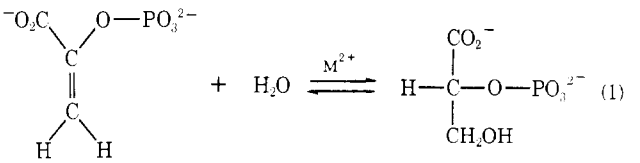
Nuclear Relaxation and Kinetic Studies of the Role of Mn^{2+} in the Mechanism of Enolase†

Thomas Nowak,* Albert S. Mildvan, and George L. Kenyon‡

ABSTRACT: When Mn^{2+} is bound at the active site of enolase, two rapidly exchanging water molecules are coordinated as indicated by the effect of the enolase–Mn^{2+} complex on the longitudinal relaxation rate ($1/T_1$) of water protons determined at six frequencies. The exchange rate ($1/\tau_M$) of these water molecules is greater than 7.7×10^6 sec^{-1}, and the correlation time for the Mn^{2+}–water interaction is 2.2×10^{-9} sec, a value which is shown to be dominated by τ_s, the electron spin relaxation time of Mn^{2+}. Ternary complexes of the substrates (phosphoenolpyruvate and α-(dihydroxyphosphinylmethyl)-acrylate (CH_2-PEP)) and inactive substrate analogs (D-phospholactate, L-phospholactate, and phosphoglycolate) all affect $1/T_1$ of water protons two–three times less than the binary enolase–Mn^{2+} complex at 24.3 MHz. From $1/T_1$ and $1/T_2$ values of the carbon-bound protons of CH_2-PEP at two frequencies and of the phosphorus at one frequency, the correlation times (τ_c) in the active ternary CH_2-PEP complex are essentially unchanged from that of the binary complex. Similar τ_c values are obtained by measurements of $1/T_1$ and $1/T_2$ of the protons and phosphorus of the inactive analogs, indicating that the decrease in $1/T_1$ of water in the ternary complexes is not due to a change in τ_c. The distances from the enzyme-bound Mn^{2+} to the carbon-bound protons (6.5–7.5 Å) and the phosphorus atoms (5.5–6.2 Å) of the active and inactive analogs are too great for inner sphere complexes, but are consistent with second sphere complexes in which a water molecule intervenes between the bound Mn^{2+} and the bound substrate analog. From the τ_c values of the analogs and $1/T_1$ of water in the ternary complexes it is calculated that 0.3–1.0 rapidly exchanging water molecule remains coordinated to Mn^{2+} in the various ternary complexes. Hence, the binding of the substrate may have "immobilized" a water molecule on the enzyme-bound Mn^{2+} such that it exchanges slower than 10^6 sec^{-1}. The exchange rate of the active analog, CH_2-PEP, into its ternary complex ($1/\tau_M = 1.1 \times 10^5$ sec^{-1}, $E_{act} = 7.5$ kcal/mol), as determined by the temperature dependence of $1/T_2$ of phosphorus, is five orders of magnitude greater than the maximal rate of hydration of this substrate (1.7 sec^{-1}), indicating that the complex forms and breaks down fast enough to participate in catalysis. The detailed geometry of the active enolase–Mn^{2+}–(H_2O)–CH_2-PEP complex, determined by measurements of distances between bound Mn^{2+} and four substrate nuclei indicates that the Mn^{2+} lies either above or below the vinyl plane of the substrate and could, therefore, place a coordinated water molecule on either the *si* or *re* face of the double bond, in molecular contact with the π-electron system. From the stereochemistry of the overall reaction (Cohn, M., Pearson, J. E., O'Connell, E. L., and Rose, I. A. (1970), *J. Amer. Chem. Soc. 92*, 4095) Mn^{2+} could therefore activate a water molecule to add a proton at C-2 of the substrate from the *si* face or a hydroxyl ion at C-3 from the *re* face. The microscopic reverse of the latter mechanism would require direct coordination of the 3-OH group of 2-phosphoglycerate by the enzyme-bound Mn^{2+}. Such coordination is suggested by a kinetic study which shows that the K_m of free Mn^{2+} is decreased sevenfold as the concentration of 2-phosphoglycerate approaches infinity and the K_m of 2-phosphoglycerate becomes immeasurably high as the concentration of free Mn^{2+} approaches zero. A carbanion mechanism consistent with the present nmr and kinetic data and the isotopic exchange data of Dinovo and Boyer (Dinovo, E. C., and Boyer, P. D. (1971), *J. Biol. Chem. 246*, 4586) is presented.

Enolase (phosphoenolpyruvate hydratase, or 2-phospho-D-glycerate hydrolase, EC 4.2.1.11) catalyzes the reversible hydration of phosphoenolpyruvate (P-enolpyruvate)[1] to 2-phospho-D-glycerate. The enzymes from both yeast and muscle require a divalent cation for activity and this requirement has been extensively studied. Wold (1971) has recently reviewed the possible mechanistic role of divalent cations and the molecular properties, the substrate specificity, and monomer–dimer activity relationships of enolases. Studies of the relaxation rate of H_2O protons have implicated the



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† From the Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111, and the Department of Chemistry, University of California, Berkeley, California 94720. *Received August 22, 1972.* This work was supported by U. S. Public Health Service Grants AM-13351, CA-06927, and RR-00542 (for use of nmr equipment located at the University of Pennsylvania), Grant RR-05539 from the National Institutes of Health, Grant GB-27739X (previously Grant GB-8579) from the National Science Foundation, and by an appropriation from the Commonwealth of Pennsylvania (T. N. and A. S. M.), and by U. S. Public Health Service Grant AM-13529 from the National Institutes of Health (G. L. K.). T. N. was also a postdoctoral fellow of the National Institutes of Health.

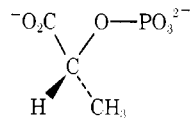
* Address correspondence to this author at: Department of Chemistry, Biochemistry and Biophysics Program, University of Notre Dame, Notre Dame, Ind. 46556.

‡ Present address: Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California—San Francisco, San Francisco, Calif. 94122.

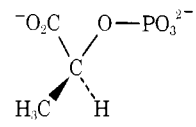
[1] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; CH_2-PEP, α-(dihydroxyphosphinylmethyl)acrylate; PRR, proton relaxation rates of water.

metal ion in coordinating the substrate at the active site of the enzyme (Cohn, 1963; Nowak and Mildvan, 1970), while ultraviolet (uv) spectral studies have suggested that the divalent cation adjusts the conformation of the enzyme to its active form (Hanlon and Westhead, 1965).

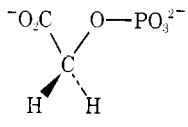
It has previously been demonstrated that although enolase catalyzes a stereospecific trans addition of H_2O (Cohn *et al.*, 1970) the saturated, enantiomeric analogs of P-enolpyruvate, D-phospholactate (I) and L-phospholactate (II), show no stereospecific inhibition and are poor competitive inhibitors of the enzyme, as is phosphoglycolate (III) (Nowak and Mildvan, 1970). Studies of the binding of these analogs and of P-enolpyruvate to enolase–Mn^{2+} by PRR[1] titrations led to the suggestion of enolase–Mn^{2+}–substrate bridge complexes, although it was not clear whether P-enolpyruvate donated a carboxyl or phosphoryl ligand or whether 2-phosphoglycerate donated a hydroxyl ligand to the enzyme-bound Mn^{2+} (Nowak and Mildvan, 1970; Mildvan, 1970). Recently, several other analogs of P-enolpyruvate including α-(dihydroxyphosphinylmethyl)acrylic acid (IV), a methylene analog of P-enolpyruvate (CH_2-PEP), have been synthesized and both IV and (*Z*)-3-fluorophosphoenolpyruvate were found to be substrates for muscle enolase (Stubbe and Kenyon, 1972). The structures of the analogs used in this study are shown below.



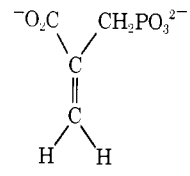
I
D-phospholactate



II
L-phospholactate



III
phosphoglycolate



IV
α-(dihydroxyphosphinylmethyl)acrylate

The present paper examines the effect of enolase-bound Mn^{2+} on the relaxation rates of water and of the hydrogen and phosphorus nuclei of these analogs of P-enolpyruvate. Similar studies with the Mn^{2+}–pyruvate kinase complex have previously shown that the enzyme-bound Mn^{2+} coordinates such analogs through their phosphoryl groups (Nowak and Mildvan, 1972b). Since enolase and pyruvate kinase catalyze different reactions of P-enolpyruvate there is no *a priori* reason, however, to believe that the Mn^{2+} may perform the same function in enolase. The present results with enolase indicate that the bound analogs form second sphere complexes with the enzyme-bound Mn^{2+}.

Experimental Section

Materials. Enolase purified from yeast (Westhead and McLain, 1964) was a generous gift from Dr. David Hanlon. The P-enolpyruvate and 2-phosphoglycerate were purchased from Sigma; 2-phosphoglycolate was purchased from General Biochemicals, Chagrin Falls, Ohio. D- and L-2-phospholactate were synthesized as previously described (Nowak and Mildvan, 1970) as was the substrate α-(dihydroxyphosphinylmethyl)acrylic acid (Stubbe and Kenyon, 1972). All other reagents were of the highest purity commercially available.

Methods. Enolase was assayed by a modification of a previously used assay (Westhead and McLain, 1964) as previously described (Nowak and Mildvan, 1970). The extinction coefficient of P-enolpyruvate at 230 nm and pH 7.5 was 2.96 mM^{-1} cm^{-1} as reported by Wold and Ballou (1957). The metal-free enzyme was prepared using Chelex-100 as previously described (Nowak and Mildvan, 1970) and was found to have <1% residual activity as compared with the enzyme assayed in the presence of added $MgCl_2$. The metal-free enzyme, when assayed after $MgCl_2$ was added, was found to have 100 ± 5% the specific activity of the purified enzyme at 25° and at 230 nm (Westhead and McLain, 1964; Wold and Ballou, 1957).

