line width (Hz)} \propto \frac{1}{T_2}$$

$$\propto \left[\frac{2I+3}{I^2(2I-1)}\right]Q^2A^2 \quad (2)$$

Equation 2 is from a theoretical treatment by Hertz (4) and is presented here in simplified form to stress the factors that contribute to the line width of a quadrupolar nucleus. Q is the aforementioned quadrupole moment; this value enters the equation as the square and an increase in Q contributes significantly to the overall line width. The expression for I, the nuclear spin, actually predicts that the line width will be narrower the larger this value becomes. This is contrary to most expectations. The term that contributes most significantly in *Eq. 2*, however, is A, the Sternheimer antishielding factor, which also enters as the square. A thorough list of these values can be found in a chapter by Lindman and Forsén (7), but two examples will be given here.

Both ^{7}Li and ^{85}Rb have $I = {}^{3}/_{2}$, but the line widths of these two cations differ by $\sim 10^4$. The difference in Q accounts for only a factor of ~35 in the line width. The critical difference is the Sternheimer antishielding factor, which is ~0.74 for $^{7}Li^+$ and ~48 for $^{85}Rb^+$. This factor alone accounts almost totally for the difference in line width and the Sternheimer antishielding factor increases as one goes down a column of the periodic table. Incidentally, the narrow line width for $^{133}Cs^+$ arises from a small value of Q and a large value of I which offsets the large value of A (111 for $^{133}Cs^+$).

PYRUVATE KINASE

Several studies have been conducted to determine how the specific activity of pyruvate kinase varies as a function of monovalent cation. Kayne (6) determined that K^+ stimulated pyruvate kinase to the greatest extent whereas ions such as Li^+ and Cs^+ gave only a few percent of the activity of K^+. Figure 1 shows a correlation of percent activity with crystal ionic radius for Li^+ (0.68 Å), Na^+ (0.97 Å), K^+ (1.33 Å), NH_4^+ (1.43 Å), Rb^+ (1.47 Å), Tl^+ (1.47 Å), and Cs^+ (1.67 Å). Nowak (9, 10) has made a systematic study of mono-, di-, tri-, and tetramethylammonium ion as activators of pyruvate kinase and has shown that only monomethylammonium ion activates (~0.5% relative to K^+). These ions would be expected to be poor activators because of the bulky methyl substituent on nitrogen.

As mentioned before, NMR measurements of the longitudinal relaxation rates ($1/T_1$) of monovalent cations present an opportunity to correlate structural data on pyruvate kinase with data on the activity caused by each monovalent cation. In an attempt to determine the exact location of the monovalent cation site relative to the sites for the other ligands of pyruvate kinase, a number of laboratories have undertaken measurements of the distance from enzyme-bound Mn^{2+} to the monovalent cation site by using NMR. This metal ion site is the structural site, not the metal-nucleotide site. Reuben and Kayne (15) have reported distances of 4.9 and 8.2 Å between $^{205}Tl^+$ and Mn^{2+} in the enzyme-Mn^{2+}-Tl^+-phosphoenolpyruvate (PEP) complex and the enzyme-Mn^{2+}-Tl^+ complex, respectively. In a ^{7}Li NMR study, Hutton et al. (5) reported distances of 5.8 and 11.0 Å for these enzyme-Mn^{2+} complexes with $^{7}Li^+$. Inasmuch as Li^+ activates only 3% as well as Tl^+, these authors proposed that the longer metal-metal distance for the Li^+ complexes compared with the Tl^+ complexes correlated with the large decrease in enzymatic activity. Ash et al. (1) pointed out that there are two interconvertible forms of the enzyme-Mn^{2+}-Li^+-PEP complex (14) that Hutton et al. did not take into account in their data analysis. Repeating the ^{7}Li NMR experiments, Ash et al. obtained distances between Mn^{2+} and Li^+ that are nearly identical with the Mn^{2+}-Tl^+ distances of Reuben and Kayne. However, the reason for the difference in the measured distances in these two ^{7}Li NMR studies is in the choice of a correlation time (τ_c) for the dipolar Mn^{2+}-Li^+ interaction. Hutton et al. used a value of 9.4 ns whereas Ash et al. used a value of 1.7 ns. These apparently disparate correlation times were obtained from a study of water proton relaxation rates. Nowak found distances between Mn^{2+} and the methyl protons of mono-, di-, tri-, and tetramethylammonium ion of 6.5, 7.8, 8.7, and 10.9 Å, respectively, in enzyme complexes with PEP (10).

