Equilibrium Measurements of the Interaction of Yeast Enolase with Activating Metal Ions*

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ABSTRACT: A study has been made of the interaction of yeast enolase (phosphoenolpyruvate hydratase EC 4.2.1.11) with two activating cations, Mn^{2+} and Mg^{2+}, and one inhibitory cation, Ca^{2+}. In the absence of substrate 2 moles of Mg and Mn are bound per 67,000 molecular weight unit, the molecular weight unit usually present and considered the native molecule. Dissociation constants of 1×10^{-5} and 5×10^{-4} were found for Mg^{2+} and 1.3×10^{-6} and 1.9×10^{-5} for Mn^{2+}. Competition studies showed that the tighter bound magnesium ion competes for the same site as the weaker bound manganese ion, although the first site filled by either ion produces the spectral shift reported earlier. In the presence of substrate, at least 2 more moles of either metal ion are bound with considerably weaker affinities. The data show that both substrate and these additional cations must bind to the enzyme alone, but that both substrate and the extra metal ions are bound more tightly in the tertiary complex. The binding of the first 2 moles of cation does not appear to be influenced by the presence of substrate. Calcium ion is found to bind to a multiplicity of sites. Over a threefold range of protein concentration, there was no concentration effect upon the metal dissociation constants.

Numerous enzyme systems involving activation by divalent cations have been investigated (*e.g.*, Malmström and Rosenberg, 1959) but, generally, these investigations have been kinetic in nature with little or no correlation made with binding of metal ion activators measured by equilibrium techniques. Where equilibrium studies are reported, workers have generally used indirect measures of metal binding. The few direct binding measurements made were often carried out under conditions of temperature, buffer concentration, and substrate concentration which were considerably different from those employed in the corresponding kinetic investigations. In order to construct a meaningful mechanism of metal ion activation it would seem essential to obtain direct binding data for the metal activator–enzyme complex in the absence and presence of substrate, using conditions essentially identical with those used for kinetic measurements.

Yeast phosphoenolpyruvate hydratase (enolase, EC 4.2.1.11) catalyzes the interconversion of PGA[1] and PEP. The enzyme has measurable activity only in the presence of certain divalent cations which include, in order of decreasing maximum velocity effect, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, and Fe^{2+} (Wold and Ballou, 1957). Ca^{2+}, Cu^{2+} and Hg^{2+} inhibit (Malmström, 1953). Cohn (1963) and Malmström (1953) have studied the interaction of yeast enolase with activating metal ions under equilibrium conditions using nuclear magnetic resonance and electron spin resonance techniques and concluded that a "bridge" mechanism is operative wherein the metal ion serves to bind substrate to enzyme. Our measurements of the interactions of yeast enolase with Mg^{2+} and Mn^{2+} using difference spectroscopy (Hanlon and Westhead, 1965) indicated that metal sites exist in addition to those shown to be present by Cohn and Malmström. It seemed most reasonable that the differences between our findings and those of others could be resolved by making direct measurements of the number of metal binding sites of yeast enolase. Therefore, in this paper we report on equilibrium dialysis and gel filtration studies performed in the absence and presence of substrate and relate our findings to optical rotatory dispersion data and difference spectroscopy data obtained under similar conditions. In the following paper we correlate the information obtained from direct binding measurements with kinetic findings in an attempt to construct a plausible mechanism for the metal ion activation of yeast enolase.

Experimental Section

Materials. Yeast enolase used in these studies was prepared by the method of Westhead and McLain (1964). The enzyme was made metal free by absorbing it to phosphorylated cellulose, as described in the reference above, and washing several times with a solution of 10^{-4} M EDTA before elution. Freshly prepared metal-free enzyme generally had a specific activity of 290–310 units under standard assay conditions. Occasionally, preparations with a specific activity as low as 270 units were used. When corrections were made for the decrease in concentration of active enzyme, the binding data obtained with these preparations were identical with those obtained with enzyme possessing the greatest specific activity. Assays made at the termination of dialysis and gel filtration experiments showed no decrease in specific activity.

Metal salts used were all in the form of their hydrated dichlorides and were Baker Analyzed reagents. Solutions were prepared using doubly distilled water. D-2-Phosphoglycerate was obtained as its barium salt (Grade B) from California Biochemical Corp. and converted into the potassium salt as described by Westhead and McLain (1964). Stock solutions

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[1] Abbreviations used are: PGA, phosphoglyceric acid; PEP, phosphoenolpyruvic acid.

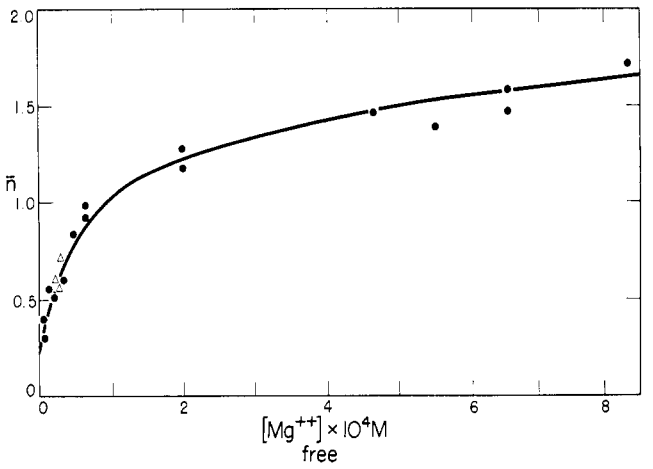

FIGURE 1: A plot of $\bar{n}$, the number of Mg^{2+} ions bound per molecule of active enolase, against the free Mg^{2+} concentration. Closed circles represent dialysis measurements. Open symbols represent Sephadex measurements. The solid line is a binding curve assuming the K_d value for Mg^{2+} and enolase in the absence of substrate listed in Table I.

