Kinetic and Magnetic Resonance Studies of Pyruvate Kinase. III. The Enzyme–Metal–Phosphoryl Bridge Complex in the Fluorokinase Reaction*

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ABSTRACT: The interactions of the substrate (F^-) and the product (FPO_3^{2-}) of the fluorokinase reaction with manganese and with the manganese–pyruvate kinase complex have been investigated. FPO_3^{2-} inhibits the pyruvate kinase reaction competitively with respect to phosphoenolpyruvate (PEP), $K_I = 3.4 \pm 1.0$ mM, but does not inhibit with respect to adenosine diphosphate (ADP). The enhancement (ϵ) of the proton relaxation rate of water was reduced from 33 in the binary E–Mn complex to 1.5 in the E–Mn–FPO_3 complex. The similar value of ϵ for the E–Mn–PEP complex, 2.2, suggests similar structures in the region of the manganese hydration sphere for the two ternary complexes. The dissociation constant K_3 of FPO_3^{2-} from the ternary E–Mn–FPO_3 complex (2.6 ± 0.3 mM), determined from titration with ϵ as the measured parameter, agrees with its K_I and approximately with the dissociation constant, K_1, of the binary Mn–FPO_3 complex (1.8 ± 0.7 mM). K_3 of the ternary complex with fluoride is an order of magnitude lower than the K_M of fluoride suggesting a preferred-order kinetic schema for the fluorokinase reaction. As measured by fluorine nuclear magnetic resonance, Mn^{2+} increased the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of fluoride. The addition of pyruvate kinase diminished these effects probably by competition for Mn^{2+}. Analogous experiments with fluorophosphate showed that Mn^{2+} also increases the $1/T_1$ and $1/T_2$ of the fluorine in this system. The addition of pyruvate kinase enhanced these effects by factors as much as 7.9 and 1.7, respectively, indicating the formation of an E–Mn–FPO_3 bridge structure. The addition of phosphoenolpyruvate to E–Mn–FPO_3 reversed the effect of E–Mn on the fluorine nucleus of FPO_3^{2-} by competition between FPO_3^{2-} and phosphoenolpyruvate for E–Mn. The distance between manganese and fluorine in the binary Mn–F in solution, determined from $1/T_1$, is 2.1 A, in agreement with crystallographic data. The manganese to fluorine distance in Mn–FPO_3 and E–Mn–FPO_3 in solution, determined from $1/T_1$, lies between 3.5 and 5.0 A; hence fluorophosphate is bonded to manganese through one or two oxygens. The rate of dissociation of Mn–FPO_3, determined from $1/T_2$, is increased on combination with pyruvate kinase due to an increase in the entropy of activation of this reaction. The rate of dissociation of E–Mn–FPO_3 (3.4×10^4 sec^{-1}) meets the necessary criterion for this complex to participate in the manganese-activated fluorokinase reaction, since it exceeds V_{max} by three orders of magnitude ($V_{max} = 18$ sec^{-1}). The rates of formation of Mn–FPO_3 and E–Mn–FPO_3 are too low to fit the general SN1-type coordination mechanism proposed by M. Eigen and K. Tamm (*Z. Elektrochem. 66*, 107 (1962)). Alternative mechanisms are proposed.

A study of the interaction of divalent cations (Mildvan and Cohn, 1965) and substrates (Mildvan and Cohn, 1966) with rabbit muscle pyruvate kinase using kinetic and magnetic resonance methods has provided suggestive evidence for an enzyme–metal–phosphoenolpyruvate bridge structure as an intermediate in the pyruvate kinase reaction (Mildvan and Cohn, 1966). The enzyme–metal linkage has been demonstrated directly by Mn^{2+} and Mg^{2+} binding studies using electron paramagnetic resonance (epr), proton relaxation rate (prr) (Mildvan and Cohn, 1965), the kinetic protection method (Mildvan and Leigh, 1964), and protein difference spectroscopy (Suelter and Melander, 1963). Dissociation constants from binding studies were in agreement with kinetically determined activator constants (Mildvan and Cohn, 1965; Mildvan and Leigh, 1964). The proposed linkage between the enzyme-bound metal and the substrate was based on indirect evidence consisting of the diminution by substrates of the enhanced effect of enzyme-bound manganese on the prr of water and the decreasing order of affinities for phosphoenolpyruvate shown by EMn > EMg > E, and for ATP[1] shown by EMn > EMg (Mildvan and Cohn, 1966).

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[1] Abbreviations used: PEP, phosphoenolpyruvate; ADP and ATP, adenosine di- and triphosphates; TFA, trifluoroacetic acid; T_1, longitudinal relaxation time; T_2, transverse relaxation time.

Because the phosphoryl donors (PEP and ATP) reduced the enhancements of the prr more than did the corresponding phosphoryl acceptors (pyruvate and ADP), it was suggested that the phosphoryl group undergoing transfer provided a ligand to the enzyme-bound divalent activator (Mildvan and Cohn, 1966).

Fluoride and fluorophosphate are the substrate and product, respectively, of the fluorokinase reaction (Flavin *et al.*, 1957), a side reaction catalyzed by pyruvate kinase (Tietz and Ochoa, 1958).

$$ATP^{4-} + F^- \xrightarrow[HCO_3^-]{Mn^{2+}, K^+} FPO_3^{2-} + ADP^{3-} \quad (1)$$

In the present investigation the interaction of F^- and FPO_3^{2-} with manganese and with the manganese–pyruvate kinase complex is examined by fluorine nuclear magnetic resonance (nmr), by kinetics, and by proton relaxation rate studies for the purpose of providing direct evidence for the existence of a short-lived enzyme–metal–fluorophosphate bridge structure.

Experimental Procedure

Materials

Pyruvate kinase was prepared from rabbit muscle by the method of Tietz and Ochoa (1958) or was purchased from Boehringer Mannheim Corp. In all, six preparations were used which ranged in specific activity from 78 to 100% of that reported for the recrystallized enzyme (Tietz and Ochoa, 1958). No significant differences in the experimental results were observed with the various preparations used. All other compounds used were reagent grade or of the highest purity commercially available as previously described (Mildvan and Cohn, 1965). Disodium monofluorophosphate (Na_2FPO_3) was purchased from K & K Laboratories, Inc., Plainview, N. Y. It was partially converted to the potassium salt by ion-exchange chromatography on Dowex 50. The K^+:Na^+ ratio, determined by atomic absorption spectroscopy, was found to be 1.23. Hence, this salt was considered to be the mixed salt $KNaFPO_3$. Potassium monofluorophosphate (K_2FPO_3) was purchased from the Ozark-Mahoning Co., Tulsa, Okla., and was purified by recrystallization (twice) from water–ethanol mixtures, as described by Hill and Audrieth (1950). The use of silver nitrate to remove pyrophosphate was omitted. Further purification of the K_2FPO_3 on Dowex 1 (Cl^-) did not significantly alter its metal binding properties suggesting that contamination by pyrophosphate was negligible. Stock solutions were assayed for FPO_3 and P_i as described by Flavin *et al.* (1957); approximately 8% of the phosphorus was found to be in the form of orthophosphate. The results of kinetic and magnetic resonance experiments obtained with all three preparations (Na_2FPO_3, $KNaFPO_3$, and K_2FPO_3) were indistinguishable.

Methods

Kinetic experiments were carried out using the coupled assay of Bücher and Pfleiderer (1955), as previously described (Mildvan and Cohn, 1965). Rates are expressed as turnover number (per minute) assuming a molecular weight of 237,000 (Warner, 1958) and that the enzyme has two active sites per mole (Reynard *et al.*, 1961) (Mildvan and Cohn, 1965).

Dissociation constants of binary (Mn–F, Mn–FPO_3, and Mn–HPO_4) complexes were determined by titration of 50 or 100 μM $MnCl_2$ with fluoride, fluorophosphate, or P_i, measuring the free manganese by epr (Cohn and Townsend, 1954) and the effect of manganese on the prr of water (Cohn and Leigh, 1962). The results were analyzed as previously described (Mildvan and Cohn, 1963) to give the dissociation constant K_1 of the 1:1 complexes and the enhancement of the effect of manganese (when bound) on the prr of water (ϵ_b) for each ligand F^-, FPO_3^{2-}, and P_i. Because of the low affinity of manganese for F^-, measurements had to be made in solutions of high ionic strength (0.2–0.3). Similar results were obtained whether the ionic strength was adjusted to constancy with KCl or KNO_3. Hence, no correction was necessary for the exceedingly weak binding of manganese by chloride ion (O'Sullivan and Cohn, 1966).