We expanded the list of monovalent cations studied for pyruvate kinase by NMR to include $^{6}Li^+$, $^{7}Li^+$, $^{14}NH_4^+$, $^{15}NH_4^+$, $^{23}Na^+$, $^{39}K^+$, $^{85}Rb^+$, $^{87}Rb^+$, and $^{133}Cs^+$ (13). Spin-lattice relaxation times (T_1) for the various monovalent cations were determined by using a 180°-τ-90° pulse sequence with a Brüker WP-200 multinuclear NMR spectrometer. A few experiments with $^{15}NH_4^+$ and $^{7}Li^+$ were also performed with a JEOL PS-100 NMR spectrometer and the Brüker WM-360. The values for the spin-lattice relaxation times of the various monovalent cations in the presence of different solutions are shown in Table 2. All experiments were performed at 30 C. As can be seen from Table 2, substantial effects on the spin-lattice relaxation times of $^{6}Li^+$, $^{7}Li^+$, $^{14}NH_4^+$, $^{15}NH_4^+$, and $^{133}Cs^+$ are caused by the addition of Mn^{2+}-pyruvate kinase to 100 mM solutions of

Figure 1. Correlation of percent activity of pyruvate kinase with crystal ionic radius of various monovalent cations.

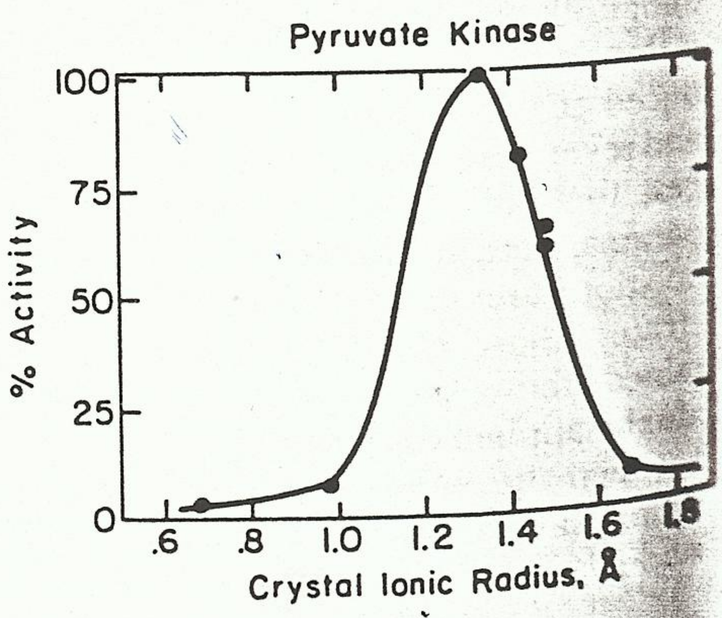


TABLE 2. NMR data for various monovalent cations[a]

Nucleus	Frequency, MHz	T_1 values			
		Buffer	Mg-PEP-enzyme	Mn^{2+}-PEP-enzyme	Mn^{2+}-enzyme
$^{6}Li^{+}$	29	151	93	2.0	17
$^{7}Li^{+}$	78	15.1	10.8	0.79	4.4
$^{133}Cs^{+}$	26	10.3	7.4	2.1	4.5
$^{14}NH_4^{+}$	14	0.51	0.52	0.32	0.41
$^{15}NH_4^{+}$	10	42	41	0.53	6.8
$^{23}Na^{+}$	53	0.043	0.041	0.040	0.041
$^{39}K^{+}$	9	0.037	0.022	0.018	—
$^{87}Rb^{+}$	65	0.0018	0.0017	0.0017	—

[a] Data from ref 13.

these ions. In agreement with the results of Reuben and Kayne (15), Ash et al. (1), and Hutton et al. (5), there is a substantially larger effect on the T_1 values in the enzyme-Mn^{2+}-M^{+}-PEP complex than in the enzyme-Mn^{2+}-M^{+} complex. This is consistent with PEP's causing the Mn^{2+}-M^{+} distance to shorten significantly.