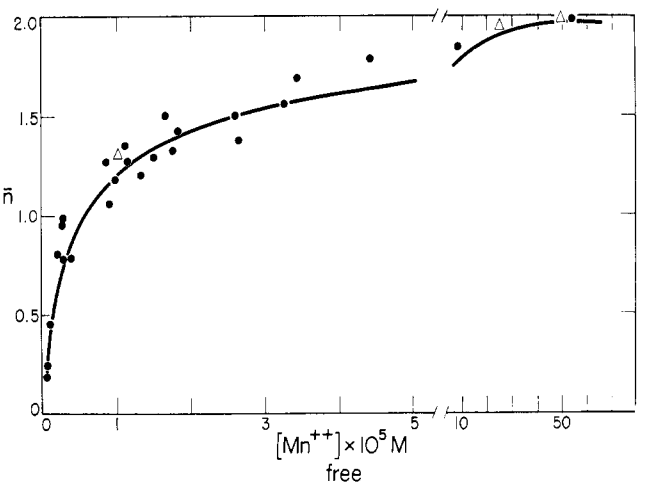

FIGURE 2: A plot of $\bar{n}$, the number of Mn^{2+} ions bound per molecule of active enolase, against the free Mn^{2+} concentration. Closed circles represent dialysis measurements. Open symbols represent Sephadex measurements. The solid line is a binding curve for enolase and Mn^{2+} in the absence of substrate assuming the K_d values listed in Table I.

of potassium 2-phosphoglycerate of known concentration were prepared using a spectrophotometric assay (Westhead, 1966). Phospholactate was a gift obtained as the cyclohexylamine salt from Dr. Clinton Ballou. 3-Phosphoglyceric acid was obtained from Sigma as the cyclohexylammonium salt. Radioisotopes were obtained from Isoserve Corp., Cambridge, Mass.

Methods. All metal binding and optical rotation studies were made in 0.5 M potassium chloride–0.05 M Tris-Cl (pH 7.5) at 22°. Measurement of free metal ion and metal bound to enolase was simplified and the sensitivity of the measurement enhanced by employing radioisotopic forms of manganese, $^{54}Mn^{2+}$, magnesium, $^{28}Mg^{2+}$, and calcium, $^{47}Ca^{2+}$.

Carrier-free $^{54}Mn^{2+}Cl_2$ (63.5 μCi/ml) was diluted with nonradioactive $MnCl_2$ in buffer to achieve the desired molarity of metal ion possessing a usable number of counts per volume assayed for radioactivity. The half-life of ^{28}Mg is 21.3 hr and the isotopic enrichment is small (8.62 μCi/mg of Mg^{2+}). Since the concentration of the solution of $^{28}MgCl_2$ was 2.24 $\times 10^{-2}$ M as obtained from Isoserve, one must use the solution of radioisotope with no further dilution by carrier Mg^{2+} in order to have a sufficient number of counts for reliable data when very low concentrations of free metal ion are desired. Although ^{28}Mg is a relatively short-lived isotope, rapid equilibrium in the microdialysis cells and the use of a gel filtration technique obviates this difficulty. $^{47}CaCl_2$ was obtained as an aqueous solution containing 209 Ci/mg of $^{56}Ca^{2+}$ and was 2.3 $\times 10^{-2}$ M in Ca^{2+}. Although the isotopic enrichment of $^{47}Ca^{2+}$ is as low as that of the Mg^{2+} isotope, the longer half-life of ^{47}Ca (4.5 days) decreases the necessity for immediate use.

Radioactivity was detected by a Nuclear-Chicago well scintillation counter.

Equilibrium dialysis studies were made using a semi-micro dialysis apparatus described by Malmström (1955). The dialysis membrane was a single layer of Visking tubing which had been previously washed as described by Westhead and McLain (1964). In a typical experiment the volume of liquid on each side of the dialysis membrane was 0.3–0.4 ml; the protein concentration was 1–10 mg/ml. After the addition of all components the five-chambered apparatus was clamped to a Burrell wrist-action shaker and shaken gently for the 2-hr period required to reach equilibrium. During this period there was little or no volume lost due to evaporation. In each dialysis chamber the total concentration of metal ion and protein was adjusted so that at equilibrium the difference in counts per unit volume assayed for radioactivity from the two sides of the membrane was at least 15%. Generally, 0.100–0.200 ml was used in counting. The total number of counts for each sample was always set so that the probable error in counting was less than 0.75% at a confidence level of 99%. Upon completion of counting, the protein aliquots were diluted as required and protein concentration was determined by making absorbance readings at 280 mμ. Absorbance was converted into molarity of enolase by assuming that 1.0 mg/ml of enzyme has an absorbance of 0.89 at 280 mμ (Warburg and Christian, 1941), and that the molecular weight of enolase is 67,000 (Malmström *et al.*, 1959). Measurements of metal binding to yeast enolase using gel filtration were performed with a column of Sephadex G-25 fine grade (2.5 $\times$ 6.0 cm) in buffer containing a known concentration of radioactive metal ion. This size column was sufficient to allow complete equilibrium between free metal ion and metal bound to enolase without excessive dilution of protein. In a typical experiment a 1.0-ml aliquot of enzyme in the metal ion buffer solution was run through the column using more metal ion buffer.

First 8.5 ml of liquid was collected at a flow rate of approximately 3 ml/min. Without changing the rate of flow, 6-drop (approximately 0.5 ml) aliquots were collected in small plastic tubes for a total of 12 samples. Carefully measured volumes from each tube were used to estimate protein and metal ion concentrations.

Optical rotatory dispersion measurements were made at 25° using a Rudolph polarimeter (Model 200) with a mercury

TABLE I: Dissociation Constants for the Interaction of Yeast Enolase with Mg^{2+} and Mn^{2+} in the Absence and Presence of an Equilibrium Mixture of 2-PGA and PEP Resulting from the Addition of 1.2×01^{-3} M 2-PGA.[a]

	Mg^{2+}		Mn^{2+}	
	No Substrate (M)	1.2×10^{-3} M Substrate (M)	No Substrate (M)	1.2×10^{-3} M Substrate (M)
$K_{d(1)}$	0.96×10^{-5}	1.0×10^{-5}	0.13×10^{-5}	0.10×10^{-5}
$K_{d(2)}$	47×10^{-5}	1.7×10^{-4}	1.9×10^{-5}	0.42×10^{-5}
$K_{d(3)}$		1.28×10^{-3}		3.5×10^{-5}
$K_{d(4)}$		1.28×10^{-3}		5.3×10^{-5}

[a] K_d values have been corrected for the interaction of metal ion with substrate using the values listed in the text.

light source. Protein solutions were centrifuged for 10 min at 8000*g* to eliminate any turbidity before polarimeter readings were taken. Rotations were read at 313, 366, 404, 436, and 546 mμ.