Dissociation constants (K_3) and enhancement parameters (ϵ_t) of ternary complexes were determined by titrations in which the concentrations of enzyme and substrate were varied at constant concentration of manganese (50 μM) and the prr of water was measured (Mildvan and Cohn, 1966). Enhancements of ternary complexes were calculated by extrapolation of the observed enhancement to infinite concentrations of substrate and then to infinite concentration of enzyme (procedure I) and conversely, by extrapolation to infinite concentration of enzyme and then to infinite concentration of substrate (procedure II). The dissociation constants, K_3 = ((EM)(S)/(EMS)), for F^- and FPO_3^{2-} were determined by the above procedures as well as by the concentration of substrate which produced half-maximal change in the enhancement of the prr at each enzyme concentration (procedure III). These analytical procedures were found to be the most convenient and accurate methods of treating prr data (Mildvan and Cohn, 1966).

Fluorine nmr spectra were recorded of 0.5-ml samples of 0.1–0.2 M solutions of KF, Na_2FPO_3, $KNaFPO_3$, or K_2FPO_3 containing 0.1–0.2 mM enzyme sites, 10–100 μM $MnCl_2$, and 0.05 M Tris-HCl (pH 7.5), using a Varian HA 60 instrument retuned to 56.4 Mcycles/sec. Heterogenous field lock was achieved with a minor modification. Normally an external sample of "doped" water is used for locking the magnetic field to the spectrometer radiofrequency. To approximate this mode of operation for fluorine resonance, an auxiliary crystal-controlled oscillator and frequency multiplier stages were constructed similar to the circuit used in the Varian V-4311 RF Unit which operated at a frequency of 60.0 MHz. The spectrometer power supply withstood the added power drain adequately. This modification allowed the reference sample to be supplied with radiofrequency energy at 60.0 MHz while the spectrometer V-4311 RF Unit operated at 56.4 MHz. Although this system is by no means ideal, in that the two oscillator frequencies are

TABLE I: Definitions and Values of Symbols Used in the Solomon–Bloembergen Equation.

Symbol	Definition	Units (cgs)	Numerical Value
T_{1M}	Longitudinal relaxation time of ligand in the first coordination sphere of metal ion	sec	
S	Electronic spin quantum number	—	$^5/_2$ (for Mn^{2+})
γ_I	Nuclear gyromagnetic ratio	rad·sec^{-1}·gauss^{-1}	2.520×10^4 (for ^{19}F)[a] 2.675×10^4 (for ^1H)[a]
h	Planck's constant	erg·sec	6.625×10^{-27}[b]
g	Electronic "g" factor	—	2.00 (for Mn^{2+})[b]
β	Bohr magneton	rad·sec^{-1}·gauss^{-1}	8.795×10^{6}[b]
r	Average electron-nuclear distance	cm	
τ_c	Dipolar correlation time	sec	$\sim 3.0 \times 10^{-11}$ ($Mn-H_2O$)[c]
τ_e	Hyperfine correlation time	sec	$\sim 1 \times 10^{-8}$ ($Mn-H_2O$)[d]
ω_I	Nuclear resonance frequency	rad·sec^{-1}	3.55×10^8 (for F at 14,092 gauss) 3.77×10^8 (for H at 14,092 gauss)
ω_s	Electron resonance frequency	rad·sec^{-1}	2.48×10^{11} (for 14,092 gauss)[a]
A	Isotropic hyperfine coupling constant	rad·sec^{-1}	2.96×10^8 for Mn–F from single crystals[e]

[a] Computed from table compiled by Bloom (1962). [b] From a table of physical constants compiled by DuMond and Cohen (1953). [c] Bloembergen and Morgan (1961). [d] Luz and Shulman (1965). [e] Shulman and Jaccarino (1957).

uncorrelated, we have found that under normal operation the frequency drift is less than 10 cycles/sec per day. In one experiment, a specially adjusted 56.4-Mcycles/sec frequency unit, kindly loaned by the Varian Corp., was used and gave similar results with a small improvement in signal-to-noise ratio.

The transverse relaxation time (T_2) was determined by measurements of line width at radiofrequency powers at least 5 decibels below saturation. T_2 is inversely proportional to the line width as given by the relationship (Pople *et al.*, 1959)

$$T_2 = \frac{1}{2\pi\Delta\omega} \tag{2}$$

where $\Delta\omega$ is the half-width of the resonance line at half-height.

The longitudinal relaxation time (T_1) was determined either by the direct method or by power saturation, using TFA containing 15 mM $Fe(NO_3)_3$ and 15% H_2O as a standard of known T_1 to calibrate the H_1 field (Pople *et al.*, 1959). The saturating radiofrequency power level, which is required to yield maximal signal amplitude, is inversely related to the longitudinal (T_1) and transverse (T_2) relaxation times as follows (Pople *et al.*, 1959)

$$\text{power at maximum signal amplitude} = \frac{1}{\omega^2 T_1 T_2} \tag{3}$$

where ω is the radiofrequency power expressed in terms of the frequency of nuclear precession. The value of ω in each experiment was determined from the TFA standard of known T_1 and T_2. The T_1 of the standard was measured by three independent methods which gave satisfactory agreement: (a) the pulsed nmr method (Carr and Purcell, 1954) at 24.3 Mcycles/sec (69 msec); (b) the direct method at 56.4 Mcycles/sec (69 msec); and (c) power saturation at 56.4 Mcycles/sec measuring ω by the ringing frequency (62 msec). The T_2 of the standard was determined by measurements of the line width as described above.

Analyses of T_1 and T_2 Data. TEMPERATURE DEPENDENCE OF T_{1p} AND T_{2p}. In an investigation of the temperature dependence of the contribution of a paramagnetic ion to the nuclear relaxation rates ($1/T_{1p}$ and $1/T_{2p}$), Luz and Meiboom (1964) found that in the temperature range where the relaxation rates of the coordinated ligands are greater than the difference in resonance frequencies between the free and coordinated ligands, $1/T_{1p}$ and $1/T_{2p}$ for an ion like Mn^{2+} are

$$1/T_{1p} = \frac{p}{\tau_M + T_{1M}} \tag{4}$$

$$1/T_{2p} = \frac{p}{\tau_M + T_{2M}} \tag{5}$$

$1/T_{1p}$ and $1/T_{2p}$ are the paramagnetic contribution to the longitudinal and transverse relaxation rates, respectively, τ_M is the residence time of a ligand in the first coordination sphere of the metal ion, T_{1M} and T_{2M} are the relaxation times in the first coordination sphere, and p is the ratio of the number of ligands in the first coordination sphere to the total number in the solution. Two regions of temperature fall within this range: (1) region A at high temperatures where the rate of ligand exchange is

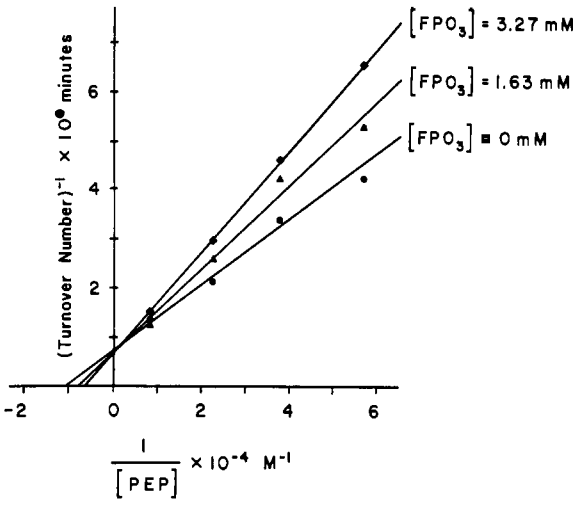


FIGURE 1: Double reciprocal plot of PEP concentration with respect to the initial velocity of the manganese-activated pyruvate kinase reaction at three concentrations of $NaKFPO_3$ showing competition between FPO_3^{2-} and PEP. Other components were 0.05 M Tris-HCl buffer (pH 7.5), 0.1 M KCl, 2 mM $MnCl_2$, 2.2 mM ADP, 150 μM reduced diphosphopyridine nucleotide, and 25 μg of crystalline lactic dehydrogenase in a total volume of 3.0 ml. The reaction was started by adding 4.9 μg of pyruvate kinase. Temperature = 25°. The reaction was followed spectrophotometrically at 340 mμ and the turnover number was calculated as described in Methods.

very rapid compared to $1/T_{1M}$ or $1/T_{2M}$, *i.e.*, $\tau_M \ll T_M$ and therefore, $1/T_{1p} = p/T_{1M}$ and $1/T_{2p} = p/T_{2M}$; and (2) region B at somewhat lower temperatures where the rate of ligand exchange is lower but still sufficiently fast to affect the relaxation of the bulk ligands by exchange, *i.e.*, $\tau_M > T_M$; in region B, τ_M dominates the observed relaxation rate. Regions A and B can be distinguished experimentally since in region A, $1/T_{1p}$ is a function of a correlation time (τ_c) (*vide infra*) and, therefore, has a negative temperature coefficient; on the other hand, in region B, $1/T_{1p}$ is a function of $1/\tau_M$ and, therefore, has a positive temperature coefficient as would be anticipated for the rate of a chemical reaction.