The data in Table 2 can be used to compute distances between Mn^{2+} and the various monovalent cations. However, a problem associated with using NMR data to compute internuclear distances between paramagnetic centers and ligands bound to a macromolecule is the determination of the correlation time τ_c for the electron-nuclear interaction (2). Practice has shown that the dipolar electron-nuclear interaction dominates the relaxation processes for several paramagnetic species and that relaxation can be described by the Solomon-Bloembergen equation (2). The simplified form of this equation applicable to longitudinal relaxation data obtained with paramagnetic centers and $\tau_c \geq 10^{-10}$ s is given by *Eq. 3*

$$r = C\left[T_{1M}\frac{3\tau_c}{(1+\omega_I^2\tau_c^2)}\right]^{1/6} \qquad (3)$$

where r is the electron-nuclear distance, C is a collection of constants whose value depends on the spin of the paramagnetic center and the gyromagnetic ratio of the nucleus, T_{1M} is the spin-lattice relaxation time attributable to the influence of the paramagnetic species, and ω_I is the nuclear Larmor precession frequency. This treatment also assumes fast exchange conditions and therefore that $1/T_{1p} = 1/T_{1M}$, where $1/T_{1p}$ is the observed relaxation rate corrected for diamagnetic effects.

Several methods have been described to estimate a value of τ_c for use in calculating distances: *1*) measurement of T_1 and T_2 values for the nuclei, *2*) a frequency dependence of T_1 of the nuclei, *3*) a frequency dependence of the relaxation rates of solvent water in the system, and *4*) measurement of the line width of the electron paramagnetic resonance spectrum of the paramagnetic species. Of these the measurement of the frequency dependence of T_1 of the nuclei under observation gives the best results, but of course requires that data be obtained using two spectrometers. Also for some ions, e.g., Mn^{2+} and Cu^{2+}, the relevant correlation time for the dipolar relaxation is the electron spin relaxation time that can itself be magnetic field-dependent, further complicating the data analysis.

It occurred to us that an unambiguous determination of the correlation time could be made by performing identical experiments with $^{6}Li^{+}$ and $^{7}Li^{+}$ at the same magnetic field strength, thus obviating many of the problems discussed above (12). A unique value of τ_c is obtained from the ratio of the observed T_{1p} (T_{1M}) values for $^{6}Li^{+}$ and $^{7}Li^{+}$, because in *Eq. 3* C and ω_I are known values for the two isotopes of lithium, and the distance r must be the same for both ions. Examination of a theoretical plot in Fig. 2 of the ratio of T_{1M} values for $^{6}Li^{+}$ and $^{7}Li^{+}$ at various magnetic field strengths versus a range of correlation times commonly found in enzyme-Mn^{2+} complexes shows that this method is very sensitive for the determination of τ_c values of $<10^{-8}$ s because in this range the T_{1M} values for $^{6}Li^{+}$ and $^{7}Li^{+}$ are quite different. The ratio of these T_{1M} values changes from $\sim$1 to $\sim$7 at all field strengths. Similar plots can also be constructed for the two isotopes of NH_4^{+} (^{15}N and ^{14}N) and Rb^{+} (^{87}Rb and ^{85}Rb).

Using the data in Table 2, the $1/T_{1M}$ values for the PEP-Mn^{2+}-enzyme complexes of $^{6}Li^{+}$ and $^{7}Li^{+}$ are 1.21×10^{3} and 2.89×10^{3} s^{-1}, respectively. From this ratio and *Eq. 3* for each isotope, a τ_c value of 3.7×10^{-9} s is calculated. Also using the relaxation data for $^{7}Li^{+}$ obtained at 78 and 39 MHz (Table 2) and the data of Hutton et al. at 24 MHz, a τ_c value of 3.4×10^{-9} s was obtained from the ratio of the slope to intercept in a plot of T_{1M} versus ω_I^2 (2).

