Interaction of Triethyltin with Pyruvate Kinase†

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ABSTRACT: Low concentrations of triethyltin activate pyruvate kinase, while higher concentrations inhibit it. Exposure of pyruvate kinase to phosphoenolpyruvate plus Mn^{2+} prior to contact with triethyltin (protected enzyme) prevents inhibition of the initial rate, but inhibition supervenes 6–7 min after the enzymatic reaction is started. When exposed to triethyltin in the absence of phosphoenolpyruvate (P-enolpyruvate) and Mn^{2+} (unprotected enzyme), the subsequent initial rate (rate 1) is 50% inhibited by 1.4 mM triethyltin; the rate then increases to a new level (rate 2) in 2–3 min. Initial rate (rate 1) kinetics reveal competition between triethyltin and Mn^{2+} as well as phosphoenolpyruvate; no homotropic cooperativity is observed with Mn^{2+} or P-enolpyruvate, but Hill coefficient n' values for triethyltin are between 2 and 4, indicating interaction between inhibitor molecules. In contrast, for rate 2, n' values approaching 2 are observed for Mn^{2+} and P-enolpyruvate, and n' for triethyltin rises to between 6 and 8. Binding studies demonstrate displacement of $^{54}Mn^{2+}$ by triethyltin under unprotected conditions, while Mn^{2+} remains bound when enzyme is protected by addition of phosphoenolpyruvate; [^{118}Sn]triethyltin binds to approximately 14 sites on the enzyme, and is displaced by Mn^{2+} plus phosphoenolpyruvate. These studies indicate that alkyltins may serve as useful probes of the active center of pyruvate kinase.

Recent studies from this laboratory have shown that phenethylbiguanide and a number of related guanidine derivatives inhibited rabbit muscle pyruvate kinase (Davidoff and Carr, 1972b). Divalent metal ion was displaced from the enzyme as the result of binding of the organic base, largely through hydrophobic interactions, presumably at the active center. Characterization of the chemical nature of the divalent metal ion binding site of pyruvate kinase therefore becomes of special interest, not only to augment our general understanding of divalent metal ion binding sites (Bachmayer *et al.*, 1968; Darnall and Birnbaum, 1970; Komatsu and Feeney, 1967), but also to help define the interaction between biologically active guanidine derivatives and proteins. The studies reported in this paper reveal triethyltin to be an effective inhibitor of pyruvate kinase, with somewhat unusual kinetic inhibitory properties; the data suggest that triethyltin binds within the active center of the enzyme, probably attaching to both the divalent metal and phosphoenolpyruvate binding loci; in addition, triethyltin appears to bind at an allosteric site removed from the active center.

Materials and Methods

Rabbit muscle pyruvate kinase (ATP, pyruvate phosphotransferase, EC 2.7.1.40) was obtained from Boehringer Mannheim Corp. and for kinetic studies was used without further purification. Polyacrylamide gel electrophoresis of this preparation at pH 8.5 and 9.5 according to the procedure of Cottam *et al.* (1969) revealed only a single component both before and after recrystallization from imidazole buffer. For studies of binding, which employed large amounts of enzyme protein, ammonium sulfate was removed from the enzyme by filtration through Sephadex G-25 in 0.05 M Tris buffer, pH 7.4; lactate dehydrogenase (EC 1.1.1.27) used in spectrophotometric assays was desalted by the same technique. Pyruvate kinase activity was assayed spectrophotometrically by measuring reduction of the reaction product, pyruvate, coupled to oxidation of NADH with lactate dehydrogenase (Mildvan and Cohn, 1965), using a Gilford recording spectrophotometer. Concentrations of free divalent metal ions in the incubation mixture were calculated by the method of Mildvan and Cohn (1965). Binding of $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and [^{118}Sn]triethyltin to pyruvate kinase was measured by the ultrafiltration technique of Paulus (1969) as described previously (Davidoff and Carr, 1972b). All activity and binding measurements were carried out at 20°. All organometal compounds were obtained from Alfa Inorganics, Beverly, Mass.; [^{118}Sn]triethyltin was purchased from Amersham/Searle, Arlington Heights, Ill., and counted according to the procedure of Rose and Aldridge (1968). Other radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.

Results

Characteristics of Pyruvate Kinase Inhibition by Triethyltin. The effect of triethyltin on the activity of pyruvate kinase was markedly dependent on (1) the conditions under which the inhibitor initially interacted with the enzyme and (2) the time following initiation of the enzymatic reaction. When pyruvate kinase was exposed to concentrations of triethyltin greater than 2 mM in the presence of Mn^{2+} plus ADP, or P-enolpyruvate[1] plus ADP, and the reaction then started by adding P-enolpyruvate or Mn^{2+}, respectively, enzyme activity was initially very low but then slowly accelerated over a period of 2–3 min and stabilized at a second rate (Figure 1, A and B). Under these conditions, maximal inhibitor effect

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[1] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; $[Mn^{2+}]_t$ and $[Mn^{2+}]_f$, concentrations of total and free manganese respectively; NADH and NAD^+, reduced and oxidized nicotinamide adenine dinucleotide, respectively.