Ultraviolet difference spectra were obtained at protein concentrations of 1–2 mg/ml as described before (Hanlon and Westhead, 1965), using a Cary 14 spectrophotometer, at 22–23°, and with the same buffer solutions used for the equilibrium dialysis measurements.

Results

Metal Binding Experiments. MEASUREMENTS MADE IN THE ABSENCE OF SUBSTRATE. The interaction of yeast enolase with Mg^{2+}, Mn^{2+}, and Ca^{2+} is depicted in Figures 1, 2, and 3 as plots of $\bar{n}$, the number of metal ions bound per molecule of enolase, *vs.* free metal ion concentration. For both Mg^{2+} and Mn^{2+}, a maximum of two metal ions are bound over the concentration range studied. The agreement between dialysis and Sephadex data in Figures 1 and 2 is good, indicating that equilibrium between free metal ion and metal bound to the enzyme has been achieved in the gel filtration experiments.

Ca^{2+} interaction with enolase is quite another story. At a free Ca^{2+} concentration of 10^{-4} M at least six atoms are bound. Ca^{2+}–enolase binding data were obtained using the Sephadex method exclusively because dialysis measurements of Ca^{2+} binding gave low and inconsistent values for $\bar{n}$.

Plots of $\bar{n}$ *vs.* $-\log [M^{2+}]$ were made to obtain temporary dissociation constants, $K_{d(temp)}$, for the interaction of Mg^{2+} or Mn^{2+} with yeast enolase. An example is shown in Figure 4, using data for the binding of Mn^{2+} to enolase in the absence of substrate. K_d values were then calculated by computer using a least-squares curve-fitting technique (Margenau and Murphy, 1956) which corrects temporary K_d values in a general expression (eq 1) for independent binding of metal ions at N sites on a protein molecule, where $\bar{n}$ is the number of metal atoms bound at a given concentration of free metal ion. $K_{d(1)}$, $K_{d(2)}$, and $K_{d(N)}$ are dissociation constants for the first, second, and Nth metals bound. K_d values for the inter-

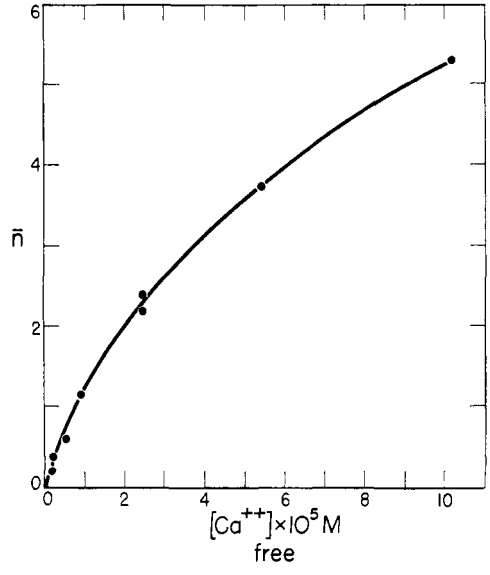

FIGURE 3: A plot of $\bar{n}$, the number of Ca^{2+} ion bound per molecule of active enolase, against the free Ca^{2+} concentration. All data were obtained by the Sephadex method. The solid line is a best-fit curve through the experimental points.

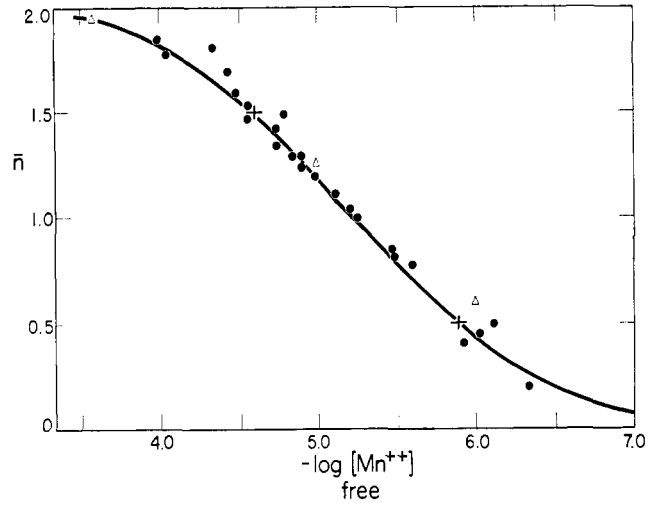

FIGURE 4: Mn^{2+}–enolase data plotted in the form of $-\log [Mn^{2+}]$ *vs.* $\bar{n}$ for the binding of Mn^{2+} to enolase in the absence of substrate. Closed circles and crosses are dialysis data and open symbols represent Sephadex binding data. The regression line is a theoretical curve derived from temporary K_d values of 10^{-6} and 2×10^{-5} M.

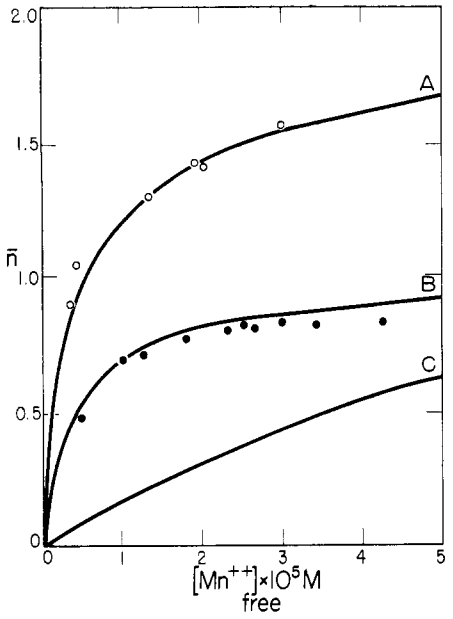

FIGURE 5: A plot of $\bar{n}$, the number of Mn^{2+} ions bound per molecule of active enolase against the free Mn^{2+} concentration in the presence of 10^{-3} M Mg^{2+}. Open circles represent control dialysis data for Mn^{2+} binding in the absence of Mg^{2+}. Closed circles represent dialysis data obtained in the presence of 10^{-3} M Mg^{2+}. Curve A is a theoretical curve for Mn^{2+} binding to enolase in the absence of Mg^{2+}, assuming the K_d values listed in Table I. Curve B is constructed assuming that the more tightly bound Mn^{2+}, with a K_d of 0.13×10^{-5} M, interacts at the same enolase site as does the weaker bound Mg^{2+}, $K_d = 4.7 \times 10^{-4}$ M, and that the weaker bound Mn^{2+}, $K_d = 1.9 \times 10^{-5}$ M, competes with the more tightly bound Mg^{2+} with a $K_d = 0.96 \times 10^{-5}$ M. Curve C is constructed assuming that the strongest metal ion binding sites of enolase and the weaker binding sites of enolase are identical for Mg^{2+} and Mn^{2+}. The equations used to derive curves B and C are given in the Results section.