Figure 2. Plot of the ratio of T_{1M} values for ^{6}Li and ^{7}Li versus correlation time. This plot derives from *Eq. 3*. The curves correspond to various magnetic field strengths with the frequency in megahertz for ^{1}H at each field strength given on the curves for ease of identification.

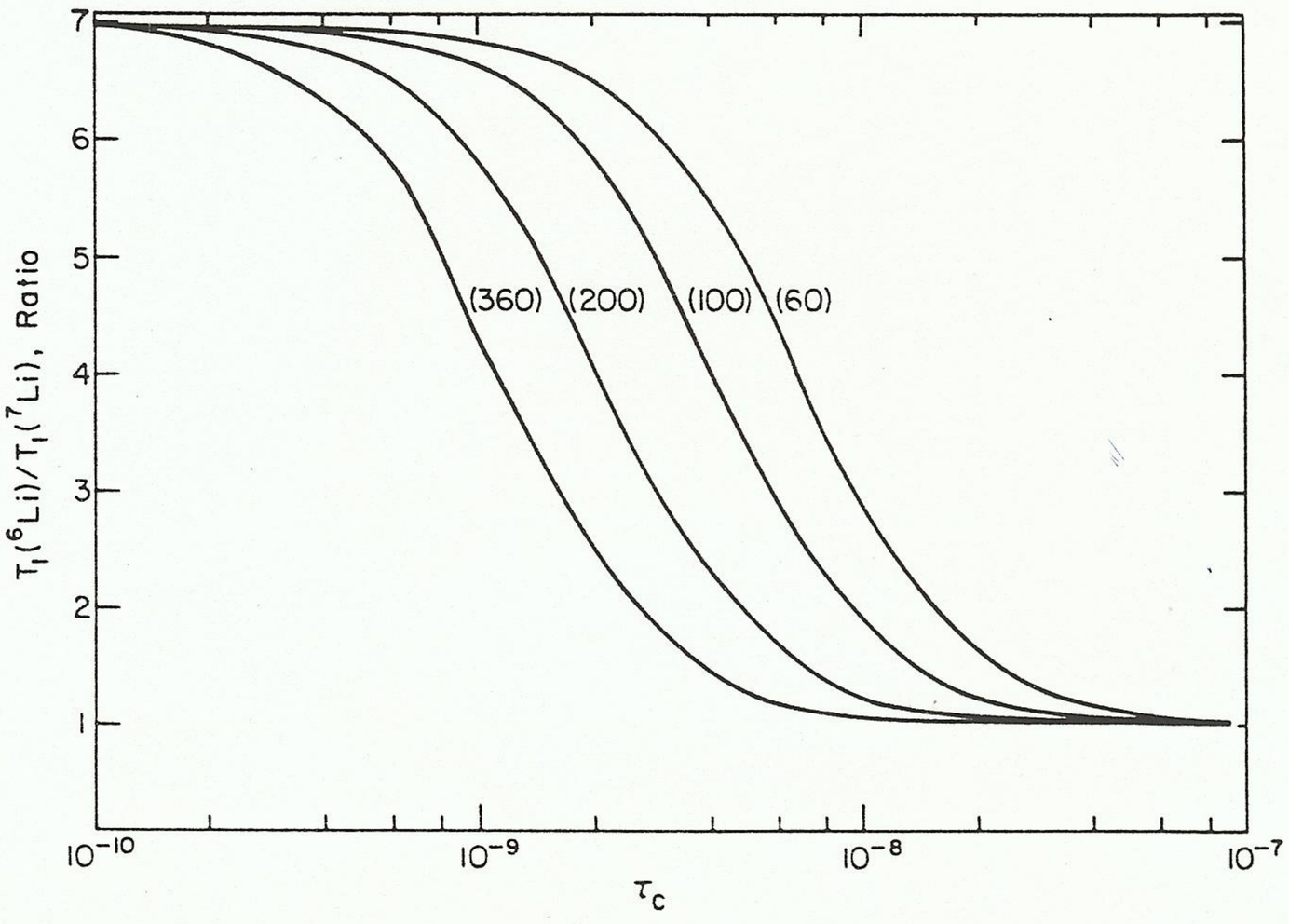


TABLE 3. Distances between Mn^{2+} and monovalent cations

Monovalent cation	E-Mn^{2+}-M^+ distance minus PEP, Å	E-Mn^{2+}-M^+ distance plus PEP, Å
Li^+ [a]	8.4 ± 0.4	5.7 ± 0.3
Na^+ [a]	—	≥4.5
Cs^+ [a]	7.7 ± 0.4	6.0 ± 0.3
Rb^+ [a]	—	≥4.1
NH_4^+ [a]	7.0 ± 0.4	4.4 ± 0.3
K^+ [a]	—	≥3.7
Tl^+ [b]	8.2 ± 0.5	4.8 ± 0.3
$CH_3NH_3^+$ [c]	8.7 ± 0.5	6.5 ± 0.3

[a] From ref 13. [b] From ref 15. [c] From ref 10.

Table 3 shows that reliable distances between Mn^{2+} and the monovalent cations were obtained from the present data for Li^+, NH_4^+, and Cs^+ whereas only lower limits for the distances to Na^+, K^+, and Rb^+ were obtained. There are two general observations to be made from the data in Table 3. The first is that there is a 2–3 Å change in the distance between Mn^{2+} and all the monovalent cations when PEP is added to the system. This is consistent with previous data. The second observation is that the cations that are better activators of the enzyme are also significantly closer to the Mn^{2+} in the complex with PEP than are the poorer activators. NH_4^+ and Tl^+ are 4.4–4.8 Å away whereas Li^+ and Cs^+ are 5.8–6.0 Å away. This suggests that the orientation of ligands on pyruvate kinase in the presence of the good monovalent activators is significantly different from that in the presence of the poor monovalent cation activators. Previous data by Nowak on monomethylammonium cation are also consistent with this idea because this cation produces about 0.5% the activity of K^+ and is 6.5 Å from Mn^{2+}. Figure 3 presents the results of the distance determinations thus far and a reasonable correlation between percent activity and Mn^{2+}-M^+ distance is demonstrated. Just how this structural change between metal ion sites alters the orientation of substrates at the active site is still unknown.