The concentration of the yeast enzyme was determined by uv absorption assuming an extinction coefficient $E_{280}^{1\%} = 8.9$ (Warburg and Christian, 1941) and a mol wt of 88,000 (Mann *et al.*, 1970). In calculating the site concentration, the enzyme was assumed to have two active sites per mole. This assumption is based on the finding that the enzyme consists of two apparently identical subunits (Mann *et al.*, 1970; Brewer and Weber, 1968; Gawronski and Westhead, 1969) and has two tight binding sites for Mn^{2+} (Hanlon and Westhead, 1969a). The enzyme from muscle has been found to have two binding sites for phosphoglycolate (Cardenas and Wold, 1968) and for the affinity label glycidol phosphate (Rose and O'Connell, 1969).

Binding studies were carried out by titrations of the enolase–Mn^{2+} complex with ligands as previously described (Nowak and Mildvan, 1970). The longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of water protons were measured at 24 ± 1° with a Nuclear Magnetic Resonance Specialties PS60W pulsed nmr spectrometer adapted for operation at 3, 8, 15, 24.3, 40, and 60 MHz.[2] The dissociation constants K_3 and K_s are defined as

$$K_3 = \frac{[E\text{–}Mn][S]}{[EMnS]} \quad (2)$$

$$K_s = \frac{[E][S]}{[ES]} \quad (3)$$

The dissociation constants and enhancements of the ternary complexes, ϵ_T, were determined at 24.3 MHz by a computer fit to the PRR titration data which minimized the per cent standard deviation (% SD) of ϵ_T (Reed *et al.*, 1970). The values reported are the averages obtained from several titrations.

For measurements of the relaxation rates of the nuclei of the analogs, enolase was exchanged into D_2O by repeated concentration of the solutions by vacuum dialysis and dilution in 50 mM Tris-Cl (pD 7.5)[3] in D_2O. The ^1H nmr spectra were taken on a Varian XL-100-15 or a Varian HR-220 spectrometer, operated at 30 ± 1°. For the ^{31}P studies the spectrometer was operated at 40.5 MHz. Broad-band noise decoupling of the protons and 12-mm sample tubes were used to increase the ^{31}P signal level as previously described (Nowak and Mildvan, 1972b). Prior to the ^{31}P experiments, the sam-

[2] The modifications were designed and built by J. L. Engle and R. Taylor.

[3] The values for pD of solutions in D_2O are equal to the meter reading (pH) + 0.4 (Glascoe and Long, 1960).

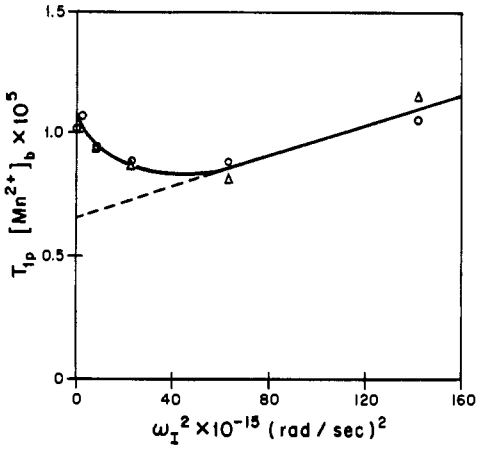


FIGURE 1: The paramagnetic contribution to the molar relaxation time (T_{1p}) of water protons as a function of frequency squared (ω_I^2) for the enolase-Mn^{2+} complex. The solution in 50 mM Tris-Cl and 0.5 M KCl, pH 7.5, contained 60 (△) or 120 (○) μM enzyme sites and 143 μM $MnCl_2$ in a volume of 0.05 ml. The values of T_{1p} were corrected to those due to bound Mn^{2+} from the dissociation constants of Mn^{2+}-enolase (Hanlon and Westhead, 1969a), and the measured effects of free Mn^{2+} at each frequency; temperature = 22.5 ± 0.5°

ples were passed through a small column of Chelex-100 to remove residual paramagnetic metal contaminants. D_2O (≥90%) was used as the solvent to permit deuterium locking.

The $1/T_2$ values were obtained from measurements of the width of the resonance signal at half-height at 5 decibels or more below saturation. The $1/T_1$ values were obtained at 220 MHz with the Varian HR-220 nmr spectrometer by a pulsed Fourier transform method (McDonald and Leigh, 1973) which measures the rate of recovery of the signal after demagnetization of the nuclei. The demagnetization is accomplished by a 90° pulse followed by the transient insertion of a field gradient. At variable time intervals after demagnetization, the recovery of magnetization is monitored by a second 90° pulse. The Fourier transform of the decay following this monitoring pulse constitutes the partially relaxed spectrum. Spectra from at least ten such intervals were recorded and $1/T_1$ values were calculated as the first-order rate constant for the recovery of the signals. The advantage of this method over the conventional 180°–τ–90° pulse sequence is that the present method requires no waiting period for signal recovery following the monitoring pulse. The $1/T_1$ values of protons at 100 MHz and of ^{31}P at 40.5 MHz were determined by measuring the power at which the signal saturates as previously described (Nowak and Mildvan, 1972b). The paramagnetic contributions to the relaxation rates ($1/T_{1p}$, $1/T_{2p}$) were calculated from the slope of the linear plot of relaxation rate against Mn^{2+} concentration. With CH_2-PEP, at Mn^{2+} concentrations below 30 μM, decreases in the line widths of all of the carbon-bound protons were observed with increasing Mn^{2+} concentration due to chemical exchange spin decoupling (Frankel, 1969a,b; Villafranca and Mildvan, 1972). Above 30 μM Mn^{2+} the resonances became singlets and the line widths increased linearly with Mn^{2+} concentration, and it is from this portion of the curve that the transverse relaxation rates were determined. The values $1/T_{1p}$ and $1/T_{2p}$ were normalized by the factor p = $[MnCl_2]$/[ligand] as previously described (Mildvan and Cohn, 1970; Mildvan and Engle, 1972).

TABLE I: Dissociation Constants (K_s) and Enhancements (ϵ_T) of Ternary Complexes of Yeast Enolase and Mn^{2+} with P-Enolpyruvate and Its Analogs Determined by Computer Analysis of PRR Titrations.[a]

Ligand	K_s (μM)	ϵ_T	% SD Range
L-Phospholactate	35 ± 10	8.39 ± 2.20	3.7–7.5
D-Phospholactate	25 ± 15	8.76 ± 1.86	4.0–4.4
Phosphoglycolate	45 ± 30	8.45 ± 2.47	2.8–9.1
CH_2-PEP[b]	8 ± 5	4.45 ± 0.49	0.8–1.6
P-Enolpyruvate[b]	0.5 ± 0.2	7.50 ± 0.70	4.5–8.4

[a] For the computer analysis, the dissociation constants and enhancement factors of the binary Mn^{2+}–ligand complexes were those previously used (Nowak and Mildvan, 1972b). The values for CH_2-PEP were assumed to be identical with those of P-enolpyruvate. For Mn^{2+}–enolase the dissociation constant 1.3 μM (Hanlon and Westhead, 1969a) and the enhancement factor 15.0 (Nowak and Mildvan, 1970) were used. The K_s values could be varied from 10^{-4} to 5 M without effect as described in the text. [b] An equilibrium mixture consisting of ~80% vinylic substrate as determined by nmr for CH_2-PEP[7] and by uv absorption for P-enolpyruvate (Wold and Ballou, 1957).

Results

Binding of Phosphoenolpyruvate and Its Analogs to Enolase–Mn^{2+} as Studied by $1/T_{1p}$ of Water Protons. Previous binding studies of P-enolpyruvate (Cohn and Leigh, 1962; Cohn, 1963) and its analogs (Nowak and Mildvan, 1970) to the enolase–Mn^{2+} complex by PRR titrations have been reported. The titrations which were previously analyzed by graphical methods, when reevaluated by computer analysis, yielded the parameters summarized in Table I. The values reported by graphical analysis were obtained by extrapolation to infinite concentration of ligand, neglecting the presence of significant amounts of binary Mn^{2+}–ligand complexes which had formed. The computer evaluation of these constants, which takes into account all possible equilibria, is deemed more reliable than the graphical analysis. The dissociation constants differ by an order of magnitude and the ϵ_T values differ by a factor ≤2 from those estimated graphically (Nowak and Mildvan, 1970). The quality of the computer fits expressed by the per cent standard deviation of ϵ_T was very sensitive to the K_s values assumed but was insensitive to the K_s values. Thus, equally good theoretical fits to the data could be obtained assuming K_s values of 1 × 10^{-4} to 5 M with little variation in the optimal values obtained for K_s or for ϵ_T. Thus, in the case of enolase, the PRR method does not detect a tight binary enzyme–analog complex (K_s < 10^{-4} M) in the absence of the divalent cation. In binding studies Wold (1971) had failed to detect complexes of enolase with substrates and substrate analogs in the absence of the divalent cation. In contrast with enolase, titrations of the pyruvate kinase–Mn^{2+} complex with P-enolpyruvate and its analogs required the assumption of stable binary enzyme–substrate and enzyme–analog complexes to obtain a theoretical fit to the experimental data (Nowak and Mildvan, 1972b).

Determination of the Correlation Time (τ_c) and the Coordination Number (q) for H_2O on Enolase-Bound Manganese.