$$\bar{n} = \frac{[M^{2+}]}{K_{d(1)} + [M^{2+}]} + \frac{[M^{2+}]}{K_{d(2)} + [M^{2+}]} + \cdots\cdots + \frac{[M^{2+}]}{K_{d(N)} + [M^{2+}]} \quad (1)$$

action of Mg^{2+} and Mn^{2+} with yeast enolase are listed in Table I. No correction has been made for Tris or chloride binding of Mg^{2+} and Mn^{2+} since neither metal ion forms a stable complex with chloride (Bjerrum *et al.*, 1957) and there is no evidence for the interaction of Tris with either ion under conditions used in these studies (Hanlon *et al.*, 1966). No attempt was made to calculate the K_d values for the interaction of Ca^{2+} with enolase since the shape of the binding curve in Figure 3 indicates that more than one atom is binding at the lowest concentration of free Ca^{2+} which could be measured with accuracy. An approximate minimum value for $K_{d(1)}$ is 3×10^{-6} M.

A study of the interaction of $^{54}Mn^{2+}$ with enolase was made in the presence of nonradioactive Mg^{2+}. The results of this competition experiment are shown in Figure 5. Curves B and C are theoretical binding curves for Mn^{2+}–enolase interaction in the presence of 10^{-3} M Mg^{2+} and were constructed with the assumption that the K_d values listed in Table I for Mg^{2+} and Mn^{2+} are correct and that the metal ions bind independently at each site. Curve B describes Mn^{2+}

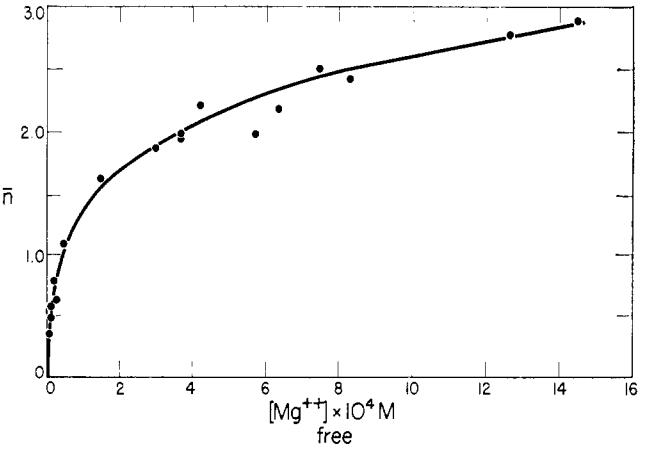

FIGURE 6: A plot of $\bar{n}$, the number of Mg^{2+} ions bound per active enolase molecule, against the concentration of Mg^{2+} *not bound* to the enzyme in the presence of an equilibrium mixture of 2-phosphoglycerate and phosphoenolpyruvate resulting from the addition of 1.2×10^{-3} M 2-phosphoglycerate to the medium. The solid line is a theoretical binding curve derived from the calculated K_d values listed in Table I.

binding to enolase assuming that the tighter bound Mn^{2+} competes with the more weakly bound Mg^{2+} and that the weaker bound Mn^{2+} competes with the more tightly bound Mg^{2+} according to the expression

$$\bar{n} = \frac{[Mn^{2+}]}{K_{d(1)Mn}\left[1 + \frac{[Mg^{2+}]}{K_{d(2)Mg}}\right] + [Mn^{2+}]} + \frac{[Mn^{2+}]}{K_{d(2)Mn}\left[1 + \frac{[Mg^{2+}]}{K_{d(1)Mg}}\right] + [Mn^{2+}]} \quad (2$$

Curve C describes the binding of Mn^{2+} to enolase in the presence of 10^{-3} M Mg^{2+} if the more tightly bound Mn^{2+} competes with the more tightly bound Mg^{2+} and that the two weaker metal ions also compete for an identical site on the protein according to an expression with the same form as eq 2 but with $K_{d(2)Mg}$ and $K_{d(1)Mg}$ interchanged.

The function $[1 + [Mg^{2+}]/K_{dMg}]$ has the value 101 for the tight magnesium site and 3.13 for the weak magnesium site at a free Mg^{2+} concentration of 10^{-3} M. These values are the factors by which either of the apparent dissociation constants for Mn^{2+} will be modified if there is competition by Mg^{2+} for the site. It is clear from Figure 5 that the tighter bound Mg^{2+} ion competes with the weaker bound Mn^{2+} ion. It also seems likely that the weaker Mg^{2+} site is the same as the tighter Mn^{2+} site, but a threefold change of K_d for the more tightly bound Mn^{2+} ion would be difficult to establish with certainty.

MEASUREMENTS MADE IN THE PRESENCE OF EQUILIBRIUM CONCENTRATIONS OF 2-PHOSPHOGLYCERATE AND PHOSPHOENOLPYRUVATE. The interaction of Mn^{2+} and Mg^{2+} with yeast enolase was also measured in the presence of an equilibrium mixture of 2-PGA and PEP resulting from the addition of 1.2×10^{-3} M 2-PGA. Dialysis data presented in Figures 6 and 7 indicate that at least four Mn^{2+}'s and three Mg^{2+}'s are bound at the highest concentration of free metal ion and

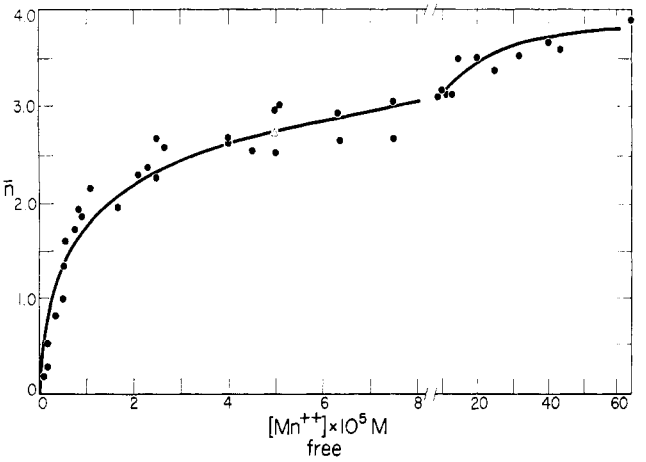

FIGURE 7: A plot of $\bar{n}$, the number of Mn^{2+} bound per active enolase molecule, against the concentration of Mn^{2+} *not bound* to the enzyme in the presence of an equilibrium mixture of 2-phosphoglycerate and phosphoenolpyruvate resulting from the addition of 1.2×10^{-3} M 2-phosphoglycerate. The solid line is a theoretical binding curve derived from the calculated K_d values listed in Table I.