CALCULATION OF MANGANESE TO FLUORINE DISTANCES IN MnF, $MnFPO_3$, AND EMn–FPO_3 FROM T_1. The relaxation rates of a nucleus in the coordination sphere of a paramagnetic ion ($1/T_{1M}$ and $1/T_{2M}$) are formulated in the Bloembergen–Solomon equations (Solomon and Bloembergen, 1956; Solomon, 1955). The expression for the longitudinal relaxation rate consists of two terms, a dipolar term and a hyperfine term

$$\frac{1}{T_{1M}} = \frac{1}{r^6} \times \frac{2}{15} S(S+1)\left(\frac{h\gamma_I g\beta}{2\pi}\right)^2 f(\tau_c) + \frac{2}{3} S(S+1)A^2 j(\tau_e) \quad (6)$$

TABLE II: Dissociation Constants (K_1) and Enhancements (ϵ_a) of Prr of Binary Manganese Complexes.[d]

Ligand	Ionic Strength	K_1 (mM)	ϵ_a
Fluoride	0.2[a]–0.3[b]	400 ± 140	0.88 ± 0.07
Fluorophosphate	0.1[b]	1.8 ± 0.7	0.99 ± 0.04
Orthophosphate	0.1[b]	4.7 ± 0.6[c]	1.12 ± 0.02

[a] KNO_3. [b] KCl. [c] Corrected for pH (7.5) assuming the pK_2 of orthophosphate = 6.8. The observed K_1 was 5.6 ± 0.6 mM. Temperature is 25 ± 2°. [d] Conditions were as follows: 0.05 M Tris-HCl buffer (pH 7.5) and 50–100 μM $MnCl_2$; the ionic strength was adjusted with KCl or KNO_3 as discussed in the text.

where

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \quad (7)$$

and

$$j(\tau_e) = \frac{\tau_e}{1 + \omega_s^2\tau_e^2} \quad (8)$$

The definitions of the symbols and the numerical values of the constants are given in Table I. Evaluation of the terms in eq 6 from the values of the constants in Table I for manganese–fluorine interaction reveals that the second term contributes less than 0.3%. Hence, eq 6 may be simplified by neglecting the second term. The longitudinal relaxation rate, $1/T_{1M}$, is proportional to $1/r^6$, where r is the distance between the paramagnetic ion and the relaxing nucleus.

Results

Kinetic Competition between FPO_3^{2-} and PEP in the Pyruvate Kinase Reaction. FPO_3^{2-} is a simple competitive inhibitor of manganese-activated pyruvate kinase with respect to phosphoenolpyruvate as shown in Figure 1. The average value of K_I obtained from five experiments is 3.4 ± 1.0 mM.

At saturating levels of phosphoenolpyruvate (1 mM) and low levels of ADP (33–200 μM, K_M = 200 μM), no inhibition was observed of the pyruvate kinase reaction by 3 mM FPO_3, indicating that FPO_3^{2-} interacts only with the site for phosphoenolpyruvate and not with the site for ADP. Thus fluorophosphate is more specific than orthophosphate (with which it is isoelectronic) since orthophosphate competes with both phosphoenolpyruvate and ADP in the magnesium-activated

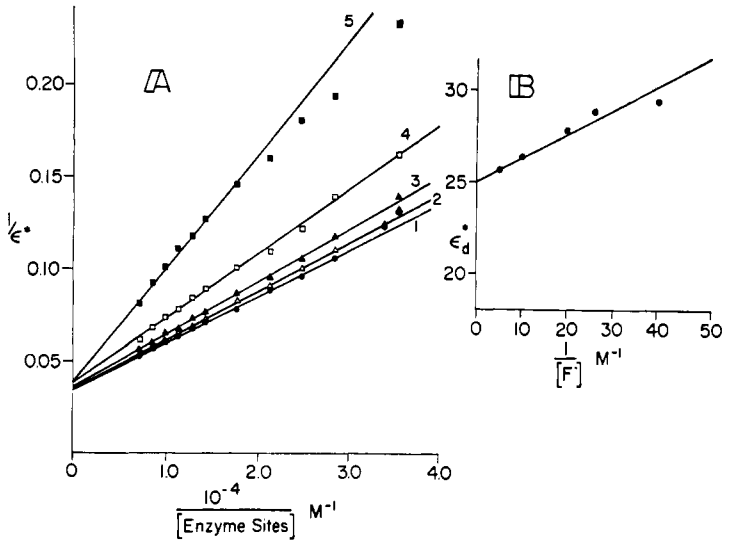


FIGURE 2: Fluoride interaction with E–Mn by prr. (A) Titration of $MnCl_2$ (50 μM) with pyruvate kinase at five concentrations of potassium fluoride. The reciprocal of the enhancement of the prr (ϵ^*) is plotted against the reciprocal of the concentration of enzyme sites for analysis according to procedure II (Mildvan and Cohn, 1966). The concentrations were 0.04 M Tris-HCl (pH 7.5); [KF] (mM), curve 1, 200; curve 2, 100; curve 3, 50; curve 4, 40; and curve 5, 25. [K^+] was held constant at 200 mM by additions of KCl. Temperature = 26°. (B) Secondary plot of the enhancements at infinite enzyme concentrations, ϵ^*_d, against the reciprocal of the fluoride ion concentration. From the intercept on the ordinate $\epsilon_t = 25$.

(Rose, 1960) and manganese-activated[2] pyruvate kinase reactions.

Dissociation Constants and Enhancements of the Prr of Water Protons of Binary Manganese–Substrate Complexes. Table II summarizes the dissociation constants (K_I) and the enhancement factors (ϵ_a) of the water protons of the binary MnF, Mn–FPO_3, and Mn–HPO_4 complexes as described in the sections on Methods. The stability constant of Mn–FPO_3 is two orders of magnitude greater than that of MnF and is similar to that of the Mn–HPO_4 complex suggesting that manganese is bound to oxygen(s) rather than to the fluorine of FPO_3^{2-}. Proof of this hypothesis is presented in a later section.

Fluoride produces a significant reduction of the effect of manganese on the prr of water. The value of ϵ_a is ~0.83 which is expected in a structure $Mn(H_2O)_5F$ in which five-sixths of the water molecules remains coordinated, provided the correlation time has not changed. This may be seen by the approximate relationship (Eisinger *et al.*, 1962)

$$\epsilon \doteq \frac{p^*\tau^*_c}{p\tau_c} \qquad (9)$$

where p is the mole fraction of exchangeable protons in the first coordination sphere of manganese and τ_c is the correlation time for the interaction between the electron spin of manganese and the nuclear spin of the protons The starred symbols refer to the complex. The ϵ_a value for Mn–FPO_3 and for Mn–HPO_4 suggest that τ_c has increased by ~20 and ~34%, respectively, in these complexes.

Dissociation Constants and Prr Enhancements of Ternary Enzyme–Manganese–Substrate Complexes. Manganese (50 mM) was titrated with variable concentrations of F^- at several levels of pyruvate kinase and the prr of each solution was measured. The observed enhancement was extrapolated to infinite enzyme and infinite substrate concentrations according to procedure II (Mildvan and Cohn, 1966) in Figure 2A,B.

A similar analysis of a titration of manganese with pyruvate kinase and $KNaFPO_3$ is shown in Figure 3A, B. The dissociation constants, K_3 = ((EM)(S)/(EMS)), and the enhancements of the ternary complexes (ϵ_t) are summarized in Table III and compared with kinetically determined constants. The K_3 value of FPO_3^{2-} from prr data is seen to be in good agreement with the K_I value obtained by competition with phosphoenolpyruvate in the pyruvate kinase reaction. Moreover, both FPO_3^{2-} and phosphoenolpyruvate drastically diminish the effect of enzyme-bound manganese on the prr of water from a value of 32.7 (Mildvan and Cohn, 1965) to a value of ~2.

The dissociation constant of the ternary fluoride complex is an order of magnitude lower than K_M of fluoride in the manganese-activated fluorokinase reaction, suggesting that a random kinetic scheme and Michaelis–Menten kinetics do not apply to the fluorokinase reaction. We have previously suggested (Mildvan and

[2] Orthophosphate competes with ADP (K_I = 3.7 ± 1.2 mM) in manganese-activated pyruvate kinase (A. S. Mildvan, unpublished observations).