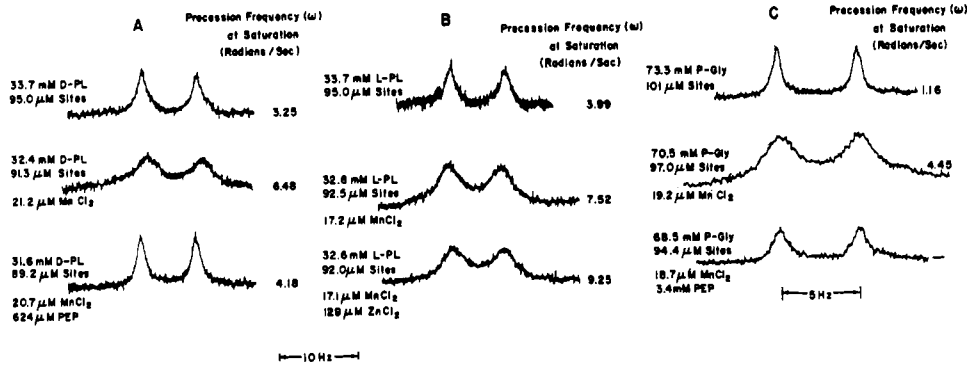


FIGURE 2: The effect of Mn^{2+}–enolase on the proton magnetic resonance spectra of D-phospholactate (A), L-phospholactate (B), and phosphoglycolate (C) at 100 MHz. The analogs were present as their potassium salts in D_2O, pH 7.5, in a 0.5 M KCl solution and the enzyme was added as a solution in 0.05 M Tris-Cl, pH 7.5, with 0.5 M KCl in D_2O. The $MnCl_2$ was added as a solution in D_2O and either P-enolpyruvate (potassium salt, pH 7.5) (A and C) or $ZnCl_2$ (B) were also added from solutions in D_2O. The initial volumes were 0.30 ml and the final volumes were 0.31–0.32 ml; temperature = 30 ± 1°.

In each of the ternary complexes studied (Table I) the enhancement factor, ϵ_T, is less than that of the binary complex, $\epsilon_b = 15.0 \pm 1.5$ (Nowak and Mildvan, 1970). As pointed out elsewhere (Mildvan and Cohn, 1970) such type II enhancement behavior ($\epsilon_T < \epsilon_b$) can be explained either by direct coordination of the ligand to the enzyme-bound Mn^{2+}, displacing a water ligand from the metal, or by a structural change about the Mn^{2+}-bound water elicited by the substrate, which could either decrease the correlation time for Mn^{2+}–H_2O interaction or increase the residence time of a coordinated water ligand. To help distinguish among these alternatives, the correlation time, τ_c, and the coordination number, q, for H_2O on enolase-bound Mn^{2+} were determined by a study of $1/T_{1p}$ of water protons in the binary complex as a function of frequency.

The Solomon–Bloembergen equation describing the Mn^{2+}–H_2O dipolar interaction (Solomon, 1955; Solomon and Bloembergen, 1956) is

$$1/pT_{1p} = [H_2O]/[Mn]T_{1p} = (812/r)^6 q(f(\tau_c)) \quad (4)$$

where r is the Mn^{2+}–water proton distance, 2.87 ± 0.05 Å from crystallographic data (see Reuben and Cohn, 1970), and q is the coordination number for H_2O. The correlation function, $f(\tau_c)$, is given by

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \quad (4a)$$

where τ_c is the correlation time for the dipolar interaction, ω_I is the nuclear resonance frequency, and ω_s is the electron resonance frequency. Equation 4 contains two unknown quantities, q and τ_c. Thus, if τ_c is determined from the frequency dependence of $1/T_{1p}$, then q can be evaluated (Peacocke *et al.*, 1969; Reuben and Cohn, 1970).

The values of T_{1p} for enolase–Mn^{2+} determined at 3, 8, 15, 24.3, 40, and 60 MHz, when plotted against ω_I^2 (Peacocke *et al.*, 1969), reveal a minimum between 24.3 and 40 MHz (Figure 1), indicating that τ_c itself is frequency dependent. Such behavior establishes that τ_s, the electron spin relaxation time of bound Mn^{2+}, dominates τ_c as has been found for several enzyme–Mn^{2+} complexes (Reuben and Cohn, 1970; Reed *et al.*, 1972). From the linear portion of the curve, $\tau_c = 2.18 \pm 0.55 \times 10^{-9}$ sec, a typical τ_s value for Mn^{2+} is calculated over the range of frequencies from 40 to 60 MHz. Using this value of τ_c in eq 4 with $r = 2.87 \pm 0.05$ Å, it is calculated that the coordination number $q = 2.1 \pm 0.4$ for water ligands with exchange rates greater than the largest relaxation rate measured. From the present study, the lower limit to the water exchange rate $1/\tau_M$ is greater than $1/pqT_{2p}$ (at 60 MHz) which is 7.7×10^6 sec^{-1}.

Since it was not feasible to determine the frequency dependence of ϵ_T, the structures of the ternary complexes were studied more directly by measurements of $1/T_1$ and $1/T_2$ of the protons and phosphorus nuclei of the analogs.

Effects of Enolase–Mn^{2+} on the Proton Relaxation Rates of Inactive Phosphoenolpyruvate Analogs. The proton nmr spectra of the three saturated analogs, D- and L-phospholactate (I, II) and phosphoglycolate (III), have previously been described (Nowak and Mildvan, 1972a,b). The effect of enolase-bound Mn^{2+} on the methyl or methylene protons of these analogs is shown in Figure 2. The enzyme-bound Mn^{2+} increases the relaxation rates of the protons of the three analogs as observed by the increase in line width of the proton resonances and by the increase in precession frequency at saturation.[4] A summary of the relaxation rates of the methyl or methylene protons of each of the analogs in the binary Mn^{2+}–ligand and ternary enzyme–Mn^{2+}–ligand complexes is given in Table II. When compared with the effects of Mn^{2+}, the

[4] Under the conditions of the nmr experiments, most of the Mn^{2+} is in the ternary enzyme–Mn^{2+}–analog complex and very little is present as free Mn^{2+} or as the binary Mn^{2+}–analog complex. This may be shown in three ways. First, a calculation of the distribution of Mn^{2+} indicates that at least 94% of the Mn^{2+} is in the ternary complex, less than 1% is free, and less than 6% is in the binary Mn^{2+}–analog complex. For these calculations the dissociation constants K_A' of Mn^{2+} from the ternary enzyme–Mn^{2+}–analog complexes were required. These were obtained from the relationship $K_A' = K_3K_D/K_s$ (Mildvan and Cohn, 1966). The values of K_D, the dissociation constants of the binary enzyme–Mn^{2+} complex, were determined by Hanlon and Westhead (1969a,b) to be 1.3 and 19 μM, the values of K_3 are given in Table I, and the values of K_s are $>10^{-4}$ (Table I). Second, displacement experiments with P-enolpyruvate (*e.g.*, Figure 3 and the text) also show that most of the Mn^{2+} is in the ternary complex and less than 5% is in the binary Mn^{2+}–analog complex. Otherwise P-enolpyruvate, at the low levels used, could not have removed the relaxation effects on the analogs since this substrate binds free Mn^{2+} no tighter than the analogs. Third, in our studies of ^{31}P relaxation, increasing the enzyme concentration by 40% had no detectable effect on $1/pT_{1p}$ of phosphorus indicating that the distribution of Mn^{2+} did not change significantly. Such behavior would be expected only if the Mn^{2+} were essentially all in the ternary complex.

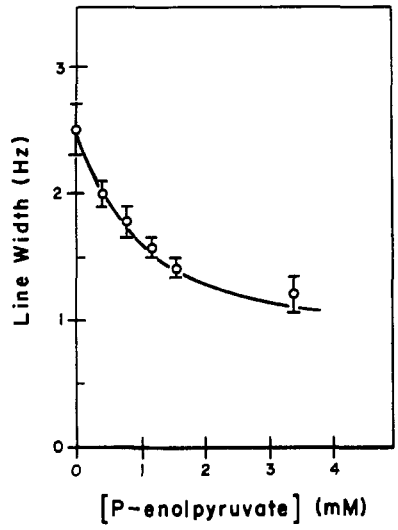


FIGURE 3: P-Enolpyruvate competition with phosphoglycolate. The effect of P-enolpyruvate on the line width of the methylene protons of phosphoglycolate (70.5 mM) was measured in the presence of enolase (74 μM sites) and $MnCl_2$ (19.2 μM) in a solution of 50 mM Tris-Cl and 0.5 M KCl in D_2O, pH 7.5; temperature = 30°. The points represent the experimental line widths measured after each addition of P-enolpyruvate and the curve is computed assuming simple competition between P-enolpyruvate (K_3 = 0.5 μM) and phosphoglycolate (K_3 = 35 μM) for enolase-Mn^{2+}.

effects of the enzyme-bound Mn^{2+} on the relaxation rates of these protons are de-enhanced with the exception of $1/pT_{2p}$ of the methylene protons of phosphoglycolate.

With both the phosphoglycolate and D-phospholactate complexes (Figures 2A and 2C, respectively), the addition of P-enolpyruvate diminishes the effect of enzyme-Mn^{2+} on the line width of the proton resonances, consistent with competition for the same Mn^{2+} site. A titration of the phosphoglycolate complex by P-enolpyruvate can be fit by assuming simple competition for the same site and using dissociation constants, K_3, for P-enolpyruvate (K_3 = 0.5 μM) and phosphoglycolate (K_3 = 35 μM) in agreement with values obtained by direct binding studies (Table I) (Figure 3). The addition of the diamagnetic cation Zn^{2+} to the enzyme-Mn^{2+}-L-phospholactate complex increases the paramagnetic effect of Mn^{2+} (Figure 2B), presumably by displacing the bound Mn^{2+}, since free Mn^{2+} is more effective in relaxing the methyl protons than is enzyme-bound Mn^{2+} (Table II).