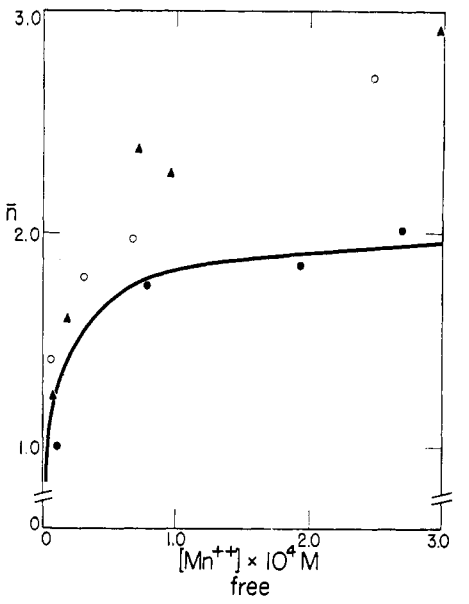

FIGURE 8: A plot of $\bar{n}$, the number of Mn^{2+} ions bound per molecule of active enolase, against the concentration of Mn^{2+} *not bound* to the enzyme in the presence of phosphate-containing enolase inhibitors. The closed triangles represent data obtained in the presence of 3.0×10^{-2} M P_i, the open circles represent data obtained in the presence of 2.0×10^{-3} phospholactate and the closed circles represent data obtained in the presence of 2.5×10^{-2} M 3-phosphoglycerate. The solid curved line represents a plot for the interaction of Mn^{2+} with enolase in the presence of 2-phosphoglycerate assuming the K_d values listed in Table I.

substrate employed. K_d values were calculated by computer using the curve-fitting technique described above and are listed in Table I along with K_d values corrected for metal ion bound to substrate. Corrections were made assuming an equilibrium ratio of 5 (Wold and Ballou, 1957) for [PEP]/[2-PGA] under our conditions. We used association constants of 180 and 280 for the interaction of Mg^{2+} with 2-PGA and PEP, an association constant of 530 for the interaction of Mn^{2+} with PEP, and an association constant of 1225 for the interaction of Mn^{2+} with 2-PGA. All of these values are taken from the paper of Wold and Ballou (1957).

We have also measured the interaction of Mn^{2+} with enolase under conditions in which the molarity and total moles of substrate and enzyme are varied. Our findings are summarized in Table II and III.

THE INTERACTION OF Mn^{2+} WITH YEAST ENOLASE IN THE PRESENCE OF PHOSPHATE-CONTAINING ENOLASE INHIBITORS. Dialysis studies were made in the presence of 2.0×10^{-3} M phospholactate, 2.5×10^{-2} M 3-phosphoglycerate, and 3.0×10^{-2} M P_i. These concentrations are five times greater than K_I for each inhibitor. The findings are shown in Figure 8. At least three metal ions are bound at 3.0×10^{-3} M Mn^{2+} when P_i or phospholactate is present but only two in the presence of 3-phosphoglycerate.

Difference Spectra. Data previously published by us (Hanlon and Westhead, 1965) contain quantitative errors in the dissociation constants because the stock enzyme solutions were not sufficiently free of magnesium ion. Dissociation constants have been redetermined by again observing the increase in absorbance at 295 mμ when the enzyme is titrated with divalent metal ion. The more correct dissociation constants are given in Table IV. PEP, which has a peak absorbance below 220 mμ, does not absorb significantly at 295 mμ where the difference spectroscopy measurements were made.

Optical Rotatory Dispersion Measurements. The optical rotation of a metal-free yeast enolase solution was compared with rotations of solutions of enzyme containing the following reagents: 2×10^{-3} M $MgCl_2$, 2×10^{-4} M $MnCl_2$, 10^{-4} M $CaCl_2$, 10^{-3} M 2-phosphoglycerate plus 2×10^{-3} M $MgCl_2$, and 10^{-3} M EDTA. All solutions had a_0 values of 240 and b_0 values of 190, similar to previously reported values (Westhead, 1964).

Discussion

The manner in which the findings presented in this paper relate to a plausible mechanism for yeast enolase catalysis will be discussed in detail in the following paper. For the present we will confine our statements to the metal ion binding properties of enolase in the absence and presence of substrate as measured under equilibrium conditions.

TABLE II: The Effect of Total Enolase Concentration on $\bar{n}$ in the Range Measuring the Interaction of Enolase with Mg^{2+} and Mn^{2+} at Their Tightest Metal Ion Binding Sites.[a]

	$[Enolase]_{total}$ (M)	$[M^{2+}]_{free}$ (M)	$\bar{n}$
Mg^{2+}	4.7×10^{-5}	2.45×10^{-5}	0.66
	1.06×10^{-5}	2.45×10^{-5}	0.68
	1.61×10^{-5}	2.45×10^{-5}	0.71
Mn^{2+}	3.34×10^{-6}	10^{-6}	0.62
	5.00×10^{-6}	10^{-6}	0.58
	6.77×10^{-6}	10^{-6}	0.60

[a] Data listed here are from gel filtration experiments.

TABLE III: Dissociation Constants for the Interaction of Yeast Enolase with Mn^{2+} in the Presence of Various Concentrations of an Equilibrium Mixture of 2-PGA and PEP Measured by Equilibrium Dialysis.[a]

$[Substrate]_{total}$ (M)	Total μmoles of Substrate	$[Enolase]_{total}$ (M)	Total μmoles of Enolase	$K_d \times 10^5$ M			
				$K_{d(1)}$	$K_{d(2)}$	$K_{d(3)}$	$K_{d(4)}$
1.2×10^{-3}	0.600	0.6×10^{-5} – 8.0×10^{-5}	0.005–0.025	0.11	0.42	3.5	5.8
1.5×10^{-4}	0.075	0.9×10^{-5} – 4.0×10^{-5}	0.005–0.010	0.13	0.46	4.6	7.4
7.5×10^{-5}	0.0375	1.5×10^{-5}	0.0013	0.13	0.46	4.8	7.7

[a] K_d values have been corrected for the interaction of Mn^{2+} with substrate.