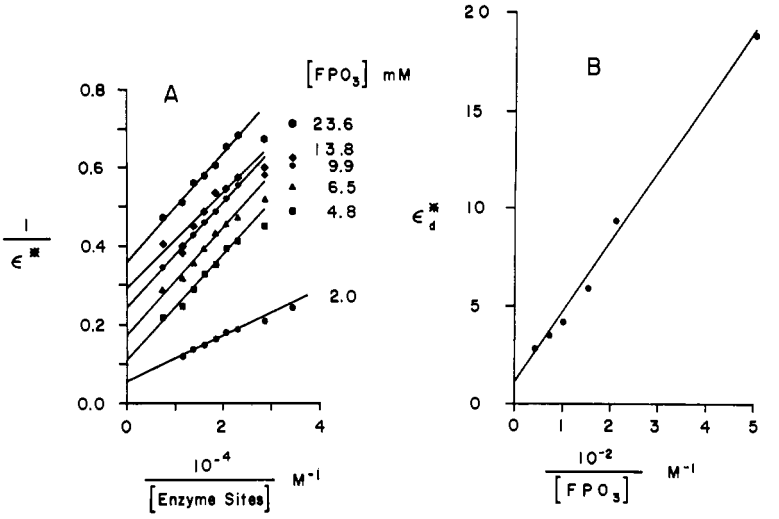


FIGURE 3: Fluorophosphate interaction with E–Mn by prr. (A) Titration of 50 μM $MnCl_2$ with pyruvate kinase at the six indicated concentrations of $KNaFPO_3$. The reciprocal of the enhancement of the prr (ϵ^*) is plotted against the reciprocal of the concentration of enzyme sites for analysis as in Figure 1. The concentrations of other components were: 0.04 M Tris-HCl; $[K^+]$ was maintained at 100 mM with additions of KCl; and $[Na^+]$ at 60 mM with additions of NaCl. Temperature = 26°. (B) Secondary plot of the enhancement at infinite enzyme concentration, ϵ^*_d, against reciprocal of the fluorophosphate concentration. From the intercept on the ordinate ϵ_t = 1.5.

TABLE III: Enhancement, Binding, and Kinetic Parameters of Ternary EMS Complexes.[a]

Substrate	Proton Relaxation Rate Values		Substrate Kinetics Values		
	ϵ_t	K_3 (mM)	K_M (mM)	K_I (mM)	K_3 (mM)
Fluoride	25.0 ± 1.0	28 ± 5	600 ± 300	—	—
Fluorophosphate	1.5 ± 0.4	2.6 ± 0.3	—	3.4 ± 1.0	—
PEP[b]	2.2 ± 0.2	0.015 ± 0.005	—	—	0.027 ± 0.005

[a] Conditions are given in the legends to Figures 2A and 3A. [b] From Mildvan and Cohn (1966).

Cohn, 1966) (Kerson *et al.*, 1967) that a preferred-order kinetic schema in which ATP binds to the enzyme first, may describe the pyruvate kinase catalyzed phosphorylation of pyruvate by ATP.

Fluorine Nmr Spectra. THE INTERACTION OF FLUORIDE WITH MANGANESE AND PYRUVATE KINASE. The nmr spectrum of 0.1 M fluoride ion in 0.05 M Tris-HCl (pH 7.5) and 0.1 M KCl is shown in Figure 4. The narrow F^- line is 40.69 ± 0.07 ppm upfield from TFA, an external standard.

The addition of 10 μM $MnCl_2$ does not alter the position of the resonance, but broadens the line, indicating a marked decrease in T_2. The saturating power level necessary to produce maximum signal amplitude is disproportionately increased indicating an even greater decrease in T_1 than in T_2.

The addition of pyruvate kinase (0.1 mM sites) which binds about one-half of the manganese in the MnF solution as determined from the dissociation constant of manganese–pyruvate kinase (Mildvan and Cohn, 1965) diminishes the effect of manganese on the line width and the saturating power level of fluoride. The control experiment, showing the effect of pyruvate kinase alone on F^-, indicates a small effect which may be due to trace amounts of paramagnetic contaminants in the enzyme. The paramagnetic contributions to the longitudinal and the transverse relaxation rates of fluorine, $1/T_{1p}$ and $1/T_{2p}$, respectively, obtained from four experiments with fluoride are summarized in Table IV. The relaxation rates have been normalized by dividing each by p = total manganese concentration/total ligand concentration (Luz and Meiboom, 1964). In Table IV it may be seen that pyruvate kinase consistently decreases the effect of manganese on the relaxation rates of fluoride. The results are not qualitatively altered by the addition of HCO_3^-, a necessary cofactor for the fluorokinase reaction. Because the pH has been raised from 7.5 to 8.0 in this experiment, pyruvate kinase binds 70% of the manganese (Mildvan and Cohn, 1965). The average error in T_{1p} and T_{2p} of F^- was 17%.

TABLE IV: Effect of Pyruvate Kinase on the Relaxation Rates of Fluoride in the Presence of Manganese.[a]

Additions	pH	Ionic Strength	Pyruvate Kinase Sites (M × 10^4)	$1/pT_{1p}$ (sec^{-1} × 10^{-3})	$1/pT_{2p}$ (sec^{-1} × 10^{-3})		$\epsilon(1/T_1)$	$\epsilon(1/T_2)$
None	7.5 ± 0.1	0.1	0	72.7[b]	398	755	—	—
			1.0	49.0[b]	109	—	0.6[b]	0.3
			1.6	41.7	—	359	0.6	0.5
0.1 M $KHCO_3$	8.0 ± 0.1	0.3	0	128	560		—	—
0.1 M $KHCO_3$			1.0	45.6	133		0.36	0.24
0.1 M $KHCO_3$ + ADP (0.2 mM)			1.0	68.2	119		0.53	0.21
0.1 M $KHCO_3$ + ADP + P_i (0.9 mM)			1.0	19.2	95.2		0.15	0.17

[a] The solutions contained 0.04 M Tris-HCl (pH 7.5), 0.1 M KF, and 10–20 μM $MnCl_2$. The temperature was 28°.
[b] Determined by direct method.

The further addition of ADP and P_i also failed to produce significant enhancements of the effect of manganese on the relaxation rates of the fluorine nucleus of fluoride. Thus, under all of the conditions examined, the effect of the enzyme is quantitatively consistent with the partitioning of the manganese between two binary complexes (E–Mn and Mn–F) with no indication of the formation of a ternary E–Mn–F bridge structure.

THE INTERACTION OF FLUOROPHOSPHATE WITH MANGANESE AND PYRUVATE KINASE. The data from an analogous experiment showing the effects of manganese and pyruvate kinase on the fluorine nucleus of FPO_3^{2-} is shown in Figure 5. The fluorine nmr spectrum of FPO_3 consists of a doublet split by 868 ± 5 cycles/sec. The upfield resonance is 2.39 ± 0.08 ppm above the external standard, TFA. Spectra were recorded of either line with identical results. As seen in Figure 5, 100 μM manganese produces a smaller effect on the line width and saturating power level of FPO_3^{2-} than was achieved with F^-. Thus the fluorine of FPO_3^{2-} is less sensitive to manganese than is the fluorine of fluoride ion although the effects are qualitatively similar. However, in contrast with the findings for fluoride, the addition of pyruvate kinase, *enhances* the effect of manganese on the line width and saturating power level of the

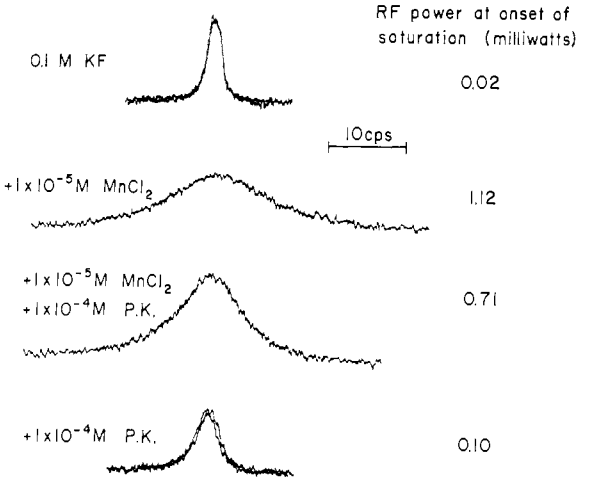


FIGURE 4: Effect of manganese and pyruvate kinase on the line width and radiofrequency power at maximal signal amplitude of the nmr spectrum of fluoride ion. The buffer was 0.03 M Tris-HCl (pH 7.5), and the total volume was 0.5 ml. Temperature = 27 ± 1°.