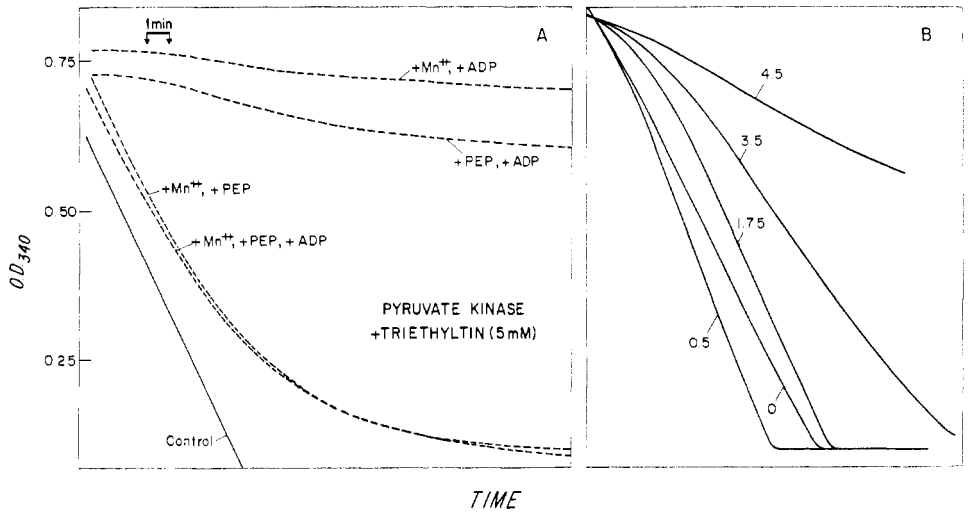

FIGURE 1: Activity of pyruvate kinase exposed to triethyltin in the presence of various reactants. (A) The reaction was carried out in 1.5 ml total volume in a cuvet containing 37.5 mM Tris-HCl buffer, pH 7.4, 40 mM KCl, 0.15 mM NADH, and 0.03 mg of beef heart lactic dehydrogenase. Prior to addition of pyruvate kinase, 0.8 mM phosphoenolpyruvate, 3.3 mM ADP, or 0.17 mM $MnCl_2$, in the combinations indicated in the figure, were added to the mixture. Triethyltin was added to a final concentration of 5 mM, in 50 μl of 50% ethanol; ethanol alone in this concentration did not affect the activity of the enzyme. (B) The reaction mixture was the same as in A, and contained ADP plus Mn^{2+}; the reaction was started with P-enolpyruvate (PEP). Triethyltin was added in the concentrations indicated (mM) prior to initiating the reaction.

was apparent upon initiation of the enzymatic reaction after exposure of pyruvate kinase to triethyltin for a period as short as 10 sec. As is evident from Figure 1A, high levels of triethyltin ($\sim$5 mM) were associated with a further late fall-off in rate, following the second rate. However, at lower triethyltin concentrations, the second rate continued in linear fashion for at least 6–7 min (Figure 1B); rate determinations during this phase were accurate and highly reproducible. The reaction rate for unprotected enzyme observed immediately (during the first 30 sec) after initiating the reaction will be referred to as rate 1, while the second rate will be termed rate 2.

When the initial contact between enzyme and triethyltin occurred in the presence of Mn^{2+} plus P-enolpyruvate, the reaction subsequently being initiated with ADP, or when pyruvate kinase was added to the complete reaction mixture (Mn^{2+}, P-enolpyruvate, and ADP) containing triethyltin, enzyme activity was initially the same as the control; inhibition became apparent only after 4–5 min had elapsed (Figure 1A) and increased progressively. After an additional 6–7 min the rate had slowed to essentially equal rate 2 found with enzyme preincubated in the absence of either Mn^{2+} or P-enolpyruvate. In the presence of Mn^{2+} plus P-enolpyruvate, therefore, the enzyme appeared initially to be protected from the inhibitory effect of triethyltin; this protection was not overcome by preincubation of enzyme with inhibitor for periods up to 1 hr prior to initiating the reaction with ADP. Enzyme exposed to triethyltin in the presence of Mn^{2+} plus P-enolpyruvate will be referred to as protected, while enzyme exposed to triethyltin in the absence of either Mn^{2+}, P-enolpyruvate, or both will be referred to as unprotected.

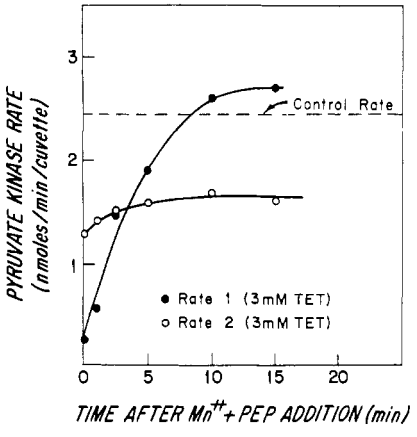

FIGURE 2: Conversion of pyruvate kinase from unprotected to protected form. Concentrations of reagents were as in Figure 1. Pyruvate kinase was added to a mixture containing 3 mM triethyltin but no Mn^{2+}, P-enolpyruvate, or ADP. Within 1 min, Mn^{2+} plus P-enolpyruvate were added simultaneously, and at increasing time intervals thereafter, the reaction was started with ADP; rate 1 was measured by drawing tangents to the slope of NADH oxidation between 0 and 30 sec after starting the tracing; rate 2 was measured after the second linear rate was established (about 3–5 min).

Pyruvate kinase could be converted from the unprotected to the protected form prior to initiating the enzymatic reaction by adding Mn^{2+} plus P-enolpyruvate to the mixture of pyruvate kinase and triethyltin (Figure 2); this reversal was much slower than the original induction of triethyltin inhibition under unprotected conditions, the time required for 50% conversion from unprotected to protected form being about 2 min.

The degree of pyruvate kinase inhibition was studied as a function of increasing triethyltin concentration at both non-saturating and saturating concentrations of Mn^{2+}. At the lower Mn^{2+} concentration ($[Mn^{2+}]_t$ = 0.167 mM, $[Mn^{2+}]_f$ = 0.022 mM), three separate activity curves were described (Figure 3A): the initial rate observed with unprotected enzyme (rate 1), the second rate observed with unprotected enzyme (rate 2), and the initial rate with protected enzyme. Rate 2 was always measured using enzyme which was initially unprotected, since with protected enzyme the second, inhibited linear rate was not approached until the NADH in the reaction mixture was nearly exhausted, particularly with small degrees of triethyltin inhibition. Furthermore, by not waiting sufficiently long with protected enzyme before measuring the inhibited