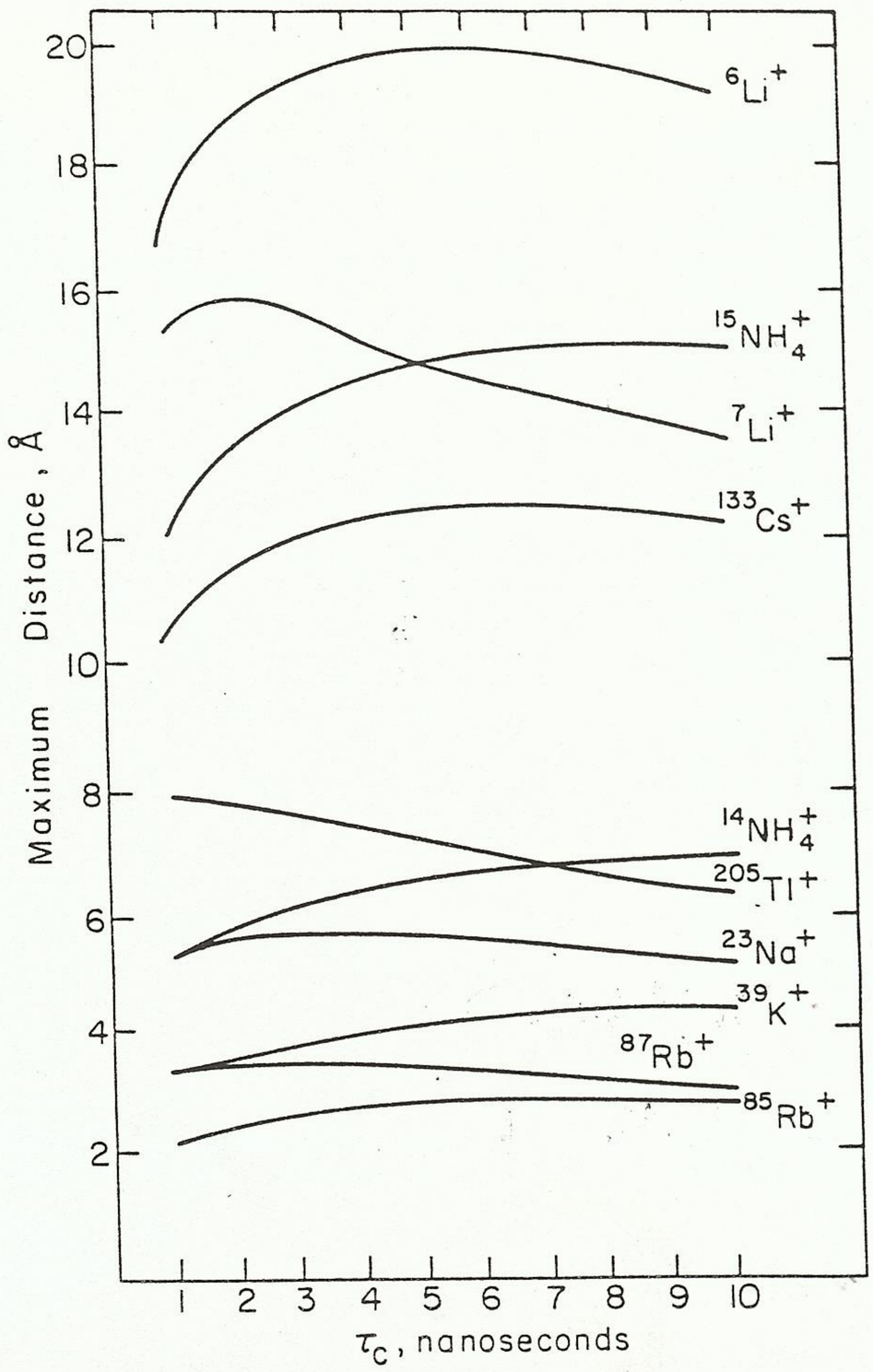