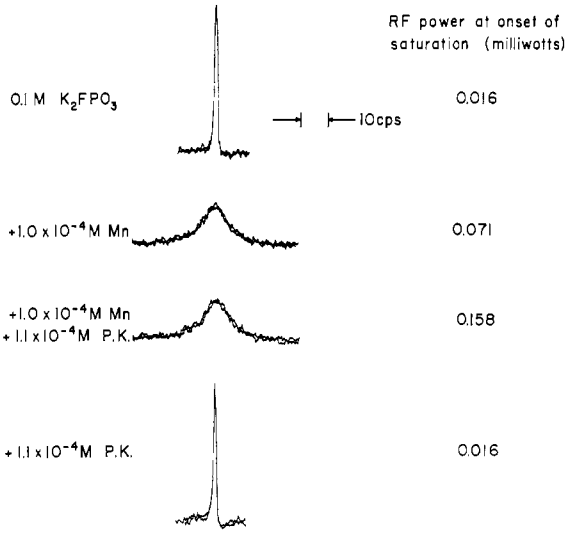


FIGURE 5: Effect of manganese and pyruvate kinase on the line width and power at maximal signal amplitude of the nmr spectrum of the fluorine nucleus of K_2FPO_3. Only the high-field component of the doublet is shown. Conditions are otherwise as described in Figure 4.

TABLE V: Effect of Pyruvate Kinase on the Relaxation Rate of Fluorine in the Mn–FPO_3 Complex.[e]

Temp (°C)	Pyruvate Kinase Sites (M × 10^4)	$1/pT_{1p}$ (sec^{-1} × 10^{-3})	$1/pT_{2p}$ (sec^{-1} × 10^{-3})	$\epsilon^*(1/T_1)$	$\epsilon^*(1/T_2)$
4	0	2.22	17±6	—	—
	1.2	5.87	24.1	2.7	1.4
14	0	1.2	19	—	—
	1.2	6.06	28.3	5.1	1.5
20	0	0.54 0.90	30	—	—
	1.2	— 7.07	34.5	7.9	1.15
	2.0	3.8 —	40.0	7.0	1.3
27	0	1.85[f] 0.7	31.8	—	—
	1.0	3.2[f] —	32.3	1.7[f]	1.03
	2.0	— 3.2	39.1	4.5	1.2
31	0[a]	1.6 0.24	15	—	—
	1.0[a]	— 0.48	20.5	2.0	1.4
	1.9[b]	3.0 —	29.8	1.8	2.0
34	0	0.91	33.3	—	—
	1.0	3.35	41	3.7	1.7
27	0[c]	0.69	17.9	—	—
	1.1[c]	1.37	24.2	2.0	1.35
28	0[d]	1.74	29.4	—	—
	1.2[d]	6.86	38.1	4.0	1.3

[a] Na_2FPO_3 instead of K_2FPO_3. [b] $KNaFPO_3$ instead of K_2FPO_2. [c] pH 8.1. [d] pH 8.1, 0.1 M $KHCO_3$ in addition to 0.1 M K_2FPO_3, ionic strength = 0.4. [e] The solutions contained 0.04 M Tris-HCl buffer (pH 7.5) at 25°, and 0.1–0.2 mM $MnCl_2$, 0.1 M K_2FPO_3, ionic strength = 0.3 except as noted. [f] Determined by direct method.

fluorine of FPO_3^{2-}. No significant change in the position of the resonance was detected.

The relaxation rates obtained under a variety of conditions with six preparations of pyruvate kinase are summarized in Table V. The average errors in T_{1p} and T_{2p} of FPO_3^{2-} were found to be 41 and 14%, respectively. In Table V it is seen that pyruvate kinase consistently enhances the effect of manganese on the $1/T_{1p}$ and $1/T_{2p}$ parameters of FPO_3^{2-}, analogous to its effect on the protons of water (Mildvan and Cohn, 1965), *i.e.*, the dipolar interaction of the fluorine nucleus of FPO_3^{2-} with manganese is stronger when the manganese is bound to pyruvate kinase.

That these enhancements are due to interaction with the paramagnetic manganese, rather than to direct interaction with the protein, *i.e.*, immobilization as a consequence of binding FPO_3^{2-} to pyruvate kinase, is established by replacement of manganese with the nonparamagnetic magnesium ion. Spectroscopically pure $MgCl_2$ (up to 5 mM) had no significant effect on the position, line width, or saturation value of the nmr spectrum of FPO_3^{2-} in the presence or absence of 0.1 mM pyruvate kinase sites. Hence, the only explanation for the enhancements is the formation of an enzyme–manganese–fluorophosphate bridge complex.

COMPETITION BETWEEN FPO_3^{2-} AND PEP BY FLUORINE NMR. In order to determine whether the enhanced effect of EMn on the relaxation rates of the fluorine of FPO_3^{2-} was due to combination of FPO_3^{2-} with the PEP binding site of pyruvate kinase, a competition experiment was done. The effect of pyruvate kinase (130 μM) and manganese (90 μM) on the line width of the fluorine resonance of FPO_3^{2-} (0.175 M) was studied at various concentrations of PEP. The results are shown in Figure 6, where it is seen that PEP markedly narrows the line width of FPO_3^{2-} which had been broadened by enzyme and manganese. From the data of Figure 6, assuming simple competition between FPO_3^{2-} and PEP for the same site, and using the value of 3.0 mM for the dissociation constant of EMn–FPO_3 (average of K_3 and K_I from Table III), one obtains a value of 1.2×10^{-5} M for the dissociation constant of EMn–PEP. The latter value obtained by fluorine nmr of FPO_3^{2-} is in good agreement with the K_3 of PEP as determined by proton relaxation rate and kinetics (Table III). Since the binding of phosphoenolpyruvate to the enzyme is quantitatively the same whether measured by fluorine resonance or by kinetics, the binding site of FPO_3^{2-} may be equated with the active site.

At infinite levels of PEP the line width approaches a value which is still 2 cycles/sec greater than that observed in absence of manganese. This corresponds to a $1/pT_{2p}$ value of 10^4 sec^{-1} (Figure 6B). This residual effect may be due to outer sphere relaxation mechanisms (Luz and Meiboom, 1964).[3]

The Effect of Temperature on the Relaxation Rates of Mn–FPO_3 and E–Mn–FPO_3. The plot in Figure 7 presents data on the effect of temperature (0–70°) on the

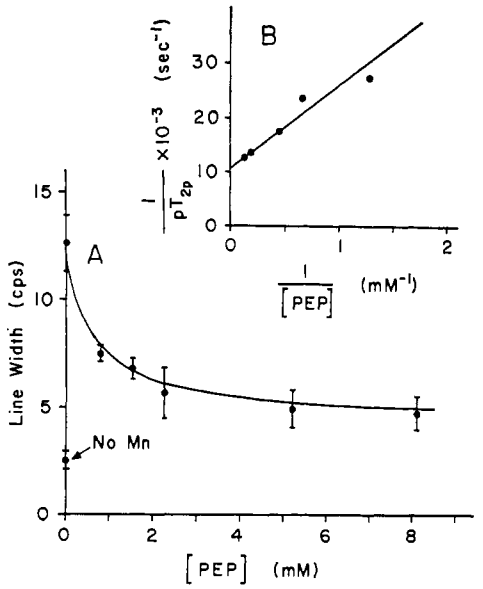


FIGURE 6: Phosphoenolpyruvate competition with FPO_3^{2-}. (A) The effect of phosphoenolpyruvate on the line width of the fluorine resonance of K_2FPO_3 (0.175 M) in the presence of pyruvate kinase (130 μM sites), $MnCl_2$ (90 μM), and Tris-HCl buffer (pH 7.5) (41 mM). Temperature = 26°. The points represent the experimental line widths plus and minus the mean deviation. The curve is drawn assuming simple competition between phosphoenolpyruvate (K_3 = 12 μM) and FPO_3^{2-} (K_3 = 3 mM) for EMn. (B) Reciprocal of phosphoenolpyruvate concentration *vs.* the paramagnetic contribution to the transverse relaxation rate ($1/pT_{2p}$). Extrapolation to infinite phosphoenolpyruvate yields $1/pT_{2p} = 10.6 \times 10^3$ sec^{-1}. The concentration of phosphoenolpyruvate (695 μM) required to produce a value of $1/pT_{2p}$ halfway between 10.6 and 55.5 $\times$ 10^3 sec^{-1} when divided by $(1 + ([FPO_3]/K_3(FPO_3)))$, gives K_3 (phosphoenolpyruvate) = 12 μM.