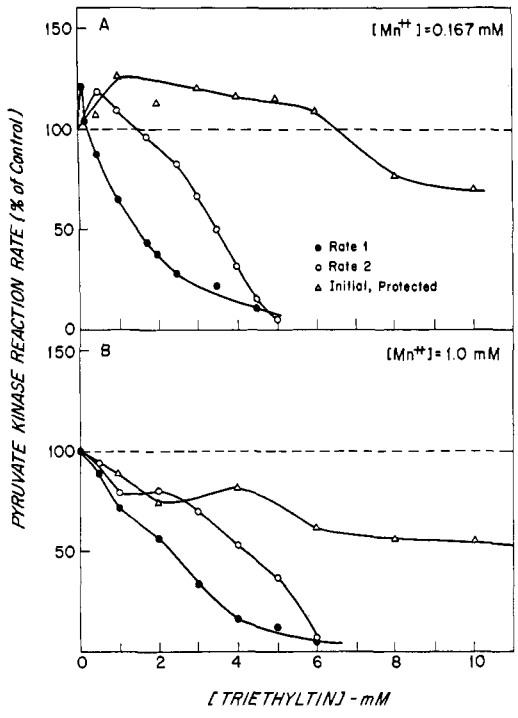

FIGURE 3: Initial and second rates of protected and unprotected pyruvate kinase at increasing triethyltin concentrations. Incubation conditions were as in Figure 1. To determine rate 1 of unprotected enzyme, pyruvate kinase was added to the mixture containing triethyltin in the absence of Mn^{2+} or P-enolpyruvate, and the reaction was initiated by adding these two reagents simultaneously. Protected initial rates were determined by adding both Mn^{2+} and P-enolpyruvate before triethyltin, the reaction then being initiated with ADP. Rates were determined for both these curves as described in Figure 2. For determination of rate 2, the reaction was started with Mn^{2+} plus P-enolpyruvate and rates were measured as in Figure 2. Data shown are from one representative experiment for each condition.

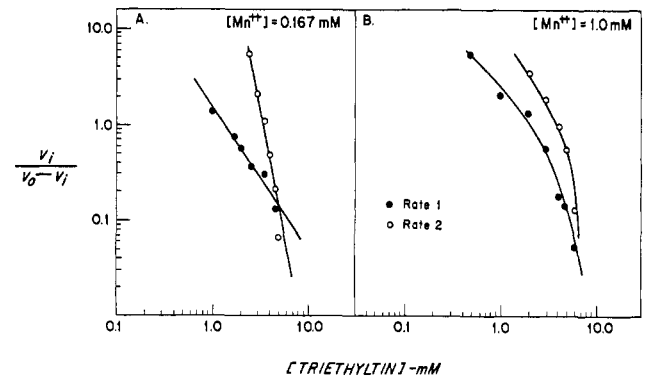

FIGURE 4: Determination of apparent n values for triethyltin inhibition of pyruvate kinase. Data were calculated according to eq 1 in the text where V_i = rate of inhibited enzyme, V_0 = rate of enzyme reaction without inhibitor. Data in A represent rates at $[Mn^{2+}]$ = 0.167 mM, and in B at $[Mn^{2+}]$ = 1.0 mM; closed circles are initial rates (rate 1) for unprotected enzyme, open circles are rate 2. Each point represents the average of data from three experiments.

second rate, the inhibited rate would be significantly overestimated.

The shape of these inhibition curves was of considerable interest: with nonsaturating levels of Mn^{2+}, the rate 1 for unprotected enzyme (Figure 3A) showed a small but consistent increase above control at concentrations of triethyltin below 0.5 mM, but then fell off with increasing concentrations, 50% inhibition occurring at about 1.4 mM triethyltin. The initial rate of protected enzyme, on the other hand (Figure 3A), was increased over that of the control by triethyltin in concentrations up through about 7 mM; above that level, there appeared to be some degree of initial inhibition, although the subsequent inhibition then occurred so rapidly and completely that the slope of the initial rate became very difficult to measure accurately.

Rate 2 of unprotected pyruvate kinase activity was also reproducibly increased by low triethyltin concentrations, reaching a maximum of about 20% above the control level (Figure 3A) at 0.5 mM triethyltin, then falling off to reach 50% inhibition at about 3.5 mM.

At saturating levels of Mn^{2+} ($[Mn^{2+}]_t$ = 1 mM, $[Mn^{2+}]_f$ = 0.164 mM), the inhibition curves with increasing triethyltin were significantly different than at low Mn^{2+} concentrations (Figure 3B). Activation of the enzyme was no longer observed at lower triethyltin concentrations; the concentration of triethyltin needed to inhibit rate 1 of unprotected enzyme by 50% rose from 1.4 at low Mn^{2+} to 2.4 mM at the higher Mn^{2+} concentration (about a 70% increase), while the 50% inhibitory level of triethyltin for rate 2 increased from 3.5 to 4.2 mM (increase of 20%).

The unusual shape of these plots for enzyme activity *vs.* inhibitor concentration suggested the possibility of cooperative interaction of inhibitor molecules (Taketa and Pogell, 1965). When the criterion of the ratio of inhibitor concentrations necessary to change enzyme activity from 60 to 40% (Taketa and Pogell, 1965) was applied to the data in Figure 3, the resultant values for n' (n' = apparent n) for the order of triethyltin inhibition of rate 1 were 1.60 and 1.80 for low and high Mn^{2+} concentrations, respectively, while the corresponding values for n' for rate 2 were 4.75 and 2.85. When data from three experiments similar to the one shown in Figure 3 were plotted according to eq 1[2] (Jensen and Nester, 1966) a

$$\log \frac{V_i}{V_0 - V_i} = \log K_{diss} - n' \log [I] \qquad (1)$$

value of 1.6 was obtained for n' for inhibition of rate 1 at the lower Mn^{2+} concentration (Figure 4A); at higher Mn^{2+}, however, the slope of the plot was not constant, and at high triethyltin concentrations the n' value approached 4 (Figure 4B). For rate 2, n' was close to 5.4 at the lower Mn^{2+} concentration, while at high Mn^{2+}, the slope began at a value of 2–3, but approached 8 at high levels of triethyltin. The maximal values for n' at high Mn^{2+} concentration, obtained by averaging the results of both methods of calculation, were 2.9 and 5.4 for rates 1 and 2, respectively, which suggest binding of triethyltin to a minimum of two cooperative sites during the initial phase of the reaction with unprotected enzyme and to at least four sites during the later phase of activity.