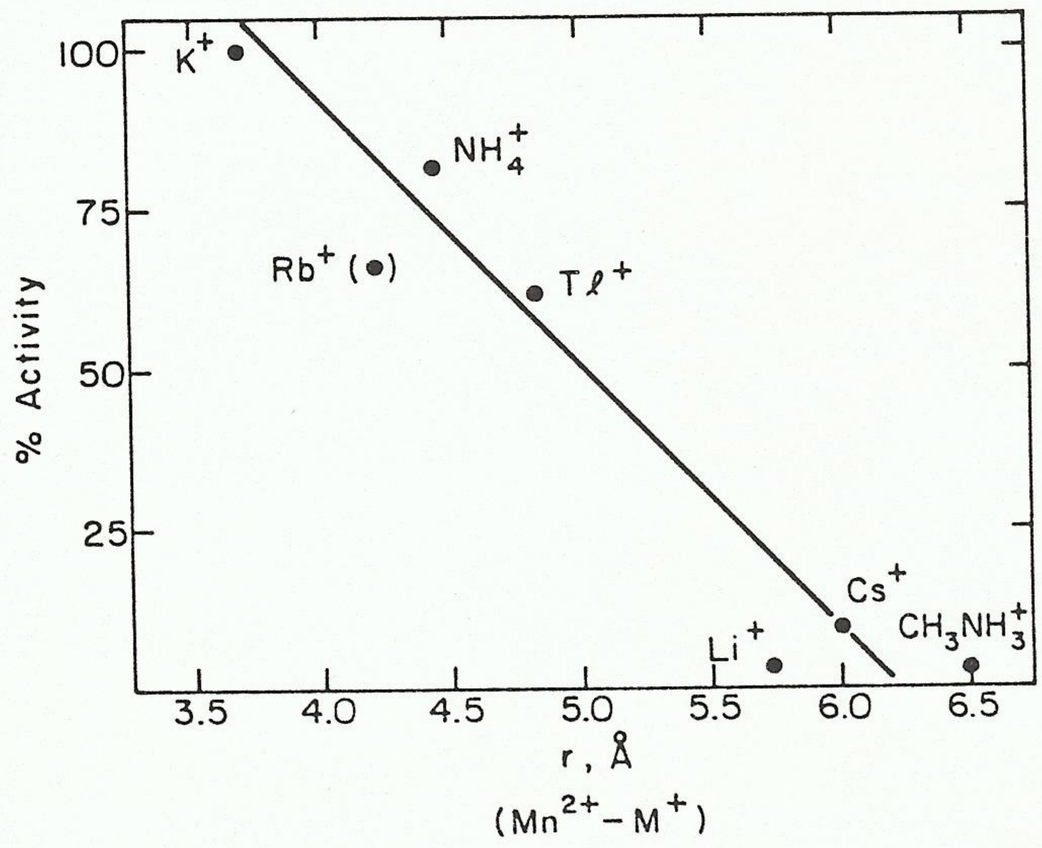