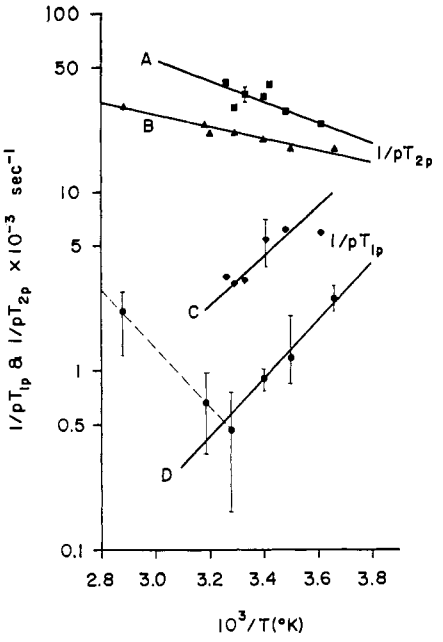


FIGURE 7: Arrhenius plot of the effect of temperature on the paramagnetic contribution to the relaxation rates of fluorine in the ternary pyruvate kinase–manganese–fluorophosphate complex (A and C) and in the binary manganese–fluorophosphate complex (B and D). Conditions are as described in Table V. The activation energies (in kcal/mole) are curve A, 2.6 ± 0.9; curve B, 1.5 ± 0.3; curve C, 6.3 ± 2.1; curve D (solid), 7.3 ± 1.8; and curve D (dashed), 7.5 ± 2.8.

paramagnetic contribution to the relaxation rates ($1/pT_{1p}$ and $1/pT_{2p}$) of fluorine in the Mn–FPO_3 complex. For comparison the results from Table V obtained when the enzyme was present are also included in Figure 7. The temperature range used in the presence of pyruvate kinase was restricted to 4–34° to avoid denaturation of the enzyme at higher temperatures.

From the positive temperature coefficient of $1/pT_{2p}$, it follows that $1/T_{2p}$ of Mn–FPO_3 and E–Mn–FPO_3 are governed by $1/\tau_M$ (region B) (eq 5) and from the negative temperature coefficient of $1/pT_{1p}$, it follows that $1/pT_{1p}$ of both complexes is dominated by $1/T_{1M}$ (region A) (eq 4) in the temperature range from 4 to 34°.[4] Therefore, $1/pT_{1p}$ may be used to calculate r and gain insight into the geometry of the Mn–FPO_3 and E–Mn–FPO_3 complexes, and $1/pT_{2p}$ may be used to determine kinetic parameters of the ligand-exchange process.

Calculations of Manganese to Fluorine Distances in MnF, $MnFPO_3$, and E–$MnFPO_3$ in Solution from $1/T_{1p}$. Evaluating the terms in eq 6 for the manganese–fluorine interactions from the values of the constants in Table I, yields

$$r^6 = 2.67 \times 10^{-31} T_{1M} f(\tau_c) \qquad (10)$$

The effective τ_c for the Mn–H_2O interaction has been evaluated (Bloembergen and Morgan, 1961) as $\sim 3 \times 10^{-11}$ sec at 27°. Since ϵ_a of water protons in the $(H_2O)_5Mn^{2+}F^-$ complex is about five-sixths (Table I), it appears reasonable that τ_c for the Mn–F interaction may be taken as being the same value as that for Mn–H in the $Mn(H_2O)_6$ complex since τ_c of the latter is determined by the tumbling time of the whole complex. The molecular geometry of the complex $(H_2O)_5Mn^{2+}F^-$ is thus assumed to be similar to that of $(H_2O)_6Mn^{2+}$. The value of T_{1M} for fluoride in the manganous complex, as measured in this

[3] Using the simple equation for outer sphere relaxation derived by Luz and Meiboom (1964), one may calculate the distance of closest approach of the fluorine of FPO_3^{2-} to the manganese in E–Mn–PEP to be ~10 A which is a reasonable value.

[4] The negative slope of $1/pT_{1p}$ of Mn–FPO_3 at high temperatures is unexplained. It may be due to the formation of a new chemical species.

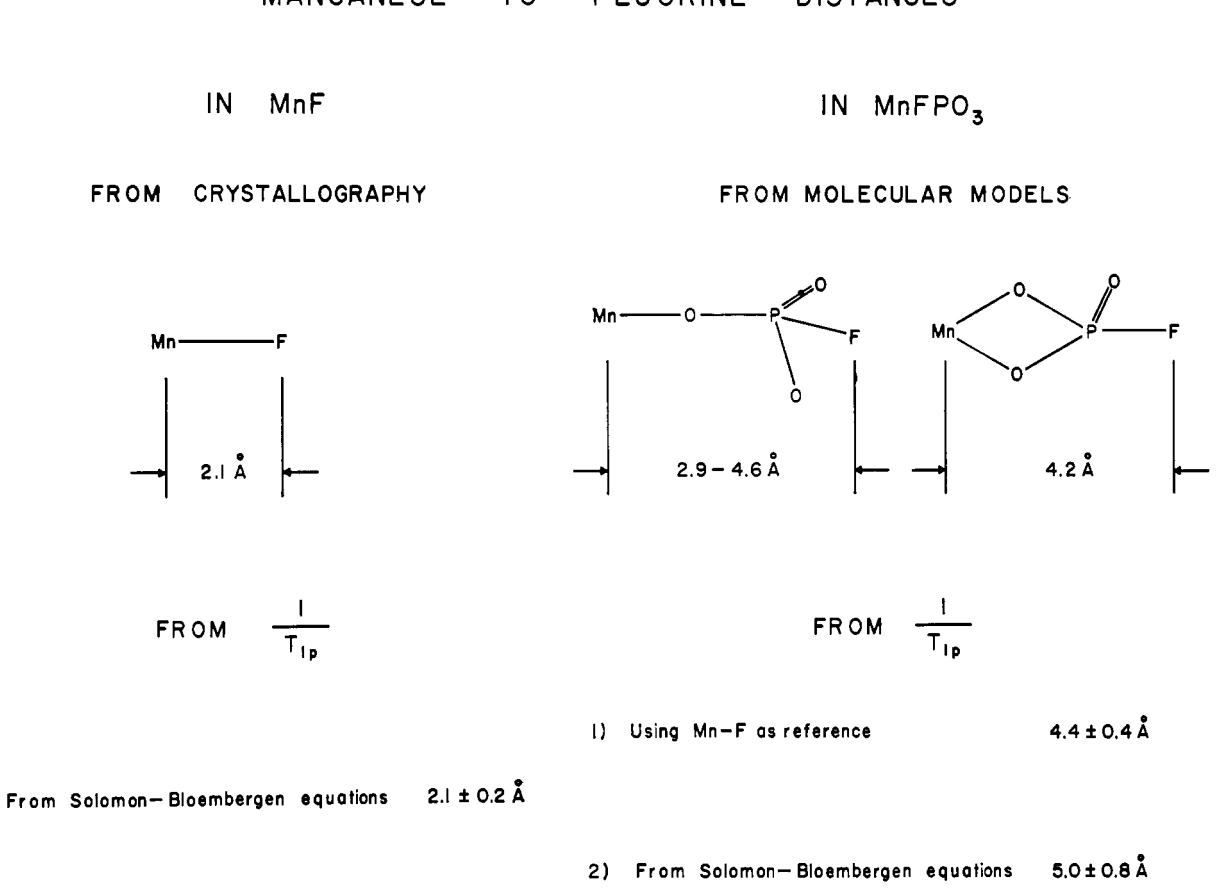


FIGURE 8: Managanese to fluorine distances in Mn–F and in $MnFPO_3$, in solution, determined by $1/T_{1p}$ compared with values from crystallographic (Griffel and Stout, 1950) and molecular model studies.

Paper gives $T_{1M} = 3.70 \times 10^{-6}$ sec when the observed value of $1/pT_{1p}$ (72.7×10^3 sec^{-1}) (Table IV) is corrected for the proportion of uncomplexed species using the dissociation constant in Table II. Evaluation of r(Mn–F) then gives 2.12 A. This is in excellent agreement with the crystallographically determined value of 2.12 A as the average Mn–F distance observed in the MnF_2 crystal (Griffel and Stout, 1950) (see Figure 8).

In the Mn–FPO_3 complex, ϵ_b for the bound water is 0.99 ± 0.04 (Table II). This is consistent with an increase in τ_c to approximately 3.6×10^{-11} sec compensating for the reduction from six to five water molecules in the hydration sphere. Using the value of $T_{1M}(MnFPO_3) = 5.4 \times 10^{-4}$ sec calculated from the observed value of $1/pT_{1p}$ (1850 sec^{-1}, Table V) the evaluation of eq 10 yields 5.0 ± 0.8 A for the manganese–fluorine distance. A separate calculation using the distance in the Mn–F complex as 2.12 A and assuming the same τ_c for the two complexes in the following approximation

$$T_{1p}(F)/T_{1p}(FPO_3) = [r(F)/r(FPO_3)]^6 \qquad (11)$$

gives a value of 4.4 ± 0.4 A. These distances are of the right magnitude for a manganese–fluorophosphate complex in which the manganese is bonded to one or two oxygens, rather than directly to the fluorine (see Figure 8).

The enhancement by pyruvate kinase of the effect of manganese on $1/T_{1p}$ (Figures 5 and 7 and Table V) is probably due to an increase in τ_c, that is τ_c(E–Mn–FPO_3) > τ_c(Mn–FPO_3). However, even if the enhancement were due to a shortening of the manganese to fluorine distance when Mn–FPO_3 is bound to the enzyme, the maximum enhancement we have observed (ϵ 7.9) corresponds to a lower limit of the manganese to fluorine distance of ~3.5 ± 0.6 A, still well above the value expected if manganese were directly bonded to fluorine, but consistent with one or two Mn–O bonds, in the ternary complex.

