Phosphoenolpyruvate Carboxykinase (Guanosine 5′-Triphosphate) from Rat Liver Cytosol. Divalent Cation Involvement in the Decarboxylation Reactions†

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ABSTRACT: The presence of a divalent metal ion together with a catalytic amount of inosine 5′-diphosphate (IDP) is essential for the formation of pyruvate from oxalacetate catalyzed by purified rat liver cytosol phosphoenolpyruvate carboxykinase (PEPCK). With decreasing order of effectiveness, this pyruvate-forming activity was supported by micromolar levels of Cd^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+}. At the same concentrations, Mg^{2+} or Ca^{2+} was not effective. Combinations of Cd^{2+} with either Zn^{2+}, Mn^{2+}, or Co^{2+} were not additive with respect to the pyruvate-forming activity of PEPCK. Kinetic determination, with Cd^{2+} as the supporting cation, showed a 1:1 stoichiometry of interaction between each enzyme molecule and the nonconsumable substrate IDP. With 10 μM added Cd^{2+}, the apparent K_m for oxalacetate was 41 μM, and the apparent K_a for IDP was 0.25 μM. With Zn^{2+} or Mn^{2+}, the apparent K_a for IDP was 0.2 or 0.13 μM, respectively. The effect of divalent transition-metal ions on PEPCK-catalyzed formation of phosphoenolpyruvate from oxalacetate was also investigated. Under steady-state conditions, the basal activity with MgITP was effectively enhanced with micromolar levels of Mn^{2+}, Cd^{2+}, or Co^{2+} included in the assay. The V_m increased 7- and 3.6-fold, and the apparent K_m for MgITP changed by about a factor of 2 with the optimal concentrations of Mn^{2+} and Co^{2+}, respectively. The most striking changes were in the apparent K_m values for oxalacetate, which decreased to one-third and one-tenth when either Mn^{2+} or Co^{2+} was present in the assay together with Mg^{2+}. The possible physiological importance of this kinetic effect is discussed.

In addition to the reversible decarboxylation of oxalacetic acid (OAA)[1] to form PEP (eq 1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) catalyzes, in some instances, the irreversible decarboxylation of OAA to pyruvate (eq 2). In

$$\text{OAA} + \text{ITP (GTP)} \overset{Me^{2+}}{\rightleftharpoons} \text{PEP} + \text{IDP (GDP)} + CO_2 \quad (1)$$

$$\text{OAA} \xrightarrow{\text{IDP (GDP)}} \text{PYR} + CO_2 \quad (2)$$

animal species, a large body of physiological evidence indicates that the enzyme's important role in gluconeogenesis is to synthesize PEP from OAA (Lardy et al., 1964). PEPCK occurs in large amounts in liver and kidney cortex (Tilghman et al., 1976). The enzyme increases with fasting and in diabetes, and it is responsive to dietary and hormonal stimuli in vivo (Shrago et al., 1963; Lardy et al., 1965; Foster et al., 1966). The rapid increase in hepatic gluconeogenesis caused by glucagon results in a metabolite crossover pattern consistent with regulation at the level of PEPCK (Exton & Park, 1969; Exton et al., 1973a,b). Even though physiological manipulations result in changes of enzyme activity, no modulators or fine controls have been clearly established. A large number of naturally occurring intermediates have been found not to affect PEPCK activity (Foster et al., 1967; Holten & Nordlie, 1965; Ballard, 1970; Utter & Kolenbrander, 1972). Thus far, the only effectors that might directly regulate the catalytic activity of PEPCK appear to be the divalent transition-metal ions. PEPCK, from all sources examined, requires a divalent cation to catalyze the reaction in the physiological direction, and in some cases the maximal rate of PEP synthesis is achieved with a combination of two metals [see Utter & Kolenbrander (1972) for a review]. With cytosol from rat liver, Foster et al. (1967) and Snoke et al. (1971) reported that in the direction of PEP synthesis micromolar levels of Mn^{2+}, Co^{2+}, Fe^{2+}, or Cd^{2+} greatly enhanced the basal activity with MgITP as substrate. More recently, the Fe^{2+} and ferroactivator stimulation of PEP synthesis by PEPCK has been proposed as a controlling factor of the enzyme in the intact liver (Bentle et al., 1976; Bentle & Lardy, 1976, 1977). The inhibition of gluconeogenesis by quinolinic acid (Veneziale et al., 1967) and 3-mercaptopicolinic acid (Di Tullio et al., 1974) appears to be related to the interaction of these compounds with a metal-activated form of PEPCK (Snoke et al., 1971; Jomain-Baum et al., 1976).