Since the $1/pT_{2p}$ values for the protons of the analogs are greater than the respective $1/pT_{1p}$ values in the ternary complexes (Table II), the $1/pT_{1p}$ values are not influenced significantly by chemical exchange and hence, as has previously been shown (Nowak and Mildvan, 1972b), can be used to calculate Mn^{2+}-proton distances in the ternary complexes.

Effect of Enolase-Mn^{2+} on the Phosphorus Relaxation Rates of Inactive Phosphoenolpyruvate Analogs. The ^{31}P nmr spectrum of phosphoglycolate has been described (Nowak and Mildvan, 1972a,b) and is shown in Figure 4. As has previously been found (Nowak and Mildvan, 1972a,b), decoupling of the protons from the phosphorus resonance simplifies the spectrum to a singlet and improves the signal-to-noise ratio without affecting the measured relaxation rates of the phosphorus nucleus. Hence the proton-decoupled spectrum was used to measure the ^{31}P relaxation rates in the presence of enolase and Mn^{2+} (Figure 4, Table III). From Table III it is seen that enolase de-enhances the effect of Mn^{2+} on the ^{31}P relaxation rates of phosphoglycolate, similar to the effects observed with the protons of the analogs (Table II).

As pointed out under Methods, yeast enolase has two active sites per mole, presumably with one Mn^{2+} at each site. The detection of catalytically active monomers supports this view (Keresztes-Nagy and Orman, 1971; Holleman, 1971), as does a recent kinetic study with the Mg^{2+}-activated enzyme (Hanlon and Feins, 1969). From the dissociation constants for Mn^{2+} in the enolase-Mn^{2+}-substrate ternary complex it is calculated that 80% of the Mn^{2+} is on the tighter of the two sites (site I) and 20% of the Mn^{2+} is on the weaker site (site II) under the conditions of the present experiments (Figure 4). Mg^{2+} shows the opposite site preference from Mn^{2+} (Hanlon and Westhead, 1969a,b). Hence, the addition of $MgCl_2$ at a level (125 μM) which is 2.7 times the concentration of site II would be expected to shift the Mn^{2+} binding almost entirely to site I. The addition of $MgCl_2$ at this level, under the conditions described in Figure 3, produced no detectable changes in $1/pT_{1p}$ (<6%) or in $1/pT_{2p}$ (<3%) of phosphorus despite a 20% change in the calculated distribution of Mn^{2+}. These results indicate that Mn^{2+} exerts a very similar paramagnetic

TABLE II: Effect of Enolase-Mn^{2+} on the Relaxation Rates of the Protons of the Inactive Analogs of P-Enolpyruvate at 100 MHz.

Complex	$1/pT_{1p}$ (sec^{-1} × 10^{-3})	$1/pT_{2p}$ (sec^{-1} × 10^{-3})
Mn^{2+}-L-phospholactate (methyl protons)	14.9 ± 1.7[a]	15.4 ± 0.9[a]
Enolase-Mn^{2+}-L-phospholactate (methyl protons)	4.39 ± 0.38	9.70 ± 0.32
Enolase-Mn^{2+}-D-phospholactate (methyl protons)	3.45 ± 0.90	5.12 ± 0.35
Mn^{2+}-phosphoglycolate (methylene protons)	13.0 ± 0.5[a]	13.0 ± 1.0[a]
Enolase-Mn^{2+}-phosphoglycolate (methylene protons)	7.01 ± 0.25	19.7 ± 0.3

[a] From Nowak and Mildvan (1972b).

TABLE III: Effect of Enolase-Mn^{2+} on the Relaxation Rates of the Phosphorus Nuclei of P-enolpyruvate Analogs at 40.5 MHz.

Complex	$1/pT_{1p}$ (sec^{-1} × 10^{-3})	$1/pT_{2p}$ (sec^{-1} × 10^{-3})
Mn^{2+}-phosphoglycolate	11.3 ± 0.7[a]	3360 ± 240[a]
Enolase-Mn^{2+}-phosphoglycolate	3.8 ± 0.5	910 ± 150
Enolase-Mn^{2+}-CH_2-PEP	3.4 ± 0.4	59.4 ± 10.0

[a] From Nowak and Mildvan (1972b).

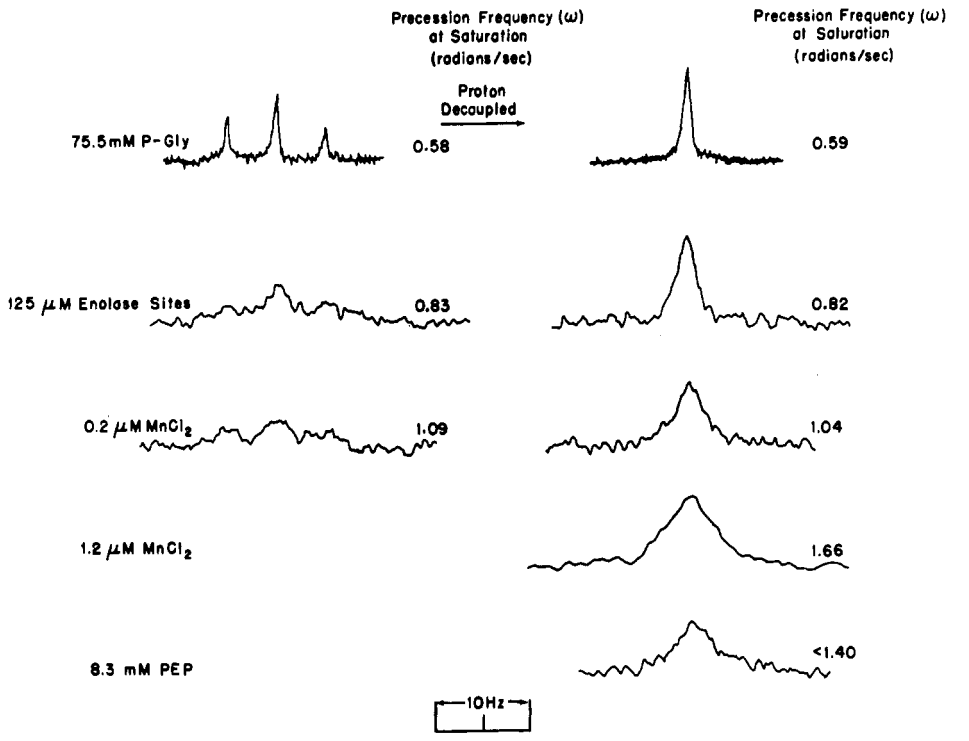


FIGURE 4: The effect of Mn^{2+} and enolase–Mn^{2+} on the ^{31}P nmr spectrum of phosphoglycolate at 40.5 MHz. The phosphoglycolate was present as the potassium salt in a solution of 0.5 M KCl–0.05 M Tris-Cl in D_2O, pH 7.5. Proton decoupling of the sample simplified the spectrum, increasing the signal-to-noise ratio, but did not affect the relaxation rates. Enolase was added as a solution in 0.05 M Tris-Cl–0.5 M KCl, pH 7.5. $MnCl_2$ was then added as a solution in D_2O. Proton decoupling again did not affect the relaxation rates within experimental error. P-Enolpyruvate was added to the solution as the potassium salt, pH 7.5. The effect of P-enolpyruvate on the line width of the ^{31}P nucleus of phosphoglycolate was titrated and the results were fit by assuming simple competition between P-enolpyruvate (K_3 = 0.5 μM) and phosphoglycolate (K_3 = 30 μM) for enolase–Mn^{2+}; temperature = 30 ± 1°.

effect on the phosphorus of phosphoglycolate whether it is on site I or on site II.

Since the $1/pT_{1p}$ is much less than the $1/pT_{2p}$, again $1/pT_{1p} = 1/T_{1M}$, the relaxation rate of the bound analog, and can be used to calculate the distance from the enolase-bound Mn^{2+} to the ^{31}P nucleus in the ternary complex.

Kinetic and Binding Properties of the Methylene Analog of P-Enolpyruvate with Enolase–Mn^{2+}. α-(Dihydroxyphosphinylmethyl)acrylic acid (IV), a methylene analog of P-enolpyruvate (CH_2-PEP), has been shown to be a substrate for muscle enolase (Stubbe and Kenyon, 1972). Under conditions described under Methods, this analog was found to be a substrate for the yeast enzyme. From initial velocity measurements at 200 μM $MnCl_2$ and at eight concentrations of CH_2-PEP, over the range 13.2–265 μM, the K_m of this analog was 12.8 ± 3.8 μM and the maximal turnover number was 1.7 sec^{-1}, a value which is 4.5% of the rate measured with saturating levels of 2-phosphoglycerate (200 μM) under otherwise identical conditions.[5] The Michaelis constant of CH_2-PEP is in reasonable agreement with the dissociation constant of this substrate determined by PRR titration (8 ± 5 μM, Table I). The ϵ_T value of the substrate is approximately half that of the more active substrate P-enolpyruvate and of the inactive analogs (Table I).

Effect of Enolase–Mn^{2+} on the Proton and Phosphorus Relaxation Rates of the Nuclei of the Methylene Analog of P-Enolpyruvate. The proton nmr spectrum of CH_2-PEP at pD 7.5[3] consists of the resonances of the methylene protons centered at 3.19 ppm downfield from the external Me_4Si standard (J_{P-H} = 20.5 Hz; $J_{H-H} \sim$ 1 Hz), the vinyl proton trans to the phosphonate group at δ 6.40 ppm (J_{P-H} = 4.7 Hz; J_{H-H} = 1.5 Hz; $J_{H-H} \sim$ 1 Hz), and the vinyl proton cis to the methylene phosphinate at δ 6.65 ppm (J_{P-H} = 4.3 Hz; J_{H-H} = 1.5 Hz). The ^{31}P multiplet was found at 22.9 ppm downfield from the resonance of a separate sample of H_3PO_4 (64%) in D_2O (20% by volume) similarly locked on deuterons. The P–H and methylene H–vinyl H couplings were verified by both heteronuclear and homonuclear spin decoupling, respectively. There is an uncertainty in the assignments of the vinyl protons which were based on the observation that the more upfield of the two vinyl protons had significantly greater coupling to phosphorus and to the methylene protons than the downfield proton. Trans coupling is generally greater than cis coupling in such systems (Brugel *et al.*, 1960); however, there are some exceptions (Stubbe and Kenyon, 1971). In any case, the uncertainty in these assignments does not alter the conclusions of this work.