Kinetic Parameters of the Mn–FPO_3 and E–Mn–FPO_3 Complexes from $1/T_{2p}$. The temperature dependence of $1/T_{2p}$ (Figure 7) indicates that the relaxation rate is dominated by $1/\tau_M$, the reciprocal of the residence time of FPO_3^{2-} in the coordination sphere of manganese. Under our experimental conditions, T_2 and therefore τ_M have been found to be independent of the concentration of the ligand. Therefore, we are dealing with a process which is kinetically first order. For simple ionization reactions

TABLE VI: Kinetic Parameters of Exchange Reactions of Manganese Complexes.[d]

$$Mn\text{-}L \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} Mn^{2+} + L$$

Parameters	Units	Complexes				
		EMn–FPO_3	Mn–FPO_3	Mn–SO_4[a]	Mn–H_2O[b]	EMn–H_2O[c]
k_{off} $(1/\tau_M)$	sec^{-1}	3.4×10^4	2.1×10^4	2×10^7	3.1×10^7	1.7×10^8
k_{on}	M^{-1} sec^{-1}	1.3×10^7	1.2×10^7	—	—	—
E_a	kcal/mole	2.6	1.54	6.0	8.7	19.9
$\Delta H^{\ddagger}$	kcal/mole	2.0	0.95	5.4	8.1	19.3
$\Delta S^{\ddagger}$	cal/°Kmole	−30.9	−35.6	−6.5	3.0	44.0
$-T\Delta S^{\ddagger}$	kcal/mole	10.2	12.7	1.9	−0.9	−13.1
k_{34}	sec^{-1}	3.3×10^5	2.9×10^5	4×10^6	—	—

[a] Eigen and Tamm (1962), 20°, ultrasonic absorption. [b] Swift and Connick (1962), nmr of ^{17}O-labeled H_2O. [c] Cohn (1967). [d] Values are at 25° except as noted. k_{34} is defined in eq 15.

$$Mn\text{-}FPO_3 \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} Mn^{2+}(aq) + FPO_3^{2-} \qquad (12)$$

and

$$EMn\text{-}FPO_3 \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} EMn^{2+} + FPO_3^{2-} \qquad (13)$$

Under our experimental conditions, essentially all of the manganese is bound to FPO_3^{2-}. Hence, the residence time is related to the first-order rate constant k_{off} in the following way

$$k_{off} = \frac{1}{\tau_M} \qquad (14)$$

From k_{off} and the dissociation constant of $MnFPO_3$ (Table II) and EMn–FPO_3 (Table III) one can determine k_{on}. The Arrhenius plot of Figure 7 gives the activation energy (E_a) from which one can calculate the enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation for $1/\tau_M$. The various kinetic parameters of the dissociation of Mn–FPO_3 and EMn–FPO_3 are summarized in Table VI which also contains data on the dissociation of $MnSO_4$ (Eigen and Tamm, 1962), $Mn(H_2O)_6^{2+}$ (Swift and Connick, 1962), and $EMn(H_2O)_4$ (Cohn, 1967).

Discussion

Since the original proposal of an enzyme–metal–substrate bridge structure by Hellerman and Stock (1938), this hypothesis has been invoked to explain the mechanism of action of many enzymes (Smith, 1951; Klotz and Ming, 1954; Malmstrom and Rosenberg, 1959; Vallee and Coleman, 1964; Mildvan and Cohn, 1966; Mildvan *et al.*, 1966). However, previous evidence for the existence of such complexes has been indirect.

The observed enhancement, by pyruvate kinase, of the effect of manganese on the longitudinal $(1/T_1)$ and transverse $(1/T_2)$ relaxation rates of the fluorine nucleus of fluorophosphate can be explained only by a direct interaction between enzyme-bound manganese and fluorophosphate, *i.e.*, the formation of an enzyme–metal–fluorophosphate bridge structure.

Several independent lines of evidence indicate that the oxygen(s) rather than the fluorine of fluorophosphate is coordinated to the manganous aquocation and to enzyme-bound manganese in solution. These are: (1) The similar affinities of E–Mn and Mn^{2+} for orthophosphate and fluorophosphate, which are much greater than the affinities for fluoride (Tables II and III and footnote 2). (2) The large negative value of $\Delta S^{\ddagger}$ for the dissociation of Mn–FPO_3 and EMn–FPO_3 which is characteristic of ionization reactions (Table VI) (Frost and Pearson, 1961). (3) The failure to detect an E–Mn–F bridge structure (Table IV). (4) The distance between manganese and fluorine in the binary Mn–FPO_3 and ternary E–Mn–FPO_3 complexes in solution lies between 3.5 and 5 A; well above the Mn–F distance in solution (2.1 A) (Figure 8). The results do not permit one to distinguish between monodentate coordination (Figure 9A) and bidentate coordination (Figure 9B). The latter structure, although strained, has been suggested for certain manganous phosphate crystals (Mays, 1963).

Tietz and Ochoa (1958) found the fluorokinase reaction and the pyruvate kinase reaction to have similar metal ion requirements and that the fluorokinase reaction was strongly inhibited by phosphoenolpyruvate. The present results show that fluorophosphate inhibits the pyruvate kinase reaction by specifically competing with phosphoenolpyruvate and not with ADP. Orthophosphate, although isoelectronic with fluorophosphate, is less specific since it competes with both ADP and phosphoenolpyruvate[2] (Rose, 1960). Moreover, the similar enhancement values, ϵ_t, of the proton relaxation rate in the presence of enzyme–manganese–phosphoenolpyruvate and enzyme–manganese–fluorophosphate suggest a similar structure of these ternary complexes (Table III). The most cogent evidence that the pyruvate

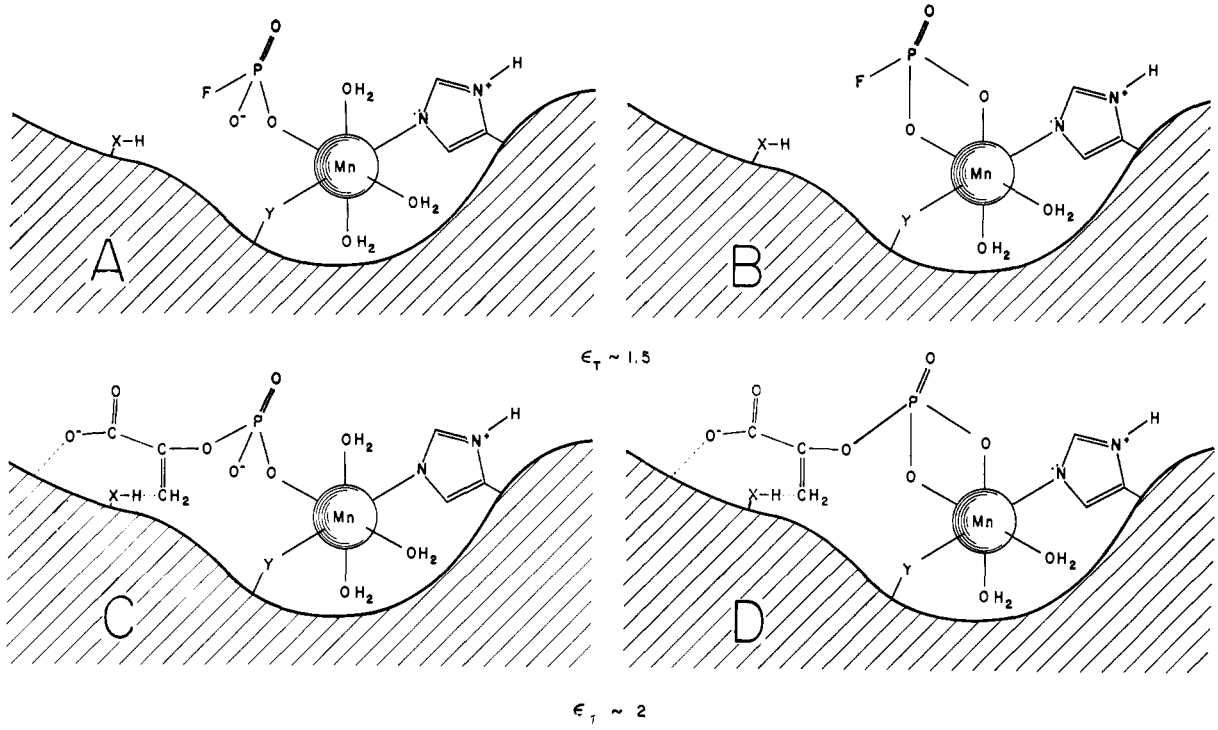


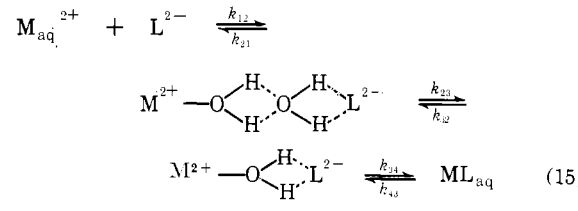
FIGURE 9: Alternative E–M–S bridge structures for the ternary complexes of pyruvate kinase and manganese with fluorophosphate (A and B) and with phosphoenolpyruvate (C and D).

kinase and the fluorokinase reactions occur at the same active site of the enzyme is the displacement of fluorophosphate from the E–Mn–FPO_3 complex by PEP as demonstrated in the fluorine nmr experiment (Figure 6). Hence, the structures shown in Figure 9C,D for the ternary complex with phosphoenolpyruvate are proposed (Mildvan and Cohn, 1966) by analogy with the structure 9A or B established for the ternary fluorophosphate complex.