One of the chief difficulties in obtaining meaningful metal ion binding data is the likelihood of contamination by endogenous metal ion in the enzyme preparation. The reduction of the level of endogenous metal ion is therefore of prime importance prior to metal interaction studies. We have been able to reduce contaminating Mg^{2+} and Mn^{2+} levels to less than 0.5 atom/molecule of enolase by absorption to phosphorylated cellulose, and washing with dilute EDTA solution. Remaining M^{2+} contamination was estimated by performing difference spectroscopy titrations with EDTA or by following the loss of radioactivity on phosphorylated cellulose treatment of enolase solutions containing $^{54}Mn^{2+}$. Our experience with the difficulty of obtaining metal ion free enzyme makes it seem reasonable that gross contamination by metal ions may have accounted for the results obtained in some earlier investigations of enolase–metal ion binding studies (*vide infra*).

Direct binding studies, performed in the absence of substrate show that a maximum of two metal ions is bound over the concentration range of Mg^{2+} and Mn^{2+} investigated. If further binding does occur with either ion, K_d for the third metal ion bound must be at least 100 times greater than $K_{d(2)}$.

Since two Mg^{2+}'s and two Mn^{2+}'s are bound there must be two metal binding sites, or four sites assuming Mg^{2+} and Mn^{2+} *do not* compete for binding sites. Our findings from the competition experiment depicted in Figure 5 clearly show that the more tightly bound Mg^{2+} ion competes with the more weakly bound Mn^{2+} but do not firmly establish competition at the second Mn^{2+} site. Thus, Mn^{2+} and Mg^{2+} interact with enolase at one binding site with nearly identical K_d values, but Mn^{2+} is bound far more strongly at the second site (at least 470-fold more strongly).

The nearly equal binding capacities of Mg^{2+} and Mn^{2+} at one site suggest that the ligand groups here are predominantly carboxylate groups while those at the second site probably involve both amino and carboxylate functions. However, the order of binding strength and the absolute magnitude of the dissociation constants are determined by the steric accessibility of ligand groups as well as their chemical nature. The observation that Mg^{2+} protects against photooxidation of a single histidine (Westhead, 1965) certainly suggests that the metal ion binds nearby, if not to, the imidazole group of the histidyl residue in question. A single, unrepeated experiment performed by us indicates that photooxidized enolase, prepared under conditions which result in the oxidation of a critical histidyl group and complete inhibition, can bind one Mn^{2+}. A K_d of 1.0×10^{-6} M indicates that the weaker Mn^{2+} binding site has been destroyed.

The interaction of Mn^{2+} and Mg^{2+} with yeast enolase at two sites has not been previously demonstrated. Dialysis experiments of Malmström *et al.* (1958) show only one Mn^{2+} bound with a $K_d = 2 \times 10^{-4}$ M. Their study was made under buffer, salt, and pH conditions very similar to ours, but at 4° rather than at 22°. On attempting low-temperature studies we found considerable scatter in our binding data. Although there appeared to be some increase in K_d values for both Mn^{2+} interactions, our results in no way duplicate their findings. At this point we are unable to explain the gross differences between our findings and those of Malmström *et al.*

Cohn (1963) has measured binding of Mn^{2+} to yeast enolase employing both nuclear magnetic resonance and electron spin resonance techniques. Her data yield a K_d of 8.5×10^{-6} M, assuming one metal ion is bound to the enzyme in the absence of substrate. Since the electron spin resonance technique measures the free Mn^{2+} concentration at a given concentration of enzyme and the moles of Mn^{2+} added is known, this technique should yield a correct estimate of the maximum number of Mn^{2+}'s bound. We now know that preparations of enolase such as that used by Cohn can

TABLE IV: Dissociation Constants for the Interaction of Divalent Metal Ions with Yeast Enolase in the Absence and Presence of Substrate Measured by Difference Spectroscopy at 295 mμ.[a]

Metal Ion	K_d (M)	K_d' (M)
Mg^{2+}	1.5×10^{-5}	1.25×10^{-5}
Mn^{2+}	2.0×10^{-6}	2.0×10^{-6}

[a] K_d' values are those obtained in the presence of an equilibrium mixture of PEP and 2-PGA resulting from the addition of 1.2×10^{-3} M 2-PGA and have been corrected for binding of metal ion to substrate.

be contaminated with 10–20 atoms of Mg^{2+} per molecule of enolase; a fact recently reported by Brewer and Weber (1966) and confirmed by us with several different preparations in our laboratory. Extensive Mg^{2+} contamination could give results similar to those of our Mn^{2+} binding experiment performed in the presence of Mg^{2+} (Figure 5). Recent nuclear magnetic resonance (Hanlon *et al.*, 1967) are in excellent agreement with the data of this present paper.

The K_d value for the interaction of Mn^{2+} with enolase measured by difference spectroscopy reported earlier by us is now known to be erroneously high (Hanlon and Westhead, 1965). Measurements made more recently using enolase with known, low amounts of Mg^{2+} contamination yield the K_d values in Table IV. It now appears that the conformation change resulting in a tryptophan perturbation spectrum is due to binding of Mg^{2+} and Mn^{2+} at their strongest sites of interaction. Competition experiments depicted in Figure 5 indicate that these sites are not identical. It seems therefore that interaction of either site with metal ion gives rise to essentially identical difference spectra. Under these conditions one should look for evidence of cooperative effects, but widely separated K_d values in the case of either M^{2+} activator make it impossible to tell whether the observed K_d's are intrinsic or result from cooperative binding.

By measuring fluorescence changes in the enzyme upon addition of Mg^{2+}, Brewer and Weber (1966, 1968) have determined dissociation constants of 2×10^{-6} and 2×10^{-5} M at 0.05 ionic strength and in 1.0 M KCl, respectively. Our difference spectra data, obtained at an intermediate salt concentration thus seem to measure the same interaction. Both optical changes appear to result from the binding of a single atom of metal to the native enzyme. The extent of the metal ion induced conformation change, although sufficient in magnitude to be detected by sensitive techniques such as difference spectroscopy and fluorescence spectroscopy (Brewer and Weber, 1966), is too small to be observed by optical rotatory dispersion methods since identical a_0 and b_0 values were obtained for metal-free enolase and enolase in the presence of substrate. Indeed, no change in a_0 or b_0 is observed when sufficient Ca^{2+} is added to cause the binding of six atoms of this metal ion per molecule of enzyme.