As exemplified in Figure 5 the $1/T_1$ values of the protons of CH_2-PEP were measured by recovery of equilibrium using pulsed Fourier transform at 220 MHz and the progressive saturation method was used at 100 MHz as described under Methods.

In the presence of 215 μM enolase sites the relaxation rates of the protons of CH_2-PEP (90 mM) at 100 MHz were measured at varying concentrations of Mn^{2+} with phosphorus decoupling (Table IV). The paramagnetic contributions to

[5] For the second ionization, phosphonic acids are generally 1 pK unit less acidic than the corresponding phosphoric acids (Trowbridge *et al.*, 1972). Hence, at the pH of these studies (7.5) the concentration of the trianion form of CH_2-PEP would be significantly less than that of P-enolpyruvate.

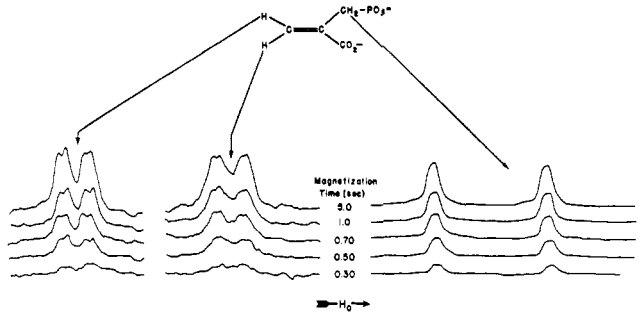


FIGURE 5: Determination of the relaxation rates of the protons of CH_2-PEP by the "return to equilibrium technique" (McDonald and Leigh, 1973) using pulsed Fourier transform nmr at 220 MHz. The relaxation rates of the protons of CH_2-PEP (50 mM) in the presence of enolase (90 μM) were measured in the presence of $MnCl_2$ (29.5 μM). The solutions contained 0.5 M KCl and 0.05 M Tris-Cl, pH 7.5, in D_2O and the temperature was controlled at 30 $\pm$ 1°; the volume was 0.5 ml.

the relaxation rates were measured, as described under Methods, at Mn^{2+} concentrations varying from 5.0 to 87.8 μM. Under the extreme concentrations of Mn^{2+} present, there is less than 0.1% free Mn^{2+} and less than 3% of the Mn^{2+}–CH_2-PEP complex. The addition of P-enolpyruvate (45 mM) to this complex halved the observed paramagnetic effects on the protons of CH_2-PEP, consistent with competition for the same site on the enzyme. The paramagnetic contributions to the relaxation rates of the protons of CH_2-PEP measured at the two frequencies are summarized in Table IV.[4,6]

Table III summarizes the relaxation rates of the phosphorus nucleus of CH_2-PEP[6] (74 mM) in the presence of 99.8 μM enolase sites and at Mn^{2+} concentrations from 0.6 to 3.4 μM.[4] While the $1/pT_{1p}$ value of CH_2-PEP is similar to that of phosphoglycolate, the $1/pT_{2p}$ value of this substrate is an order of magnitude less than that of the inhibitor, due to exchange-limited relaxation (*vide infra*).

The addition of P-enolpyruvate decreased the paramagnetic effect of enolase–Mn^{2+} on $1/T_2$ of the phosphorus of CH_2-PEP. A titration of this effect at P-enolpyruvate concentrations of 9.0, 19.9, and 39.2 mM could be fit theoretically by assuming simple competition between P-enolpyruvate (K_3 = 0.7 μM) and CH_2-PEP (K_3 = 3.5 μM) for the same site on the enzyme. These values for K_3 are in reasonable agreement with those obtained by PRR titration (Table I) and with the K_m of CH_2-PEP (12.8 $\pm$ 3.8 μM) determined in the present study, indicating that the complex detected by nmr has the dissociation constant of the kinetically active complex.

Correlation Times and Correlation Functions for the Mn^{2+}–Nuclear Interactions with the Various Enolase–Mn^{2+}–Ligand Complexes. It has previously been demonstrated that the longitudinal relaxation rate of a complexed ligand ($1/T_{1M}$) may be used in the Solomon–Bloembergen equations (Solomon, 1955; Solomon and Bloembergen, 1956) to calculate distances between paramagnetic metal ions and magnetic nuclei. These distances agree with those obtained by X-ray

[6] During the time course of the continuous wave proton nmr experiments with CH_2-PEP, only the resonances of the vinyl substrate were detected, indicating that CH_2-PEP accounted for at least 90% of the substrate present. Within 12 hr after completion of the experiments new resonances appeared, accounting for 15–20% of the total substrate present. The slow reaction was due to a limiting amount of Mn^{2+} present. Hence no corrections were made to the relaxation data for hydration of CH_2-PEP during the nmr experiments.

TABLE IV: Effect of Enolase–Mn^{2+} on the Relaxation Rates of the Protons of the Active Methylene Analog of P-Enolpyruvate.

Proton	ν_I (MHz)	$1/pT_{1p}$ ($sec^{-1} \times 10^{-3}$)	$1/pT_{2p}$ ($sec^{-1} \times 10^{-3}$)
Methylene	100	5.60 $\pm$ 1.80	25.9 $\pm$ 1.0
	220	3.08 $\pm$ 0.09	
Upfield vinyl	100	3.66 $\pm$ 1.28	18.1 $\pm$ 6.0
	220	0.86 $\pm$ 0.10	
Downfield vinyl	100	4.34 $\pm$ 1.18	30.5 $\pm$ 10.1
	220	0.76 $\pm$ 0.08	

diffraction (Mildvan and Cohn, 1970; Nowak and Mildvan, 1972b; Villafranca and Mildvan, 1972). For Mn^{2+}–proton interactions in a 1:1 complex, the appropriate form of the Solomon–Bloembergen equation is obtained from eq 4 as

$$r\ (\text{in Å}) = 812(T_{1M}f(\tau_c))^{1/6} \quad (5)$$

and for Mn^{2+}–phosphorus interactions it is

$$r\ (\text{in Å}) = 601(T_{1M}f(\tau_c))^{1/6} \quad (6)$$

where the correlation function, $f(\tau_c)$, is defined in eq 4a.

Hence, in order to use the relaxation rates of nuclei to calculate the distances between the paramagnetic probe and the nuclei from eq 5 and 6, the correlation time, τ_c, for the electron–nuclear dipolar interaction must be known from which the correlation function, $f(\tau_c)$, is calculated. Several methods have been used to estimate τ_c. The most common method has been to use the correlation time estimated for the Mn^{2+}–H_2O interaction from the ϵ_T value of the same complex as measured by pulsed nmr methods (Nowak and Mildvan, 1972b). The assumption is made that the correlation time for the Mn^{2+}–H_2O interaction is the same as for the Mn^{2+}–substrate (or inhibitor) interaction, an assumption which is justified by the observations that in the binary enolase–Mn^{2+}–(H_2O) complex (Figure 1) and in various other enzyme–Mn^{2+} complexes (Reuben and Cohn, 1970; Reed *et al.*, 1972), τ_c is dominated by the electron spin relaxation time, τ_s. A second method for estimating τ_c is from T_{1p}/T_{2p} ratios of that nucleus. This method assumes the absence of a hyperfine contribution to $1/T_{2p}$. In metal–ligand complexes in which a substantial hyperfine interaction may occur, a longer τ_c and thus an incorrect value for r would be calculated. The most satisfactory method for estimating τ_c is by a study of the dependence of the longitudinal relaxation rate on ω_I and ω_s, the resonance frequencies, since no ancillary assumptions are made. However, this method obviously requires extensive experimentation at several frequencies.

Several of these methods have been used to estimate τ_c and $f(\tau_c)$ for the enolase–Mn^{2+}–ligand interactions. A summary of the values of τ_c estimated by each of the three methods is given in Table V. The τ_c values for the carbon-bound protons show agreement to within an order of magnitude. The values of $f(\tau_c)$ (Table V) show better agreement to within a factor of 2.

The values of τ_c for phosphorus, calculated from the high T_{1p}/T_{2p} ratios, may be too great since they exceed the electron spin relaxation times of Mn^{2+} in the same complexes as re-

TABLE V: Correlation Times (τ_c) and Correlation Functions ($f(\tau_c)$) for Ternary Enolase Complexes.[a]

Complex	Interaction	$\tau_c \times 10^9$ sec			$f(\tau_c) \times 10^9$ sec		
		From ω_I	From T_1/T_2	From $1/T_1(H_2O)$[d]	From ω_I	From T_1/T_2	From $1/T_1(H_2O)$[d]
Enolase–Mn–(CH_2-PEP)	Mn–methylene protons	0.43[b]–0.83[c]	3.62	0.78	1.21[b]–1.96[c]	1.76	1.89
	Mn–upfield vinyl proton	0.73[b]–3.76[c]	3.79	0.78	1.72[c]–1.81[b]	1.72	1.89
	Mn–downfield vinyl proton	0.89[b]–3.69[c]	4.71	0.78	1.64[c]–2.03[b]	1.45	1.89
	Mn–phosphorus		>19.4	0.78		<2.29	2.26
Enolase–Mn–L-phospholactate	Mn–methyl protons		1.99	1.54		2.33	2.38
Enolase–Mn–D-phospholactate	Mn–methyl protons		1.10	1.62		2.26	2.39
Enolase–Mn–phosphoglycolate	Mn–methylene protons		2.50	1.55		2.17	2.39
	Mn–phosphorus		≥74.7	1.55		≤0.62	4.02

[a] $f(\tau_c)$ calculated for 100 MHz for protons and 40.5 MHz for phosphorus. [b] Calculated from $1/pT_{1p}$ at 100 and 220 MHz assuming maximal frequency dependence of τ_c. [c] Calculated as in footnote *b*, assuming no frequency dependence of τ_c. [d] Calculated assuming one exchangeable water molecule remaining.

flected in the respective τ_c values for the protons. The high T_{1p}/T_{2p} ratios for phosphorus may, in turn, be due to a contact or pseudocontact contribution to $1/T_{2p}$.[7] However, because of the definition of $f(\tau_c)$ as given in eq 4a and because of the sixth root relationship in eq 6, the effects of these variations in τ_c on the calculated distances are within the reported experimental errors (Table VI).