Effects of Other Organometal Compounds. Trimethyltin inhibited rate 2 of pyruvate kinase 50% at a concentration of 15 mM, *i.e.*, a potency of about four times less than that of triethyltin; as with the triethyl derivative, inhibition was maximal initially with unprotected enzyme, and the rate then accelerated to a second, inhibited level. In contrast, the activity of pyruvate kinase inhibited with tripropyltin

[2] Symbols are: V_i, velocity of inhibited enzyme; V_0, velocity of enzyme without inhibitor; K_{diss}, dissociation constant; [I], concentration of inhibitor.

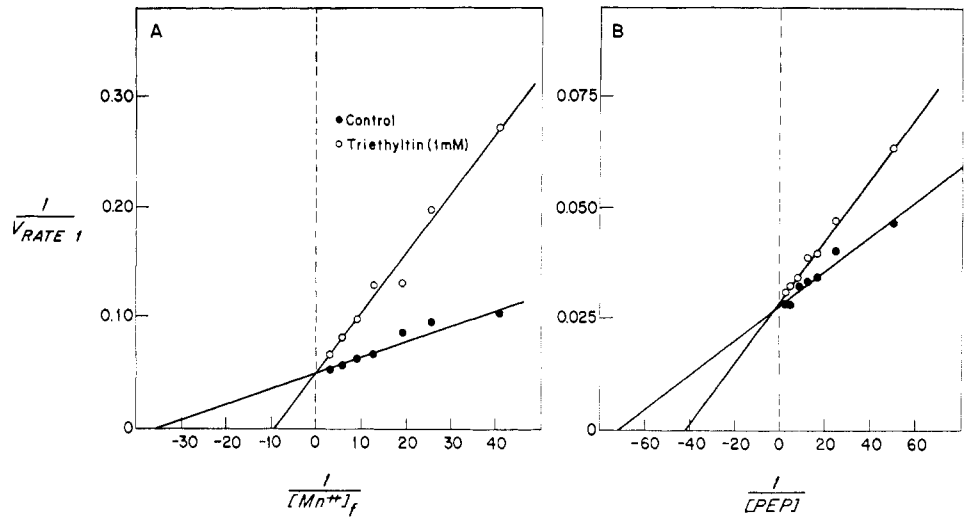

FIGURE 5: Initial velocity (rate 1) of pyruvate kinase as a function of $[Mn^{2+}]_f$ and [P-enolpyruvate]$_t$. Incubation conditions were as in Figure 1, except that KCl concentration was 75 mM, and Mn^{2+} or P-enolpyruvate concentration was varied as indicated in A and B, respectively. V is expressed in nanomoles of NADH oxidized per minute per cuvet; $[Mn^{2+}]_f$ and [P-enolpyruvate]$_t$ are in M $\times 10^{-3}$. Lines were fitted by least squares.

did not change with time. Inhibition with the tripropyl derivative rose from 10 to 90% over the concentration range of 0.6–1.1 mM, 50% inhibition occurring at 0.89 mM under the standard assay conditions; the value of n' for tripropyltin was calculated from these data (Tageta and Pogell, 1965) to be almost exactly 8. No inhibition was observed with triethylgermanium at concentrations up to 10 mM.

Control Studies. To rule out the possibility that the slow change from rate 1 to rate 2 was due to the accumulation of a reaction product, NAD^+, L-lactate, and ATP were added individually to unprotected enzyme prior to initiation of the enzymatic reaction with P-enolpyruvate; all were without effect on the reaction pattern.

Phosphate at very high concentrations has been shown to complex with triethyltin (Rose, 1969); control assays were therefore carried out using higher concentrations of the phosphorylated substrates, P-enolpyruvate and ADP. No quantitative or qualitative alterations in inhibitory effects of triethyltin were observed, thus eliminating the formation of complexes between these substrates and triethyltin as the cause of the inhibition. Similarly, appropriate controls measuring the rate of reduction of pyruvate added directly to the incubation mixture revealed no interference by triethyltin with the activity of the lactic dehydrogenase employed as the indicator enzyme in these assays, nor did increasing the lactic dehydrogenase concentration in the pyruvate kinase assay affect the patterns of inhibition found with triethyltin.

Kinetic Interactions of Triethyltin, Mn^{2+}, Phosphoenolpyruvate, and K^+ with Pyruvate Kinase. RATE 1. Using pyruvate kinase exposed to triethyltin in the unprotected state, the double reciprocal plots of initial rates (rate 1) were linear both for control and inhibited enzyme as a function of free Mn^{2+} concentration, and these plots intersected on the ordinate, indicating competitive behavior (Figure 5A). With increasing total P-enolpyruvate concentration, the plots were also linear and competitive (Figure 4B); similar linear functions were obtained when the concentration of free rather than total P-enolpyruvate was plotted, which was not unexpected since under these incubation conditions, the free Mn^{2+} concentration was mainly a function of ADP concentration, and was negligibly affected by changes in total P-enolpyruvate over the concentration ranges used.

RATE 2. In contrast to the data obtained with rate 1, plots of rate 2 for triethyltin-inhibited pyruvate kinase were nonlinear functions of $1/[Mn^{2+}]_f$ and 1/[P-enolpyruvate] (Figures 6A and B). When plotted using $1/[Mn^{2+}]^2_f$ and 1/[P-enolpyruvate]^2, straight lines were obtained; the V_{max} intercepts of these lines were different than the control V_{max} values. Hill plots of these data (Figure 7) using the extrapolated values for V_{max} gave values for n' of 1.80 for $[Mn^{2+}]_f$ and 1.78 for P-enolpyruvate with the triethyltin-inhibited enzyme; n' values for the controls were between 0.88 and 0.91. Fructose 1,6-diphosphate, an allosteric activator of the L type of pyruvate kinase (Llorente *et al.*, 1970), at a concentration of 0.1 mM, did not alter the nonlinear kinetics obtained with Mn^{2+}. Addition of triethyltin in concentrations up to 2 mM, *i.e.*, below that required to inhibit rate 2 significantly (*cf.* Figure 3A), did not alter the linear character of the Lineweaver–Burk plot for $1/[Mn^{2+}]_f$.