The present results establish the existence of an enzyme–metal–product bridge complex. The question arises whether this complex functions as an obligatory intermediate in the fluorokinase reaction. E–Mn–FPO_3 meets a necessary kinetic requirement of an intermediate, namely, its rate of decomposition (k_{off}) is larger by three orders of magnitude than the maximum velocity of the manganese-activated fluorokinase reaction (18 sec^{-1}) which we have measured, or of the magnesium-activated fluorokinase reaction (10 sec^{-1}) calculated from the data of Tietz and Ochoa (1958). Its participation as an obligatory intermediate is favored by two experimental findings. First, there are no detectable ancillary or nonspecific binding sites on pyruvate kinase for manganese, an obligatory component, under the conditions of these experiments (Mildvan and Cohn, 1965) and second, simple competition for E–Mn occurs between FPO_3^{2-} and PEP as detected by kinetics and by fluorine resonance. Moreover, the dissociation constants determined by both methods are in agreement.

It is of interest to compare the kinetic parameters of the ligand exchange of Mn–FPO_3 and E–Mn–FPO_3 with those of other exchange reactions on manganese (Table VI). The value of k_{off} of Mn–FPO_3 and of E–Mn–FPO_3 is smaller by three orders of magnitude than that of $MnSO_4$ as measured by ultrasonic absorption (Eigen and Tamm, 1962), suggesting a different mechanism of dissociation of the fluorophosphate and sulfate complexes. A different mechanism for $MnSO_4$ and Mn–FPO_3 is further supported by the different values of $\Delta H^{\pm}$ and $\Delta S^{\pm}$ for the dissociation of FPO_3^{2-} complexes of manganese or E–Mn and $MnSO_4$. The major kinetic barrier to the dissociation of FPO_3^{2-} complexes is entropic, while the barrier to the dissociation of $MnSO_4$ is largely enthalpic. (See Table VI.) The former complexes must pass through a highly ordered, hence improbable, transition state during dissociation.

Eigen and Tamm (1962) have proposed the following three-step mechanism for the formation and dissociation of metal complexes.



(15)

Application of the Eigen–Tamm (1962) treatment to the

$Mn–FPO_3$ complex gives the following relationships.

$$k_{off} = k_{43} \tag{16}$$

and

$$K_1 = \frac{k_{21}k_{32}k_{43}}{k_{12}k_{23}k_{34}} \tag{17}$$

therefore

$$k_{on} = k_{12}k_{23}k_{34}/k_{32}k_{21} \tag{18}$$

Eigen and Tamm (1962) have made theoretical calculations of the following rate constants for a divalent cation and a divalent anion.

$$k_{12} = 4 \times 10^{10}\ M^{-1}\ sec^{-1}$$

$$k_{21} = k_{23} = k_{32} = 10^9\ sec^{-1}$$

Using these values, together with our measured values of K_1 and k_{off} for $Mn–FPO_3$ and $EMn–FPO_3$, we can calculate k_{34}, the hypothetical first-order rate constant for the displacement of a coordinated water molecule in the manganous aquocation and in the E–Mn complex by a fluorophosphate anion which is located in the second coordination sphere.

Since k_{34} is thought to be controlled by the rate of release of coordinated water from the metal, according to Eigen and Tamm (1962) this rate constant should be independent of the nature of the anion (as they have observed for $MgSO_4$, MgS_2O_3, and $MgCrO_4$). However, as may be seen from Table VI, k_{34} for $Mn–FPO_3$ is an order of magnitude slower than that for $MnSO_4$ and two orders of magnitude slower than k_{off} of water from $Mn(H_2O)_6^{2+}$. Similarly, k_{34} for $EMn–FPO_3$ is much slower than k_{off} of water from $EMn–(H_2O)_4$. The reason for these discrepancies is not clear. Possible explanations are: (a) Sulfate, and to a greater degree, fluorophosphate, may hinder the departure of that water molecule which is located along the reaction coordinate, *i.e.*, the inner sphere water molecules are not equivalent when an anion is in the outer coordination sphere. (b) In contrast to the Eigen–Tamm (1962) hypothesis the ligand, while coming in from the outer sphere, may expel a water molecule from the coordination sphere, by a nucleophilic displacement on the metal ion. This theory would require the "nucleophilicity" to increase as follows, $FPO_3^{2-} < SO_4^{2-} < H_2O$. (c) The mechanisms of exchange of H_2O, SO_4^{2-}, and FPO_3^{2-} into the coordination shell of manganese differ qualitatively as is suggested by the different values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Table VI), and no single theory can explain all three processes. (d) The rate constants k_{23} and k_{32} for the formation and breakdown of the outer sphere complex are not identical for all divalent anions. There is at present no experimental basis for a choice among these hypotheses.

The enhancement by pyruvate kinase of the effect of manganese on the $1/T_{2p}$ of FPO_3^{2-} may be ascribed solely to a 50% acceleration of the rate of dissociation, k_{off} of $EMn–FPO_3$ as compared to $Mn–FPO_3$ (Table VI). This acceleration results from an increase in the entropy of activation of $1/\tau_M$ rather than a decrease in the energy of activation. Thus the effect of enzyme may be to increase the relative number of conformations of $Mn–FPO_3$ which are like the transition state for dissociation. This is most simply achieved by decreasing the total number of forms which $Mn–FPO_3$ can take. The increase in the entropy of activation of k_{off} for FPO_3^{2-} when $Mn–FPO_3$ is bound to the enzyme corresponds to $\sim{}^5/_2R$, a value which is suggestive of the loss of one translational and two rotational degrees of freedom (Tolman, 1938).

Acknowledgments

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Resolution of Multiple Ribonucleic Acid Species by Polyacrylamide Gel Electrophoresis*

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ABSTRACT: High molecular weight ribonucleic acid (RNA) from rat liver, kidney, and brain has been fractionated by polyacrylamide gel electrophoresis. A large number of new species of RNA have been resolved with electrophoretic mobilities and sedimentation coefficients intermediate between 30S and 18S RNA and intermediate between 18S and 4S RNA. The number and relative amount of each of these species were constant from preparation to preparation from the same tissue as well as from different tissues. Evidence is presented that these RNA components are present *in vivo* and are not the result of *in vitro* artifacts. The results suggest that the current classification of RNA into three major types (30, 18, and 4 S) is inadequate to describe the true heterogeneity of cytoplasmic RNA, and therefore hinders studies of the functional role played by different RNA species.

Earlier reports on the successful electrophoretic fractionation of RNA in supporting gels (Bachvaroff and McMaster, 1964; Richards and Gratzer, 1964; Tsanev, 1965; Beney and Székely, 1966; McPhie *et al.*, 1966; Bachvaroff and Tongur, 1966) had indicated that such methods might be capable of higher resolution than the more commonly used techniques of zone sedimentation and column chromatography. The first descriptions of this technique involved the use of agar gels (Bachvaroff and McMaster, 1964; Tsanev, 1965). These studies were significant in showing that high molecular weight RNAs (*e.g.*, rRNA) could be resolved and also suggested that rRNA might be composed of more than the two species resolved on sucrose gradients, *i.e.*, 18S and 30S RNA. Later, workers turned to polyacrylamide gels (Richards and Gratzer, 1964; McPhie *et al.*, 1966) or starch gels (Beney and Székely, 1966) in which even greater resolving power was observed; however, these studies were largely confined to fractionation of low molecular weight RNA (*e.g.*, sRNA (Richards and Gratzer, 1964), and nuclease digestion products of rRNA (McPhie *et al.*, 1966)).

In the present study we have used the very high resolving power of electrophoretic separations on polyacrylamide gels to examine the heterogeneity of RNA. It appears that cytoplasmic ribonucleic acid (cRNA) is composed of multiple species, and that these same species are present in rat liver, kidney, and brain in similar proportions.

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