Calculated Distances between Enolase-Bound Mn^{2+} and the Protons and Phosphorus Atoms of the Ligands in the Various Ternary Complexes. The Mn^{2+}–proton distances in the ternary complexes of enolase, Mn^{2+}, and substrate or analog calculated with eq 5 from $1/pT_{1p}$ and the individual values of $f(\tau_c)$ for each proton are given in Table VI. The Mn^{2+}–phosphorus distances (Table VI) were calculated with eq 6 using the average $f(\tau_c)$ value for the protons of the substrate or analog and of H_2O in the same complex. The estimated error in the distances is obtained from the extreme values of $1/pT_{1p}$ and of $f(\tau_c)$. Because of the sixth root relationship in eq 5 and 6 a twofold uncertainty in T_{1M} or $f(\tau_c)$ would lead to only a 12% error in a calculated distance. The distances (Table VI) are too great for inner sphere enolase–Mn^{2+}–analog bridge complexes but are consistent with second sphere enolase–Mn^{2+}–(H_2O)–analog complexes in which a water ligand can intervene between the bound Mn^{2+} and the bound analogs (Figures 6 and 7).

Kinetic Studies of the Activator Constant of Mn^{2+} and the Michaelis Constant of 2-Phosphoglycerate. Because of its low concentration in the enolase equilibrium, and its slow exchange from the enzyme, the interaction of 2-phosphoglycerate with the enolase–Mn^{2+} complex could not be studied by nuclear relaxation methods. Hence the substrate kinetics of 2-phosphoglycerate with enolase and Mn^{2+} were reinvestigated to seek evidence for a metal bridge complex. After treatment of the enzyme to remove trace metals for the kinetic study, the enzyme had less than 0.5% residual activity. A determination of the K_m of $MnCl_2$ as a function of 2-phosphoglycerate (Figure 8A), using the most general treatment for metal ion activation (Dixon and Webb, 1964), shows that the K_m of Mn^{2+} increases with decreasing 2-phosphoglycerate concentration and an activator constant, K_A, for Mn^{2+} of 30.3 ± 9.0 μM is obtained from the intersection of the lines in Figure 8A where [2-phosphoglycerate] equals zero. This value is in reasonable agreement with the dissociation constant of Mn^{2+} from site II of enolase as determined by equilibrium dialysis (19 μM) (Hanlon and Westhead, 1969a). The K_m for $MnCl_2$ extrapolated to infinite phosphoglycerate is 4.5 ± 0.5 μM, in agreement with the value of 4.2 μM obtained in a direct binding study (Hanlon and Westhead, 1969a) and in a kinetic study (K_m = 6.3 ± 0.5 μM) (Hanlon and Westhead, 1969b). A least-squares fitting of the data gave identical results. Hanlon and Westhead (1969a,b) had observed that, over the range 0.1–1.0 mM, the concentration of 2-phosphoglycerate had no effect on the K_m of Mn^{2+} or Mg^{2+}. The present study was made at lower levels and over a wider range of concentrations of 2-phosphoglycerate (2.9–57 μM). Replotting the present data to obtain K_m values for 2-phosphoglycerate

TABLE VI: Calculated Distances between Mn^{2+} and the Nuclei of P-Enolpyruvate Analogs in the Enolase Ternary Complex.

Analog	Distance	r (Å)
L-Phospholactate	Mn–CH_3	7.16 ± 0.15
D-Phospholactate	Mn–CH_3	7.45 ± 0.28
Phosphoglycolate	Mn–CH_2	6.59 ± 0.10
	Mn–P	5.47 ± 0.11
CH_2-PEP	Mn–CH[a]	7.04 ± 0.34
	Mn–CH[b]	6.81 ± 0.26
	Mn–CH_2	6.50 ± 0.30
	Mn–P	6.23 ± 0.36

[a] Upfield vinyl proton. [b] Downfield vinyl proton.

[7] Contact or hyperfine effects, which operate through chemical bonds, may also be transmitted to ligands in the second coordination sphere of metals (Alei, 1964; Brown and Drago, 1970). Pseudocontact effects have not been detected for Mn^{2+} and would be expected to be small because of the high symmetry of the ligand field and the long electron spin relaxation time of Mn^{2+}.

TABLE VII: Kinetic Parameters[a] of the Exchange Reactions of the Enolase–Mn^{2+}–Ligand Complexes at 30°.

Ligand	k_{off}[b] (sec^{-1})	E_A (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$-T\Delta S^{\ddagger}$ (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)	k_{on}[c] (M^{-1} sec^{-1})
CH_2-PEP	1.10×10^5	7.49 ± 0.61	6.90	3.84	10.74	1.38×10^{10}
L-Phospholactate	$\geq 9.70 \times 10^3$					$\geq 2.77 \times 10^8$
D-Phospholactate	$\geq 5.12 \times 10^3$					$\geq 2.05 \times 10^8$
Phosphoglycolate	$\geq 9.10 \times 10^5$					$\geq 2.02 \times 10^{10}$
H_2O	$>7.70 \times 10^6$					

[a] The rate constants are defined as

$$\text{enolase–}Mn^{2+}\text{–}(H_2O)\text{–ligand} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{enolase–}Mn^{2+}\text{–}(H_2O) + \text{ligand}$$

[b] From $1/pT_{2p}$ for each ligand at 30° (Tables II and III). [c] Calculated from the relationship $K_3 = k_{off}/k_{on}$ using K_3 for each ligand (Table I).

at varying concentrations of Mn^{2+} (Figure 8B) shows that increasing $MnCl_2$ concentrations decrease the K_m of 2-phosphoglycerate and extrapolation to infinite concentration of $MnCl_2$ gives a K_m for 2-phosphoglycerate of 4.0 ± 1.5 μM, a value much lower than that previously reported for the K_m of 2-phosphoglycerate for the Mn^{2+}-activated enzyme (Hanlon and Westhead, 1969a,b). Extrapolation to zero concentration of Mn^{2+} gives an immeasurably large K_m for 2-phosphoglycerate, suggesting little or no substrate binding in the absence of the divalent cation. Again, a least-squares fitting of the data gave identical results. It should be pointed out that the data are not precise enough, especially at low 2-phosphoglycerate concentrations, to eliminate an ES complex in which $K_s \geq 200$ μM. These data suggest a role for Mn^{2+} in the binding of 2-phosphoglycerate to enolase to form a ternary enzyme–Mn^{2+}–2-phosphoglycerate bridge complex as previously suggested (Malmström, 1961; Cohn, 1963; Nowak and Mildvan, 1970; Mildvan, 1970).

Effect of Temperature on the Transverse Relaxation Rates of the ^{31}P Nucleus of CH_2-PEP. The paramagnetic effect of the enolase–Mn^{2+} on $1/pT_{2p}$ of the phosphorus nucleus of CH_2-PEP increases with temperature in a manner predicted by the Arrhenius equation over the entire temperature range possible for enolase (0–43°) (Figure 9). From the large and positive temperature dependence of $1/pT_{2p}$ (E_{act} = 7.5 kcal/mol), it follows that the transverse relaxation rate is dominated by $1/\tau_M$, the exchange rate of the nucleus into the ternary complex of enolase, Mn^{2+}, and substrate. From these data, the kinetic parameters for $1/\tau_M$ are calculated (Swift and Connick, 1962) and are summarized in Table VII. Table VII also includes lower limits for the exchange rates of the inactive analogs of P-enolpyruvate into their respective ternary complexes. The exchange rate of CH_2-PEP into the active enolase–Mn^{2+}–(H_2O)–CH_2-PEP complex (1.1×10^5 sec^{-1}) exceeds the maximal rate of hydration of CH_2-PEP (1.7 sec^{-1}) by five orders of magnitude. Hence the complex detected by nmr forms and dissociates rapidly enough to participate in catalysis.

Discussion

It had previously been shown that when Mn^{2+} binds to enolase its effect on the relaxation rate of water is enhanced, and the addition of substrates (Cohn and Leigh, 1962; Cohn, 1963; Nowak and Mildvan, 1970) or substrate analogs (Nowak and Mildvan, 1970) decreases this effect. These results are here confirmed (Table I) by a computer analysis of the data (Reed *et al.*, 1970). The computer analysis also confirms the lack of stereospecificity in the binding of D- and L-phospholactate to the enolase–Mn^{2+} complex since there is no difference in the affinity for these analogs or in the con-

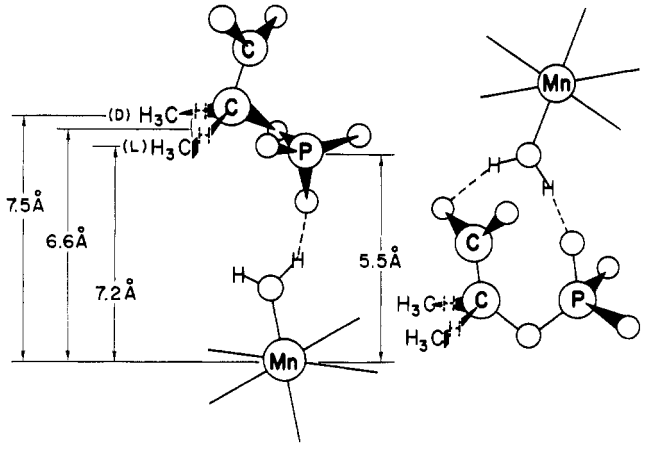


FIGURE 6: Alternate composite structures of ternary complexes of enolase–Mn^{2+} with saturated analogs. The structures are drawn consistent with the Mn^{2+}–nuclear distances calculated from $1/T_{1p}$ as described in the text (Table VI).