In contrast to the results obtained with Mn^{2+} and P-enolpyruvate, K^+ gave rise to uncompetitive kinetics with triethyltin.

Binding of $^{54}Mn^{2+}$ and [^{113}Sn]Triethyltin to Pyruvate Kinase. In the presence of 75 mM K^+, $^{54}Mn^{2+}$ bound to unprotected pyruvate kinase with a formation constant of approximately 2.8×10^4 M^{-1}, assuming four binding sites for Mn^{2+} per enzyme molecule (Davidoff and Carr, 1972b; Reuben and Cohn, 1970) (Figure 8A). The presence of 1 mM triethyltin markedly diminished the binding of $^{54}Mn^{2+}$, the decrease ranging between 80 and 40% at the increasing Mn^{2+} concentrations shown in Figure 8A. There was no clear-cut evidence for homotropic cooperativity of Mn^{2+} binding either with or without triethyltin.

$^{54}Mn^{2+}$ binding decreased progressively as a function of increasing triethyltin concentration when pyruvate kinase was equilibrated with these two cations in the absence of phosphoenolpyruvate (Figure 8B). Displacement of 50% of bound $^{54}Mn^{2+}$ occurred at about 0.7 mM triethyltin, which is in the same range as the 50% inhibitory triethyltin concentration for the initial rate of unprotected enzyme (rate 1) observed

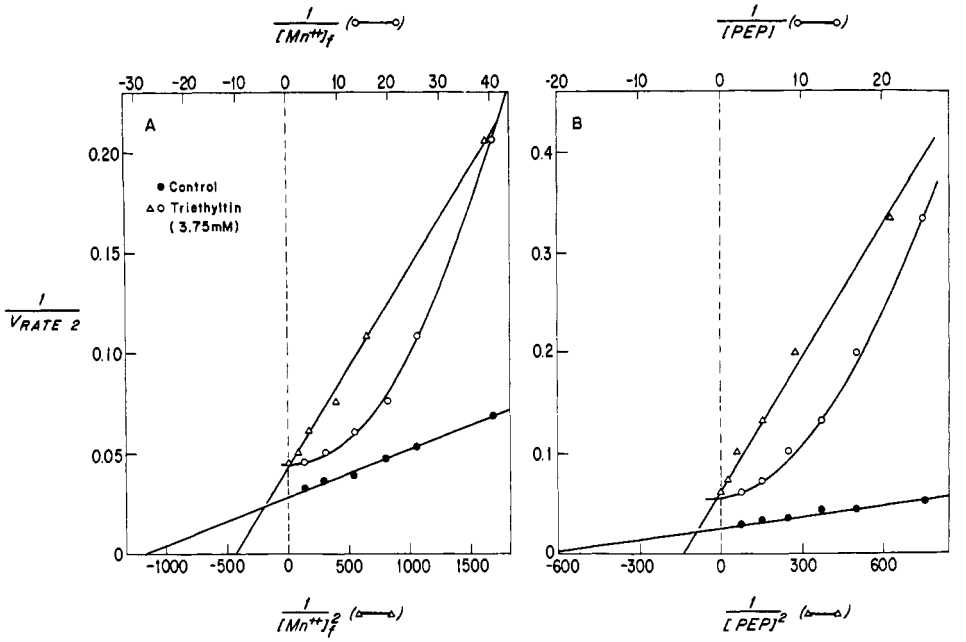

FIGURE 6: Rate 2 of pyruvate kinase as a function of Mn^{2+} and P-enolpyruvate. Incubation conditions were as in Figure 5. Closed circles: controls, $1/V$ *vs.* $1/[Mn^{2+}]_f$ or $1/[\text{P-enolpyruvate}]_t$. Open circles: 3.75 mM triethyltin, $1/V$ *vs.* $1/[Mn^{2+}]_f$ or $1/[\text{P-enolpyruvate}]_t$. Triangles: 3.75 mM triethyltin, $1/V$ *vs.* $1/[Mn^{2+}]_f^2$ or $1/[\text{P-enolpyruvate}]^2$.

in kinetic studies (see Figure 3A). Addition of phosphoenolpyruvate markedly protected the enzyme against displacement of $^{54}Mn^{2+}$ by triethyltin (Figure 8B); no increment of $^{54}Mn^{2+}$ binding above the control was observed at any level of triethyltin employed. Because of the very large amounts of pyruvate kinase needed to study Mn^{2+} binding directly, measurements of binding under the reaction conditions required to reach rate 2 were technically unfeasible; hence comparison of $^{54}Mn^{2+}$ binding in this later reaction state with binding to protected and unprotected enzyme prior to initiating turnover was unfortunately impossible.

A Scatchard plot of the data for [^{118}Sn]triethyltin binding (Figure 9A) deviated from a single linear slope. One possible explanation for this curved plot would be the presence of two binding sites containing n_1 and n_2 binding loci with intrinsic binding constants K_1 and K_2 for the higher and lower affinity sites, respectively. Using the slopes and intercepts from the data plotted in Figure 9A, the numerical values for these parameters were calculated according to the method of Klotz and Hunston (1971) with the following results: $n_1 = 14$; $k_1 = 2.15 \times 10^3\ M^{-1}$; $n_2 = 322$; $k_2 = 1.43\ M^{-1}$. The very low affinity and large number of the second set of binding sites suggested that this low affinity binding may have been nonspecific; from these data it is not possible to rule out the possibility of more than one class of higher affinity sites.

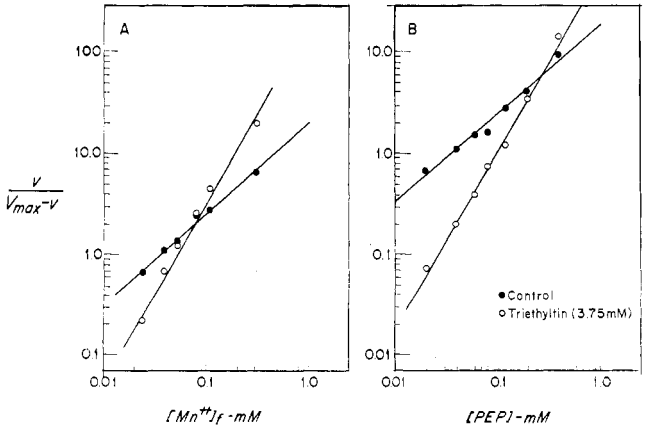

FIGURE 7: Hill plots of pyruvate kinase activity. Rate 2 is plotted as a function of Mn^{2+} and P-enolpyruvate concentration. The data of Figure 6 were recalculated and plotted by the method of Atkinson *et al.* (1965).