Figure 4. Plot of maximum distance from Mn^{2+} to various monovalent cations versus correlation time. Details are given in the text for the calculations based on *Eq. 3*.

Figure 3. Correlation of percent activity of pyruvate kinase with Mn^{2+} to monovalent cation distance derived from the NMR data.



CARBAMYL-PHOSPHATE SYNTHETASE

Experiments have just been initiated in our laboratory on structural studies of carbamyl-phosphate synthetase from *Escherichia coli*. The reaction catalyzed by this enzyme is

$$2ATP + HCO_3^- + \text{glutamine} \xrightarrow{M^{2+},\, M^+} 2ADP + P_i + \text{carbamyl-P} + \text{glutamate} \quad (4)$$

Recent work from our laboratory established that there is a structural site for divalent cations as well as the two metal ATP sites (11). In addition, a monovalent cation is required for activity.

Using an approach similar to that described for pyruvate kinase, we studied the effect of Mn^{2+} (bound at the structural metal ion site) on the longitudinal relaxation rates of $^{6}Li^{+}$, $^{7}Li^{+}$, and $^{15}NH_4^{+}$. From these experiments the $1/T_{1M}$ values in s^{-1} were 620 ($^{7}Li^{+}$), 170 ($^{6}Li^{+}$), and 170 ($^{15}NH_4^{+}$), respectively. From the ratio of $1/T_{1M}$ values for ^{6}Li and ^{7}Li a τ_c value of 3.2×10^{-9} s was obtained. Distances from Mn^{2+} to M^{+} were calculated by using *Eq. 3* and were 7.6 ± 0.4 Å (Mn^{2+} to Li) and 7.4 ± 0.5 Å (Mn^{2+} to NH_4^{+}). Further correlation of structure-reactivity parameters with this enzyme are under way to assess the influence of substrates and allosteric modifiers on the Mn^{2+}-M^{+} distance.

GENERAL APPLICABILITY OF THE NMR METHOD

One can evaluate the general applicability of the NMR method to calculate metal-metal distances in enzyme systems by computing a theoretical plot of maximum distance for each of the monovalent cations discussed in this paper. By using Mn^{2+} as our paramagnetic probe, a practical upper limit for an Mn^{2+}-M^{+} distance can be computed using the following assumptions: *1)* a change in T_1 of the monovalent cations of 33% was observed on going from enzyme-Mg^{2+} to enzyme-Mn^{2+}, and *2)* a reasonable ratio of free to bound monovalent cation could be achieved experimentally. This ratio depends on the binding constant of enzyme for M^{+} for each cation. Figure 4 shows a theoretical plot (using *Eq. 3*) for maximum distance as a function of correlation time in the range usually found for Mn^{2+} bound to enzymes. For this plot a magnetic field of 47 kG was chosen (for use in *Eq. 3*).

The monovalent cations fall roughly into three groups from this theoretical analysis. $^{6}Li^{+}$, $^{7}Li^{+}$, $^{15}NH_4^{+}$, and $^{133}Cs^{+}$ can be used to measure distances from 12 to 20 Å with reasonable accuracy. $^{14}NH_4^{+}$, $^{205}Tl^{+}$, and $^{23}Na^{+}$ are useful only for shorter distances in the range of 5–8 Å whereas $^{39}K^{+}$, $^{85}Rb^{+}$, and $^{87}Rb^{+}$ are useful only below 4 Å. Another parameter that can influence the distance determinations is the choice of magnetic field strength. Longer distance can be measured by decreasing the field because greater paramagnetic effects are seen with Mn^{2+} under these conditions. If Cu^{2+}, Fe^{3+}, or a nitroxyl radical are the paramagnetic probes, the maximum distances are much shorter.