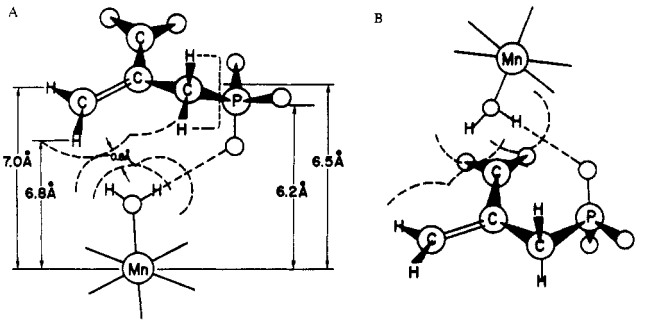


FIGURE 7: Alternate structures of the active enolase–Mn^{2+}–(H_2O)–CH_2-PEP ternary complex consistent with the Mn^{2+}–nuclear distances calculated from $1/T_{1p}$ (Table VI).

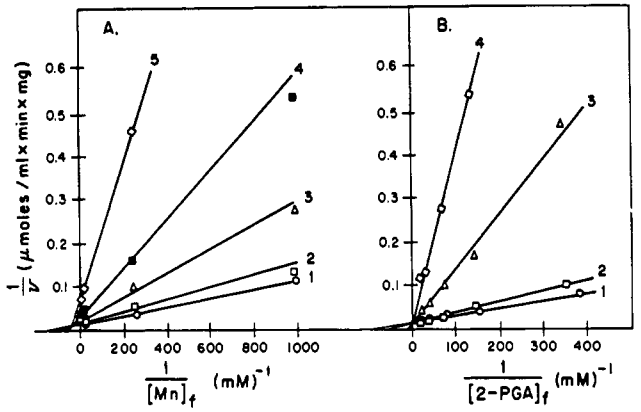


FIGURE 8: (A) A double reciprocal plot of the effect of manganese concentration on the initial velocity of the enolase reaction at varying 2-phosphoglycerate concentrations. The reactions contained the following concentrations of 2-phosphoglycerate: curve 1, 57.0 μM; curve 2, 28.5 μM; curve 3, 14.2 μM; curve 4, 7.1 μM; and curve 5, 2.29 μM. In addition the reaction mixtures contained 50 mM Tris-Cl buffer, pH 7.5, 0.5 M KCl, 0.57 μg of enolase, and $MnCl_2$ as indicated in a final volume of 1 ml; temperature = 25°. The free manganese concentration was calculated as described in the text. (B) A double reciprocal plot of the effect of 2-phosphoglycerate concentration on the initial velocity of the enolase reaction at varying manganese concentrations. The reactions contained the following concentrations of $MnCl_2$: curve 1, 100 μM; curve 2, 40 μM; curve 3, 4.0 μM; curve 4, 1.0 μM. The remaining conditions were those of Figure 8A with the concentrations of 2-phosphoglycerate as indicated. The velocity data at the lowest substrate concentration have an experimental uncertainty of ±30%; however, at all higher concentrations, an uncertainty of ±2–5% is obtained.

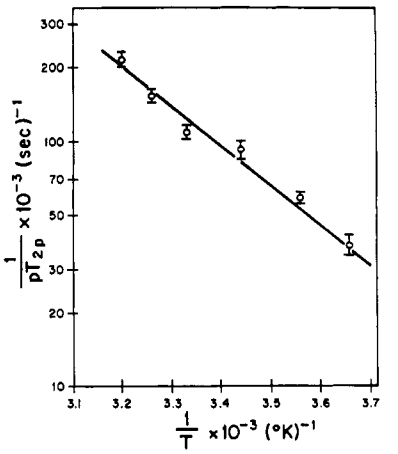


FIGURE 9: Arrhenius plot of the effect of temperature on the paramagnetic contribution to the transverse relaxation rate of the ^{31}P nucleus of CH_2-PEP in the enolase-Mn^{2+}-substrate ternary complex. The energy of activation, E_A, calculated is 7.49 ± 0.61 kcal/mol.

formation about the enzyme-bound Mn^{2+} in their respective ternary complexes as reflected in ϵ_T values (Table I). The equidistance of the methyl groups of D- and L-phospholactate from the enzyme-bound Mn^{2+} (Table VI, Figure 6) establishes this lack of stereospecificity in the ternary complex. This lack of stereospecificity must be due to the absence of the OH group at C-3 since the normal substrate is D-2-phosphoglycerate and the enantiomeric L-2-phosphoglycerate fails to interact with the enzyme (Wold, 1971).

The substrates of enolase contain three possible liganding groups for the enzyme-bound Mn^{2+}: the carboxyl and phosphate groups of both P-enolpyruvate and 2-phosphoglycerate, and the 3-OH group of 2-phosphoglycerate. Since the K_3 and ϵ_T values (Table I) afford only inconclusive evidence concerning the coordination schemes, direct experiments measuring the relaxation rates of the protons and phosphorus of the substrate analogs were carried out (Tables II and III). From these experiments, Mn^{2+}–proton and Mn^{2+}–phosphorus distances were calculated (Table VI). The distances between enzyme-bound Mn^{2+} and the methyl protons of D- and L-phospholactate and the methylene protons of phosphoglycolate are too great by 1.7–2.0 Å to permit carboxyl coordination, but are consistent with either phosphoryl coordination ($r \leq 7.2$ Å for methyl protons; $r \leq 6.2$ Å for methylene protons) or with the formation of second sphere, enolase-Mn^{2+}-(H_2O)-analog complexes. The calculated Mn^{2+}–phosphorus distance (5.47 ± 0.11 Å) (Table VI) is too great by 1.6–2.7 Å for direct coordination to the Mn^{2+} (2.9–3.8 Å), but overlaps with the correct distance for a second sphere complex (5.6–6.6 Å) in which a molecule of water intervenes between the enzyme-bound Mn^{2+} and the phosphorus of the ligand.[7] Thus, the inhibitory analogs of P-enolpyruvate form second sphere complexes with the enzyme-bound Mn^{2+}. Composite superpositions of the structures of the ternary complexes of the three inhibitors, consistent with all of the distances calculated for D- and L-phospholactate and phosphoglycolate (Figure 6), reveal two alternative second sphere enolase-Mn^{2+}-(H_2O)-analog complexes.

Hence, the decreases in enhancement on forming the ternary inhibitor complexes are not due to the displacement of water on Mn^{2+} by the inhibitors but could be due to (a) a decrease in the correlation time, τ_c, (b) the displacement of a water ligand on Mn^{2+} by a ligand from the protein, or (c) an increase in the residence time (τ_M) of coordinated water ligands. The first possibility is excluded since the τ_c values in the ternary complexes, calculated from the T_{1p}/T_{2p} ratios (Table V), are indistinguishable from that of the binary complex. Using these τ_c values, a coordination number, q, of 0.96 ± 0.35 rapidly exchanging water ligands on Mn^{2+} is obtained for the ternary inhibitor complexes. This value, which is significantly less than the corresponding value for the binary complex ($q = 2.2 \pm 0.6$), is consistent with either b or c. The similar τ_c values in the binary and ternary complexes argue against a large change in the ligand field at Mn^{2+} as would occur with b. An increase in the residence time (τ_M) of an inner sphere water molecule could well occur in a hydrogen-bonded second sphere complex (Figure 6).

More detailed information on the mechanism of action of enolase is obtained by studying the geometry of an active ternary complex. Such studies were not possible with either P-enolpyruvate or with 2-phosphoglycerate because of the tight binding (Table I) and resulting slow exchange of these substrates. Moreover, 2-phosphoglycerate is present only at a low concentration at equilibrium (~20%) (Wold and Ballou, 1957). The methylene analog of P-enolpyruvate is a substrate which is hydrated at a rate which is 1.6% that of P-enolpyruvate with muscle enolase (Stubbe and Kenyon, 1972) and 4.5% that of P-enolpyruvate with yeast enolase. This substrate binds to the yeast enzyme an order of magnitude more weakly than does P-enolpyruvate but an order of magnitude more tightly than the inhibitory analogs[5] (Table I). The distances between the bound Mn^{2+} and the vinyl and methylene protons and the phosphorus atom of CH_2-PEP in the active ternary enolase-Mn^{2+}-CH_2-PEP complex (Table VI) are too great for direct coordination to the Mn^{2+} and are consistent

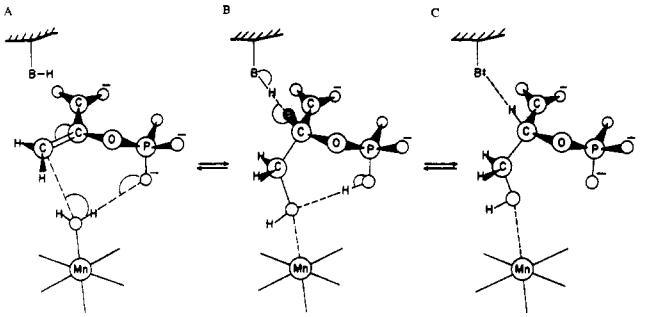


FIGURE 10: Catalytic mechanism of enolase. This mechanism is consistent with the structure of the active ternary complex (Figure 7A) as well as with kinetic studies and isotope exchange studies (Dinovo and Boyer, 1971), as discussed in the text.

only with a second sphere complex. Two alternative structures are shown in Figure 7.