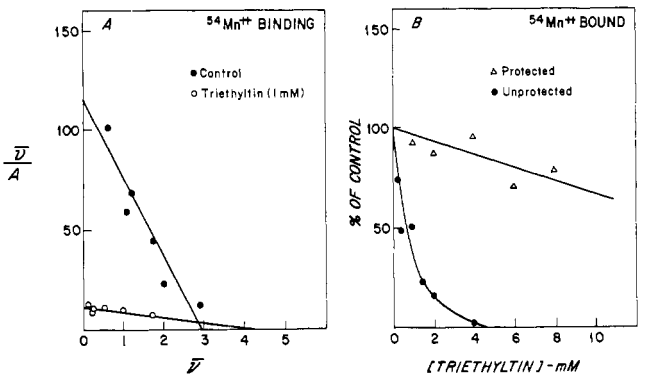

FIGURE 8: Binding of $^{54}Mn^{2+}$ to pyruvate kinase; effect of triethyltin. (A) Scatchard plots (Scatchard, 1949) of $^{54}Mn^{2+}$ binding to pyruvate kinase; binding was measured in the presence of 50 mM Tris buffer, pH 7.4, plus 75 mM KCl. Each point is the mean of two paired samples; lines were fitted by least squares. $\bar{\nu}$ = moles of ligand bound per mole of enzyme; A = concentration of unbound ligand (mM). (B) $^{54}Mn^{2+}$ binding to pyruvate kinase under unprotected (closed circles) and protected (open triangles) conditions. Unprotected samples contained 75 mM KCl and 0.1 mM Mn^{2+}; in the control samples (without triethyltin), 2.8 nmol of Mn^{2+} was bound per mol of pyruvate kinase. Protected samples also contained 0.8 mM P-enolpyruvate, and the total Mn^{2+} concentration was 0.14 mM ($[Mn^{2+}]_f$ = 0.1 mM); 3.45 nmol of Mn^{2+} was bound per mol of enzyme in the absence of triethyltin.

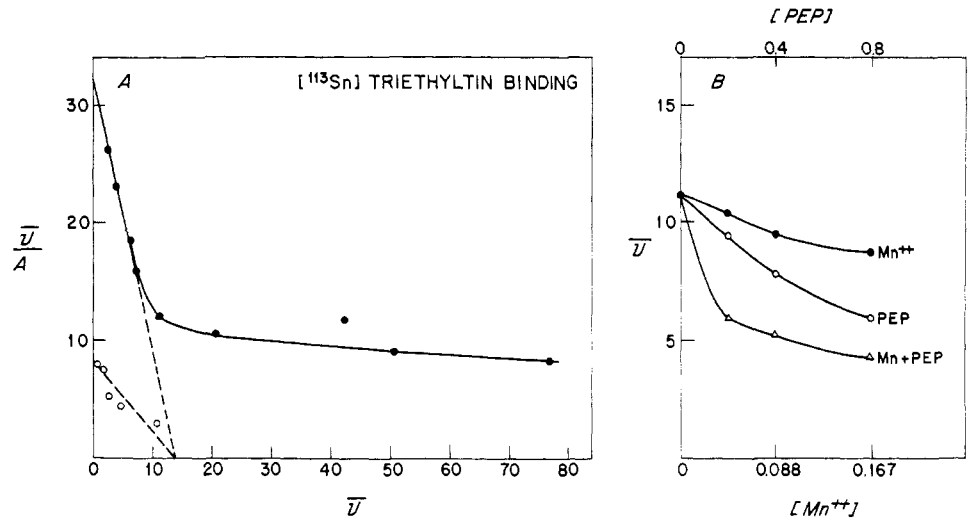

FIGURE 9: Binding of [^{113}Sn]triethyltin to pyruvate kinase. (A) Binding was measured in 50 mM Tris buffer in the presence of 75 mM K^+. Closed circles: no added reactants; open circles: 0.8 mM P-enolpyruvate plus 0.167 mM Mn^{2+}. Each point represents the mean of 2–16 individual determinations; standard errors of the mean for $\bar{\nu}$ were all less than $\pm 7\%$ of the mean value. (B) Concentration of [^{113}Sn]triethyltin was 1 mM and Mn^{2+}, P-enolpyruvate, or both were added in the concentrations indicated.

The presence of Mn^{2+} plus P-enolpyruvate diminished the amount of [^{113}Sn]triethyltin bound; low-affinity or nonspecific binding was not observed under these conditions. The apparent higher affinity binding constant for triethyltin was reduced about fivefold, to the range of 5×10^2 M^{-1} (Figure 9A). Mn^{2+} or P-enolpyruvate alone was less effective in displacing triethyltin from the enzyme than the combination; the effect of the two reagents appeared to be approximately additive (Figure 9B).

At a triethyltin concentration of 0.2 mM, the quantity of [^{113}Sn]triethyltin bound was 4.07 mol/mol of enzyme, while at 1.0 mM triethyltin, about 11.2 mol/mol was bound in the absence of Mn^{2+} or P-enolpyruvate. Addition of Mn^{2+} and P-enolpyruvate in the concentrations used during rate studies diminished the quantity of triethyltin bound; in the presence of these concentrations of Mn^{2+} and P-enolpyruvate, a triethyltin concentration of 0.2 mM, which caused maximal activation of rate 1, was associated with the binding of approximately 1.4 mol of triethyltin/mol, while 1.0 mM triethyltin, which inhibited rate 1 by 50%, gave rise to binding of 4.3 mol/mol. The presence of potassium did not detectably influence [^{113}Sn]triethyltin binding.