Ca^{2+} binding to yeast enolase, as measured by gel filtration, is characterized by a multiplicity of sites (on the enzyme). At 10^{-4} M Ca^{2+} six metal atoms are bound. The shape of the binding curve in Figure 3 in the region of 10^{-4} M Ca^{2+} implies that further binding can occur with an increase in Ca^{2+} concentration. Ca^{2+}, therefore, is similar to Zn^{2+} (Malmström, 1953) in its binding to yeast enolase. The factors which contribute to Ca^{2+} and Zn^{2+} interactions at multiple sites but which restrict Mg^{2+} and Mn^{2+} binding (to two sites) must operate on an extremely subtle level since there is no direct correlation with such parameters as atomic size, stereochemistry and coulombic potential.

Ultracentrifuge and depolarization of fluorescence data of Brewer and Weber (1968) indicate that yeast enolase will dissociate at low protein concentrations into inactive subunits in 1.0 M KCl at pH 7.5 and 25° if the solution is made 0.02 M in EDTA. If one or both of the metal ions bound in the absence of substrate is responsible for dimerization of a metal-free monomer species, $\bar{n}$ should be a function of the total enolase concentration. In Table II we show that $\bar{n}$ operates as an independent variable at a free Mn^{2+} concentration where the first Mn^{2+} is bound and $\bar{n}$ is likewise independent of enzyme concentration at a concentration where the first Mg^{2+} is bound.

Brewer and Weber (1968) and Gawronski and Westhead (1969) have showed that Mg^{2+} does shift the monomer–dimer equilibrium toward association but that dimer formation does not *require* divalent cations, especially at higher protein concentrations. The data in this paper were obtained at higher protein concentrations and lower salt concentrations than in the cited references so it is not surprising that our metal dissociation constants are independent of protein concentration.

The Interaction of Yeast Enolase with Mn^{2+} in the Presence of Inhibitors. Wold and Ballou (1957) have found that 3-PGA, phospholactate, and P_i inhibit enolase activity. We have found that 3-PGA and phospholacetate inhibit competitively with K_i's of 5×10^{-3} and 4×10^{-4} M. P_i is a noncompetitive inhibitor ($K_i = 6 \times 10^{-3}$ M). Figure 8 indicates that at higher Mn^{2+} concentrations more than two Mn's are bound in the presence of 2×10^{-3} M phospholactate or 3×10^{-2} M P_i. However the Mn^{2+} binding curve in the presence of 2.5×10^{-2} M 3-PGA is essentially identical with the Mn^{2+} binding curve in the absence of substrate (Figure 8). These facts add to the evidence that binding to the active site does not require metal ion (3-PGA data) and that there are other phosphate-binding sites that do involve a metal ion (phospholactate and P_i data).

The Interaction of Metal Ions with Yeast Enolase in the Presence of Substrate. The presence of an equilibrium mixture of 2-PGA and PEP resulting from the addition of 1.2×10^{-3} M 2-PGA causes an increase in the number of Mn^{2+} ions and Mg^{2+} ions bound to the enzyme as shown in Figures 6 and 7. The value of $\bar{n}$, the number of metal ions bound per molecule of enolase at the highest concentration of Mn^{2+} investigated, suggests that at least four Mn^{2+} ions are bound. If a maximum of four Mn^{2+}'s are bound over the concentration range studied, the K_d values listed in Table I best fit the binding data. Although $\bar{n}$ is less than 3.0 at the highest concentration of Mg^{2+} studied, we think that the data represent binding four or more Mg^{2+} ions because it is not possible to derive a theoretical curve which fits the data using any combination of three constants. If four sites are being filled by magnesium ions, then the K_d values given in Table I best fit the $\bar{n}$ data. The best possible fit derivable with three constants does not require a significant change in either $K_{d(1)}$ or $K_{d(2)}$.

It is obvious that there is no significant difference between the values of $K_{d(1)}$ obtained in the absence of substrate and those obtained in the presence of 1.2×10^{-3} M substrate, for either metal ion. This independence of $K_{d(1)}$ from substrate concentrations requires that substrate bind neither to the metal-free enzyme, nor to the 1:1 complex, *or else* that it bind to both forms with equal affinity. Difference spectroscopy measurements performed in the presence and absence of substrate also yield identical K_d values as shown in Table IV.

We have arbitrarily assigned K_d values in order of increasing numerical value and assumed that the sites designated by $K_{d(1)}$ and $K_{d(2)}$ in the absence and presence of substrate are identical. If this is so, the presence of 1.2×10^{-3} M substrate appears to cause a threefold decrease in the value of K_d for the second bound Mg^{2+} and a fourfold decrease in the value of K_d for the second bound Mn^{2+}.

These slight decreases in the second metal site dissociation constants may not be real since the apparent decrease may be due to the error associated with the calculation of the second constants in the presence of the additional binding caused by the substrate. A real difference, even of this small degree, should be detectable kinetically, but as shown in the following paper (Hanlon and Westhead, 1969), no such effect is seen. If the effect is real, it means that substrate binds somewhat more tightly to the 2:1 metal–enzyme complex than to the 1:1 complex.

In considering the binding of the third and fourth atoms of metal in the presence of substrate, the requirement for substrate is seen to contrast with the lack of effect of changing substrate concentration upon the dissociation constants that is seen in Table III. This combination of information places definite restrictions on our picture of the substrate–metal–enzyme interactions at these additional metal binding sites. References to Figures 1 and 2 allow us to see that *in the absence of substrate* the cations must have dissociation constants at sites 3 and 4 greater than about 7×10^{-3} M for Mg^{2+} and 5×10^{-3} M for Mn^{2+}; otherwise we would see evidence for this binding at the highest metal ion concentrations used. In the presence of substrate, these minimum constants are reduced by factors of at least 10 for Mg^{2+} and 100 for Mn^{2+}. A further consideration is that the data in Table III show that in the substrate range of 7.5×10^{-5}–1.2×10^{-3} M, there is no significant change in the apparent affinity of the third and fourth sites for the cations.