As with the inhibitory analogs, the decrease in ϵ_T with CH_2-PEP may be due to an increase in τ_M, the residence time of coordinated water ligands, as suggested by the value of $q = 0.31 \pm 0.03$ calculated for the number of rapidly exchanging water molecules in the active ternary complex. Such an increase in τ_M could result from the hydrogen-bonded second sphere complex shown in Figure 7. As with the inhibitory analogs, the displacement of a water ligand on Mn^{2+} by a ligand from the protein in the ternary complex cannot be rigorously excluded but is not supported by a detectable change in τ_c (Table V).

From the temperature dependence of $1/pT_{2p}$ of the phosphorus nucleus of CH_2-PEP, the relaxation rate was found to be limited by the exchange rate of the analog from the enzyme complex ($k_{off} = 1/\tau_M = 1.10 \times 10^5$ sec^{-1}). Using the dissociation constant K_3 measured for the ternary complex, the rate of formation ($k_{on} = 1.38 \times 10^{10}$ M^{-1} sec^{-1}, Table VII) of this complex is calculated. This high rate of formation of the ternary CH_2-PEP complex also argues against the direct coordination of this substrate by the enzyme-bound Mn^{2+}. The value of k_{on} is consistent with a diffusion-controlled reaction (Eigen and Hammes, 1963) and is an order of magnitude greater than the value expected for a ligand substitution reaction on Mn^{2+} in which a trivalent anion replaces a coordinated water molecule ($k = 1.6 \times 10^9$ M^{-1} sec^{-1}) (Eigen and Hammes, 1963; Mildvan, 1970).

The Mn^{2+} to substrate nuclei distances (Figure 7), consistent with a second sphere enolase–Mn^{2+}–(H_2O)–CH_2-PEP complex, strongly suggest that the bound Mn^{2+} activates the water molecule which is to attack the vinylic substrate rather than activating the substrate itself. Stereochemical studies of the hydration reaction with the muscle enzyme (Cohn *et al.*, 1970) indicate the trans addition of H_2O; a proton adds to C-2 from the *si* face and a hydroxyl ion adds to C-3 from the *re* face of the vinyl plane. Hence the two geometries possible for the second sphere complex (Figure 7) predict opposite modes of activation of water by Mn^{2+} in the hydration reaction. Thus, in Figure 7A in which Mn^{2+} is below the vinyl plane of the substrate, the divalent cation would increase the nucleophilicity of water to facilitate hydroxyl attack on C-3 of the substrate. From the distance calculations (Table VI), using appropriate van der Waals radii (Pauling, 1960) (Figure 7A), the oxygen atom of the attacking water ligand is less than 1 Å from C-3 of CH_2-PEP. Alternatively, in Figure 7B where the Mn^{2+} is above the vinyl plane of the substrate the cation could promote the electrophilicity of water and facilitate a proton transfer to C-2 of the substrate. From the distance calculations and appropriate van der Waals radii (Figure 7B) the proton of the coordinated water is in molecular contact with C-2 of CH_2-PEP. Hence, while the nmr data exclude an inner sphere enolase–Mn^{2+}–substrate complex and suggest a second sphere enolase–Mn^{2+}–(H_2O)–substrate complex, the data fail to distinguish between the two possible modes of activation of H_2O shown in Figure 7.

Other data support the structure shown in Figure 7A over that in Figure 7B. Thus, the addition of a coordinated hydroxyl group to C-3 of P-enolpyruvate as depicted in Figure 10 would result in the formation of an enolase–Mn^{2+}–2-phosphoglycerate complex directly coordinated through the 3-OH group of the substrate. The microscopic reverse of this process would require the same complex to form prior to the dehydration of 2-phosphoglycerate (Figure 10C). Our kinetic data, in which the K_m of 2-phosphoglycerate becomes very large as the concentration of Mn^{2+} approaches zero (Figure 8A), suggest the formation of such an enolase–Mn^{2+}–2-phosphoglycerate bridge complex (Figure 10C). The lower coordination number for rapidly exchangeable water molecules in the active ternary complex (0.3) as compared with that in the inactive ternary complexes ($\sim$1.0) may be due to the replacement of an exchangeable water molecule by the 3-OH group of the hydrated product, directly coordinated to the enzyme-bound Mn^{2+}.

The mechanism depicted in Figure 10 is consistent with our nmr and kinetic data and with mechanisms proposed for closely related reactions (Mildvan, 1971; Villafranca and Mildvan, 1972). It is also consistent with the rate-controlling formation of a carbanion intermediate established for muscle enolase by incisive studies of the rates of isotopic exchange and by the detection of an inverse secondary kinetic isotope effect at C-3 of P-enolpyruvate (Dinovo and Boyer, 1971). In the proposed mechanism (Figure 10), the phosphate group of the substrate is seen to function as a general base in the hydration of P-enolpyruvate and as a specific acid in the dehydration of 2-phosphoglycerate. This proposal is consistent with the pH dependence of the enolase reaction (Wold and Ballou, 1957; Shen and Westhead, 1973).

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References

Alei, M., Jr. (1964), *Inorg. Chem. 3*, 44.
Brewer, J. M., and Weber, G. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 216.
Brown, D. G., and Drago, R. S. (1970), *J. Amer. Chem. Soc. 92*, 1871.
Brugel, W., Ankel, Th., and Kruekeberg, F. (1960), *Z. Elektrochem. 64*, 1121.
Cardenas, J. M., and Wold, F. (1968), *Biochemistry 7*, 2736.
Cohn, M. (1963), *Biochemistry 2*, 623.
Cohn, M., and Leigh, J. S. (1962), *Nature (London) 193*, 1037.
Cohn, M., Pearson, J. E., O'Connell, E. L., and Rose, I. A. (1970), *J. Amer. Chem. Soc. 92*, 4095.
Dinovo, E. C., and Boyer, P. D. (1971), *J. Biol. Chem. 246*, 4586.
Dixon, M., and Webb, E. C. (1964), Enzymes, New York.

N. Y., Academic Press, p 438.
Eigen, M., and Hammes, G. (1963), *Advan. Enzymol. 25*, 1.
Frankel, L. S. (1969a), *J. Chem. Phys. 50*, 943.
Frankel, L. S. (1969b), *J. Mol. Spectrosc. 29*, 273.
Gawronski, T. H., and Westhead, E. W. (1969), *Biochemistry 8*, 4261.
Glascoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Hanlon, D. P., and Feins, R. H. (1969), Abstracts, 158th National Meeting of the American Chemical Society, New York, N. Y., Abstract BIOL 57.
Hanlon, D. P., and Westhead, E. W. (1965), *Biochim. Biophys. Acta 96*, 537.
Hanlon, D. P., and Westhead, E. W. (1969a), *Biochemistry 8*, 4247.
Hanlon, D. P., and Westhead, E. W. (1969b), *Biochemistry 8*, 4255.
Holleman, W. H. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 36 Abstr.
Keresztes-Nagy, S., and Orman, R. (1971), *Biochemistry 10*, 2506.
Malmström, B. (1961), *Enzymes 5*, 475.
Mann, K. G., Castellino, F. J., and Hargrave, P. A. (1970), *Biochemistry 9*, 4002.
McDonald, G. G., and Leigh, Jr., J. S. (1973), *J. Magn. Resonance 9* (in press).
Mildvan, A. S. (1970), *Enzymes 2*, 446.
Mildvan, A. S. (1971), *Advan. Chem. Ser. No. 100*, 390.
Mildvan, A. S., and Cohn, M. (1966), *J. Biol. Chem. 241*, 1178.
Mildvan, A. S., and Cohn, M. (1970), *Advan. Enzymol 33*, 1.
Mildvan, A. S., and Engle, J. L. (1972), *Methods Enzymol. 26C*, 654.
Nowak, T., and Mildvan, A. S. (1970), *J. Biol. Chem. 245*, 6057.
Nowak, T., and Mildvan, A. S. (1972a), *Biochemistry 11*, 2813.
Nowak, T., and Mildvan, A. S. (1972b), *Biochemistry 11*, 2819.
Pauling, L. (1960), The Nature of the Chemical Bond, Ithaca, N. Y., Cornell University Press.
Peacocke, A. R., Richards, R. E., and Sheard, B. (1969), *Mol. Phys. 16*, 177.
Reed, G. H., Cohn, M., and O'Sullivan, W. J. (1970), *J. Biol. Chem. 245*, 6547.
Reed, G. H., Diefenbach, H., and Cohn, M. (1972), *J. Biol. Chem. 247*, 3066.
Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245*, 6539.
Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244*, 6548.
Shen, T., and Westhead, E. W. (1973), *Biochemistry* (in press).
Solomon, I. (1955), *Phys. Rev. 99*, 559.
Solomon, I., and Bloembergen, N. (1956), *J. Chem. Phys. 25*, 261.
Stubbe, J. A., and Kenyon, G. L. (1971), *Biochemistry 10*, 2669.
Stubbe, J. A., and Kenyon, G. L. (1972), *Biochemistry 11*, 338.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Trowbridge, D. B., Yamamoto, D. M., and Kenyon, G. L. (1972), *J. Amer. Chem. Soc. 94*, 3816.
Villafranca, J. J., and Mildvan, A. S. (1972), *J. Biol. Chem. 247*, 3454.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Westhead, E. W., and McLain, G. (1964), *J. Biol. Chem. 239*, 2464.
Wold, F. (1971), *Enzymes 5*, 499.
Wold, F., and Ballou, C. E. (1957), *J. Biol. Chem. 227*, 301.