Discussion

Muscle pyruvate kinase (M type) is an oligomeric protein made up of four protomers, all of apparently identical molecular weight (Steinmetz and Deal, 1966) and amino acid composition (Cottam *et al.*, 1969). The binding of 4 mol of Mn^{2+}/mol of enzyme (Reuben and Cohn, 1970; Davidoff and Carr, 1972b; *cf.* also Figure 9) thus suggests binding of one Mn^{2+} per protomer, although this is obviously not the only possibility. Indeed, studies of pyruvate, P-enolpyruvate, and ADP binding (Reynard *et al.*, 1961; Betts and Evans, 1968) indicate the presence of only two active centers per enzyme molecule, suggesting that only two of the Mn^{2+} are bound within active centers, while the other two may be only indirectly related to catalytic activity, *e.g.*, bound to regulatory sites. Most of the enzymatic activity is retained when the enzyme is split into dimers (Cottam *et al.*, 1969), but binding data for Mn^{2+} under these conditions are not available.

Rabbit muscle pyruvate kinase demonstrates regulatory properties in that both its Mn^{2+} binding affinity and catalytic activity are affected by monovalent cation activators (Davidoff and Carr, 1972b; Reuben and Cohn, 1970), K^+ probably being the physiologically important cation. This effect of K^+ is accompanied by both spectrophotometric (Wilson *et al.*, 1967) and electrophoretic (Sorger *et al.*, 1965) evidence for conformational changes; the K^+ effect is probably an example of a "V"-type regulatory system (DeAsúa *et al.*, 1970) since V_{max} is strikingly affected by K^+, while the change in binding affinity for Mn^{2+} is small (Davidoff and Carr, 1972b; Reuben and Cohn, 1970) and the binding of ADP and P-enolpyruvate is not altered (Betts and Evans, 1968). However, the muscle enzyme has not previously been reported to exhibit homotropic cooperative regulatory behavior with respect to substrate or Mn^{2+} nor have heterotropic inhibitor effects been described. This is in marked contrast to the liver (L type) enzyme for which temperature-dependent cooperativity has been demonstrated with divalent metals and substrates; allosteric activation of the L-type enzyme occurs with fructose 1,6-diphosphate as well as inhibition with ATP and alanine (Llorente *et al.*, 1970). Red cell pyruvate kinase appears to be even more complex, demonstrating differential effects of Mg^{2+} *vs.* Mn^{2+} on cooperative behavior (Leonard, 1972).

Inhibition of rabbit muscle pyruvate kinase by Ca^{2+} and by guanidine derivatives is probably due to simple competition at the divalent metal binding site itself (Davidoff and Carr, 1972b; Mildvan and Cohn, 1965). Similarly, triethyltin appears to bind to unprotected enzyme at the active center in a reversible and competitive manner (Figure 5), thus displacing divalent metal cation (Figure 8); however, the observation that both P-enolpyruvate and Mn^{2+} reverse the inhibition of initial rate (Figure 5) and diminish [^{113}Sn]triethyltin binding (Figure 9) suggests that the binding domain of the triethyltin molecule may include portions of both the Mn^{2+} and the P-enolpyruvate binding loci within the active center.

Binding of triethyltin to unprotected pyruvate kinase appears to be relatively rapid (complete in less than 10 sec at 20°); however, binding of Mn^{2+} plus P-enolpyruvate to the enzyme is even more rapid, since addition of pyruvate kinase

to medium containing Mn^{2+}, P-enolpyruvate, and triethyltin gives rise to a "protected" initial rate. The slow conversion of unprotected to protected enzyme observed upon addition of Mn^{2+} plus P-enolpyruvate prior to initiation of the enzyme reaction (Figure 2) must therefore be attributed to the slow and therefore rate-limiting dissociation of the enzyme–triethyltin complex. The phenomenon of protection by Mn^{2+} plus P-enolpyruvate, which is qualitatively and quantitatively quite different than the effect of either Mn^{2+} or P-enolpyruvate alone, could possibly be ascribed to an "induced-fit" conformational change in the enzyme due to simultaneous binding of these two ligands (Koshland, 1971) which markedly diminishes its binding affinity for triethyltin at the active center (Figures 3 and 9).

Although under unprotected conditions there are initially no heterotropic triethyltin effects on substrate, or homotropic substrate effects in the controls, the inhibition curves with triethyltin for rate 1 reveal two features indicative of homotropic allosteric behavior for the inhibitor itself: (1) activation by low levels of triethyltin and (2) an n' value for triethyltin of between 2 and 4. These phenomena, as well as the rate 2 activating and cooperative inhibitory effects of triethyltin (Figures 3A, 4, 6, and 7), may be explained according to a symmetry model (Monod *et al.*, 1965), as follows.

In the presence of K^+, pyruvate kinase may be assumed to exist in the two conformational states, R and T, in an equilibrium described by eq 2, R being the enzymatically active form.

$$T \rightleftharpoons LR \tag{2}$$

In the absence of other ligands, the relative quantity of the T form of the enzyme can be assumed to be greater than the R form, *e.g.*, the coefficient L being of the order of 10 (Rubin and Changeux, 1966). Activation of pyruvate kinase by triethyltin was observed only at less than saturating Mn^{2+} concentrations. This observation suggests the mechanism of activation may be similar to activation of aspartate transcarbamylase by the substrate analog, maleate (Monod *et al.*, 1965; Gerhardt and Pardee, 1964), *i.e.*, binding of the activator within one reaction site of an enzyme containing more than one such site, thus stabilizing the R form of the enzyme. Alternatively, the activating molecules of triethyltin may be bound to regulatory rather than reaction-center Mn^{2+} sites in a manner analogous to the activation of isocitrate dehydrogenase by low concentrations of citrate (Atkinson *et al.*, 1965).