The simplest model in which substrate would produce an apparent increase in affinity of enzyme for cation would be

$$E + M \rightleftharpoons EM \overset{S}{\rightleftharpoons} EMS$$

This mechanism is the one required by the "bridge" hypothesis in which the role of metal ion is to serve as central atom in a tertiary complex. Such a model is ruled out by the Table III data since it requires the apparent enzyme–metal dissociation constant to become linearly dependent upon substrate concentrations as (S) becomes indefinitely large (see, for example, Dixon and Webb, 1964). A second scheme, in which only a metal–substrate complex binds to the enzyme, would give similar results, and is furthermore ruled out by the M–S dissociation constants (Wold and Ballou, 1957).

Finally we may consider the case in which substrate is bound to the enzyme in the absence of metal ion, but more strongly to the enzyme–metal complex. This model is set up as

$$E + M \overset{K}{\rightleftharpoons} EM \qquad ES + M \overset{K'}{\rightleftharpoons} EMS$$

$$E + S \overset{K_s}{\rightleftharpoons} ES \qquad EM + S \overset{K_s'}{\rightleftharpoons} EMS$$

Any three of the four equilibria are enough to derive the over-all equilibrium, and if we choose to work with K, K_s, and K', we can derive the equation for the degree of saturation of enzyme with metal ion (at the site under consideration). If we let total enzyme concentration, E_0, equal unity and solve for EM + EMS, where $E_0 = 1 = E + EM + ES + EMS$, we find that this fraction is given by

$$EM + EMS = \frac{\frac{M}{K} + \left(\frac{M}{K'}\right)\left(\frac{S}{K_s}\right)}{1 + \frac{M}{K} + \frac{S}{K_s}\left(1 + \frac{M}{K'}\right)} \qquad (3)$$

Under limiting conditions of high substrate and low substrate concentration, this expression becomes much simpler. At high (S), the first term in the numerator is negligible and upon dividing by the second term we get

$$EM + EMS = 1 \Big/ \left\{1 + \frac{K'}{M} + \frac{K_s}{S}\left[\frac{K'}{M} + \frac{K'}{K}\right]\right\}$$

which is seen to be insensitive to changes in (S) at high (S), and to be similar to the expression for a normal hyperbolic saturation curve. At low values of (S), the last terms in the numerator and denominator become negligible and the equation reduces to $EM + EMS = 1/(K/M + 1)$, with obvious consequences.

Qualitatively at least, this is entirely in accord with all of our results. Both metal ion and substrate bind to the enzyme independently, but are bound more tightly in the tertiary complex. We can also see that 7.5×10^{-5} M substrate is enough to approach the high substrate limiting case. If we arbitrarily let this limit be at $(M/K')(S/K_s) = 10(M/K)$, we can get an estimate of K_s. Rearranging and simplifying this limit expression gives us a maximum value of $K_s = (K/K')(S/10)$; for Mn^{2+}, this maximum value is then 7.5×10^{-4}. This calculation is also based on the requirement discussed earlier in which the binding of Mn^{2+} at site 3 is at least 100-fold stronger in the presence of substrate than in its absence. The maximum value of K_s to satisfy the available Mg^{2+} data (Table I) would be 1.2×10^{-3}.

It should be noted explicitly that in the above derivations, "E" refers only to those species of enolase free of substrate and the third and fourth M^{2+}'s and does not distinguish between enzyme alone and enzyme in the form of the 1:1 or 2:1 metal complexes.

Summary

The binding of both Mg^{2+} and Mn^{2+} appear to follow the same pattern: a tight binding site, which on interacting with metal ion gives rise to a difference spectrum indicative of tryptophan perturbation, the K_d of which is not effected by substrate; a second somewhat weaker binding site, the K_d of which changes little or not at all in the presence of substrate, and at least one or two more relatively weak sites which show considerably enhanced affinity for the cations in the presence of substrate.

The sites responsible for the perturbation of the ultraviolet spectrum are not the same sites or Mg^{2+} and Mn^{2+}, as shown by the competition experiment (Figure 5) in which both Mg^{2+} and Mn^{2+} are present. In an accompanying paper (Gawronski and Westhead, 1969) this is discussed in terms of possible subunit structures of the enzyme. In the following paper (Hanlon and Westhead, 1969) we show that the two more tightly bound cations are absolutely required for enzymic

activity, and that the more weakly bound metal ions are, to a greater or lesser extent, inhibitory in action.

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Kinetic Studies on the Activation of Yeast Enolase by Divalent Cations*

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ABSTRACT: Activation constants for Mg^{2+} and Mn^{2+} have been determined for yeast enolase at several concentrations of 2-phosphoglyceric acid in the forward reaction and phosphoenolpyruvic acid in the reverse direction. Michaelis constants for both substrates have been determined at several concentrations of the activating cations. The activation constants are shown to be independent of substrate concentration and the Michaelis constants are independent of metal ion concentration. It is concluded that activating ions and substrates bind to the critical sites independently, ruling out the possibility that the metal functions as a bridge to bind substrate and enzyme. The kinetically determined activation constants were found to be nearly the same as the binding constants for the two cations at the weak sites, determined by equilibrium dialysis. Inhibition by excess Mn^{2+} is shown to be dependent upon the concentration of substrate. The inhibition constant is similar to the binding constant for the binding of a third Mn^{2+} in the presence of substrate.

In the preceding paper (Hanlon and Westhead, 1969) we have established that the interaction of yeast enolase (phosphoenolpyruvate hydratase EC 4.2.1.11) with metal ion activators involves a number of different metal ion acceptor sites. Since the presence of divalent metal cations is an absolute requirement for enolase activity, it is obvious that one or more of these metal ion binding sites must be filled to effect catalysis.

In this paper we report enzyme kinetic studies of both the forward and reverse reaction and correlate these findings with information obtained from our investigation of the equilibrium system.

Experimental Section

Materials

Buffer, 2-D-(+)-PGA,[1] and the enzyme preparation used in these studies have been described in the preceding paper

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[1] Abbreviations used are: PGA, phosphoglyceric acid; PEP, phosphoenolpyruvate; K_A, kinetically determined activation constant for metal ion; K_m, Michaelis constant for substrate; K_S, dissociation constant for enzyme–substrate complex; K_A and K_S are constants for the dissociation of the binary enzyme–metal and enzyme–substrate com-