CONCLUSION

This paper has presented the basic properties of monovalent cations that possess quadrupole moments. Quite a few have ideal properties for high-resolution NMR studies with macromolecular systems of biochemical interest. The use of these cations in the study of metal-metal distances on two enzymes has been presented along with a theoretical analysis of the range over which these distances can be measured. The method is quite sensitive and accurate and should find applicability to several enzyme systems in the near future. FP

REFERENCES

1. **Ash, D. E.; Kayne, F. J.; Reed, G. M.** Temperature dependent conformational changes at the active site of pyruvate kinase. A ^{7}Li nuclear magnetic resonance study. *Arch. Biochem. Biophys.* 190: 571–577; 1978.
2. **Dwek, R. A.** *Nuclear magnetic resonance in biochemistry.* Oxford: Clarendon; 1973.
3. **Gupta, R. K.; Mildvan, A. S.** Structures of enzyme-bound metal nucleotide complexes in the phosphoryl transfer reaction of muscle pyruvate kinase. *J. Biol. Chem.* 252: 5967–5976; 1977.
4. **Hertz, H. G.** Magnetic relaxation by quadrupole interaction of ionic nuclei in electrolyte solutions. I. Limiting values for infinite dilution. *Ber. Bunsenges. Phys. Chem.* 77: 531–540; 1973.
5. **Hutton, W. E.; Stephens, E. E.; Grisham, C. M.** Lithium-7 nuclear magnetic resonance as a probe of structure and function of the monovalent cation site on pyruvate kinase. *Arch. Biochem. Biophys.* 184: 166–171; 1977.
6. **Kayne, F. J.** Pyruvate kinase. Boyer, P. D., ed. *The enzymes*, vol. 8. New York: Academic; 1973: 353–372.
7. **Lindman, B.; Forsén, S.** The alkali metals. Harris, R. K.; Mann, D. E., eds. *NMR and the periodic table.* New York: Academic; 1978: 129–181.
8. **Mildvan, A. S.; Sloan, D. L.; Fung, C. H.; Gupta, R. K.; Melamud, E.** Arrangement and conformations of substrates at the active site of pyruvate kinase from model building studies based on magnetic resonance data. *J. Biol. Chem.* 251: 2431–2434; 1976.
9. **Nowak, T.** Monomethylammonium ion as a magnetic resonance probe for monovalent cation activators. *J. Biol. Chem.* 248: 7191–7196; 1973.
10. **Nowak, T.** Conformational changes required for pyruvate kinase activity as modulated by monovalent cations. *J. Biol. Chem.* 251: 73–78; 1976.
11. **Raushel, F. M.; Rawding, C. J.; Anderson, P. M.; Villafranca, J. J.** Paramagnetic probes for carbamoylphosphate synthetase: metal ion binding studies and preparation of nitroxide spin-labeled derivatives. *Biochemistry* 18: 5562–5566; 1979.
12. **Raushel, F. M.; Villafranca, J. J.** A direct NMR method for the determination of correlation times in enzyme complexes involving monovalent cations and paramagnetic centers. *J. Am. Chem. Soc.* 102: 6618–6619; 1980.
13. **Raushel, F. M.; Villafranca, J. J.** A multinuclear nuclear magnetic resonance study of the monovalent-divalent cation sites of pyruvate kinase. *Biochemistry* 19: 5481–5485; 1980.
14. **Reed, G. H.; Cohn, M.** Electron paramagnetic resonance studies of manganese(II)-pyruvate kinase-substrate complexes. *J. Biol. Chem.* 248: 6436–6442; 1973.
15. **Reuben, J.; Kayne, F. J.** Thallium-205 nuclear magnetic resonance study of pyruvate kinase and its substrates. *J. Biol. Chem.* 246: 6227–6234; 1971.
16. **Suelter, C. H.** Enzymes activated by monovalent cations. *Science* 168: 789–795; 1970.