The absence of homotropic Mn^{2+} or P-enolpyruvate interactions initially (rate 1) in control and inhibited kinetics (Figure 5) or later (rate 2) in the controls (Figure 6) may be attributed to relative nonexclusivity of binding of these two ligands to the R and T conformations of the enzyme states (Rubin and Changeux, 1966). On the other hand, the n' value for triethyltin was 2 or greater under initial, unprotected conditions, which is consistent with greater exclusivity for the binding of this inhibitor, assuming the true number of inhibitor binding sites to be 4. The observations that Ca^{2+} (Mildvan and Cohn, 1965) and guanidine derivatives (Davidoff and Carr, 1972b) at low concentrations do not activate the rabbit muscle enzyme and show no evidence of homotropic cooperativity probably indicate minimal exclusivity of binding of these inhibitors to the two conformational states of the enzyme.

The slow change in enzyme kinetics from rate 1 to rate 2 includes (i) a decrease in the apparent affinity of the enzyme for triethyltin, as indicated by the increase in rate (Figure 1B); (ii) the appearance of homotropic Mn^{2+} and P-enolpyruvate interactions, with n values approaching 2 (*cf.* Figure 7); and (iii) an increase in n' for triethyltin from 2 to 3 toward 6–8 (Figure 4). The rate-limiting step in creation of the "rate 2 complex" may consist of an induced-fit conformational change (Koshland, 1971) which can only occur with the proper combination of bound ligands, *i.e.*, Mn^{2+}, P-enolpyruvate, ADP, and triethyltin at concentrations up to about 2 mM. Pyruvate kinase in this new configuration apparently exists in two forms, R′ and T′, which are in equilibrium as described in eq 3.

$$T' \rightleftharpoons LR' \tag{3}$$

The appearance of homotropic Mn^{2+} and P-enolpyruvate interactions during rate 2, as the enzyme becomes inhibited by concentrations of triethyltin above 2 mM, indicates that this inhibition may be due to the binding of triethyltin to new, allosteric binding sites, remote from the active centers or regulatory Mn^{2+} sites. Furthermore, the binding affinity for triethyltin at these allosteric sites must be much greater for the inactive (T′) form of the enzyme than the active (R′) form. As a result, triethyltin in higher concentrations stabilizes the T′ form of the enzyme, thus increasing the value of the apparent L coefficient, L', into the range of perhaps 1000, according to the relationship[3]

$$L' = L\frac{(1+\beta)^n}{(1+\gamma)^n} \tag{4}$$

Thus, with sufficiently high triethyltin concentrations, homotropic cooperativity of Mn^{2+} and P-enolpyruvate will become apparent (Rubin and Changeux, 1966). The value of n' for triethyltin during rate 2 activity presumably reflects the existence of at least four and possibly as many as eight allosteric sites for binding of triethyltin; tripropyltin may bind to this same set of allosteric sites, judging from the observed n' value of 8.

The binding energy of the alkyltins to pyruvate kinase may include a large contribution from hydrophobic interactions, judging from the increasing inhibitory effectiveness of alkyltins with increase in size of the *n*-alkyl substituent; this increase parallels the increasing effectiveness of the same compounds as inhibitors of mitochondrial function (Aldridge and Rose, 1969). In this connection it is also noteworthy that approximately 70% of the binding energy of phenethylbiguanide to pyruvate kinase is derived from hydrophobic bond (Davidoff and Carr, 1972b). The binding of triethyltin to pyruvate kinase resembles the binding of phenethylbiguanide in several other respects as well: (1) both bind competitively at Mn^{2+} binding sites; (2) intrinsic binding constants are quantitatively similar ($K_i = 5 \times 10^2$ for triethyltin in the presence of P-enolpyruvate and Mn^{2+}, *vs.* 2.4×10^2 for phenethylbiguanide); and (3) the total number of these higher affinity binding sites is equal to 14 for triethyltin and 12 for phenethylbiguanide (Davidoff and Carr, 1972b). As noted above, however, the data in Figure 9a cannot be taken to demonstrate unequivocally the presence of a single, homogeneous set of higher affinity sites. In fact, the demonstration that the lowest concentrations of triethyltin activate unprotected enzyme in association with binding of less than 2 mol/

[3] Symbols are: $\beta = [I]/K_I$ and $\gamma = [A]/K_A$, where [I] and [A] are concentrations of inhibitor and activator, respectively, and K_I and K_A are microscopic dissociation constants of inhibitor and activator for T′ and R′ forms of the enzyme, respectively.

mol of [^{118}Sn]triethyltin, then followed by inhibition of the enzyme at higher triethyltin concentrations, with 4 mol of [^{118}Sn]triethyltin bound at 50% inhibition, suggests that three subclasses of triethyltin binding sites, with similar but not identical affinities, may be present on the enzyme. In order of decreasing affinity, these sites would appear to be at or near (1) non-active-center Mn^{2+} sites, (2) active-center Mn^{2+} sites, and (3) allosteric triethyltin inhibitory sites.

Triethyltin and the related alkyl derivatives of lead and germanium from the IVb group of elements in the periodic table are extremely unreactive chemically, but trialkyltins can form polymeric coordination complexes with certain heterocyclic nitrogen-containing ring compounds such as imidazole (Poller, 1965). Rose and his coworkers have presented evidence that triethyltin coordinates with paired histidines in rat hemoglobin (Rose, 1969), although it forms complexes with very few other proteins (Rose and Aldridge, 1968). These investigators have therefore suggested that binding of triethyltin to biological materials with affinity constants in the range of 10^5 indicates binding between paired histidines. Triethyltin binds selectively to certain mitochondrial proteins with affinities of this order of magnitude, and, on this basis, Aldridge and Rose (1969) have proposed a mechanism of oxidative phosphorylation which includes interaction of the electron transport chain with an energy-conserving protein or proteins containing seven–ten paired histidines. The inability of triethylgermanium to inhibit pyruvate kinase noted in the present studies corresponds with its inability to form 5-coordination compounds in imidazole model systems, and with its very slow binding, relative to triethyltin, to rat hemoglobin (Aldridge and Rose, 1969). Thus, although the binding affinity of triethyltin for rabbit muscle pyruvate kinase appears to be lower than for rat hemoglobin or mitochondria, the present studies raise the possibility that at least some of the highest affinity triethyltin binding sites on pyruvate kinase may consist of paired histidines.

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