The decarboxylation of OAA to pyruvate (reaction 2) requires nucleoside diphosphate, which functions as an activator rather than a consumable substrate with all PEPCK's thus far found to catalyze this reaction. Reports regarding the involvement of Me^{2+} are discordant. With yeast PEPCK, the ADP-dependent formation of pyruvate in the absence of added Me^{2+} was inhibited by millimolar amounts of Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, or Mg^{2+} (Cannata & Stoppani, 1963) and was stimulated by micromolar amounts of Cd^{2+} (Cannata & Flombaum, 1974). The IDP-supported pyruvate formation with mitochondrial PEPCK from pig liver required added Mn^{2+} (Chang & Lane, 1966; Chang et al., 1966) while the chicken liver enzyme did not require, and was actually inhibited by, added Me^{2+} (Noce & Utter, 1975). With rat liver cytosol PEPCK, pyruvate formation was stimulated by low levels of Mn^{2+} in the presence of GDP (Jomain-Baum & Schramm, 1978) and inhibited by millimolar concentrations of Mg^{2+} in the presence of IDP (Colombo et al., 1978). Due

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[1] Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; PYR, pyruvate; EDTA, ethylenediaminetetraacetic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Me^{2+}, divalent metal ion; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; PK, pyruvate kinase; DTT, dithiothreitol.

0006-2960/81/0420-2758$01.25/0

to this variability, it was deemed of interest to investigate the cation specificity of this side activity with purified rat liver cytosol PEPCK, with the study focused on the cations that have been shown to act as free metal activators (Colombo et al., 1981) in both the forward and the reverse direction of reaction 1.

A reinvestigation of the effect of metals on the PEP-forming activity of the purified enzyme was also undertaken. This was based on the premises that since PEPCK's role in gluconeogenesis is to catalyze the formation of phosphoenolpyruvate from oxalacetate then (a) the metal requirement for activity in vivo is probably satisfied by Mg^{2+} because it is abundant in the cytosol (Thiers & Vallee, 1957) and (b) the activation of this basal Mg activity by micromolar levels of divalent cations should be determined at physiological levels of oxalacetate (1–10 μM in rat cytosol) rather than at the high OAA concentrations (>1 mM) used in the previous studies (Snoke et al., 1971; Bentle et al., 1976; Bentle & Lardy, 1977). Our results indicated that with MgITP and under steady-state conditions the activation by micrmolar levels of Co^{2+} or Mn^{2+} also involves a drastic reduction in the apparent Michaelis constant for oxalacetate.

Materials and Methods

Reagents of the highest purity available were from the sources previously listed (Colombo et al., 1981). PEPCK from the cytosol of rat liver was purified as previously described (Colombo et al., 1978). After chromatography on agarose–hexane–GTP, the enzyme was stored under an N_2 atmosphere and at 4 °C. Prior to use, PEPCK was freed of KCl and GSH by chromatography on Sephadex G-25 equilibrated with 10 mM Tes buffer (pH 7.2) containing 10% glycerol and 0.1 mM EDTA. This enzyme was stored under an N_2 atmosphere and at 4 °C. Enzyme protein concentration was determined spectrophotometrically at 280 nm with the use of the extinction coefficient, $E^{1\%,1\,cm}_{280\,nm}$ of 16.6. The molarity of PEPCK was calculated by using a molecular weight of 72 000 (Colombo et al., 1978).

PEPCK Activity Determinations. Enzyme activities were determined spectrophotometrically at 25 °C by the decrease in the absorbance at 340 nm. All the reactions were started by the addition of PEPCK to the otherwise complete reaction mixture.

Pyruvate-Forming Activity. The decarboxylation of oxalacetate to pyruvate catalyzed by PEPCK was measured, unless indicated otherwise, in a 1-mL reaction mixture that contained 100 mM Hepes–KOH (pH 7.0), 0.25 mM NADH, and 40 μg of LDH. The concentration of IDP, OAA, and EDTA as well as the concentration and nature of the divalent cations added is indicated in the figure legend for each experimental condition. In order to correct the data for the spontaneous and/or metal-catalyzed decarboxylation of oxalacetate, controls were always included in which the rate of NADH oxidation was determined in the presence of all the components of the reaction mixture except for the PEPCK. This decarboxylation rate was subtracted from that obtained in the presence of PEPCK. The specific activity is expressed as micromoles of pyruvate formed per minute per milligram of PEPCK.

Phosphoenolpyruvate-Forming Activity. PEP-forming activity was determined, unless otherwise stated, in a 1-mL reaction mixture that contained 100 mM Hepes–KOH (pH 7.0), 2.2 mM $MgCl_2$, 1 mM ADP, 0.25 mM NADH, 60 μg of PK (12 units), and 38 μg of LDH (20 units). The concentration of MgITP, OAA, and EDTA as well as the concentration and nature of other added divalent metal ions is indicated for each experimental condition in the respective figure legend. The rate of nonenzymatic decarboxylation of OAA was determined in the presence of all the components of the reaction mixture except the PEPCK. This rate of NADH oxidation for 1–2 min in the absence of PEPCK when OAA was not the variable substrate was subtracted from that observed after PEPCK addition (5–10 μL) to obtain the net rate of PEP formation. When OAA was the variable substrate, the nonenzymatic OAA decarboxylation was determined in a separate cuvette containing all the components of the reaction but with the PEPCK deleted and substituted by a corresponding aliquot of buffer. The rates of PEPCK-catalyzed PEP formation were then corrected for the nonenzymatic rate obtained in the absence of PEPCK. Enzyme specific activity is expressed as micromoles of PEP formed per minute per milligram of PEPCK.

When PEPCK activity was determined in a reaction mixture as described above for the formation of PEP from OAA, an additional control was included in which the NADH oxidation rate was determined in the presence of PEPCK, LDH, and all the components of this assay system except for the PK which was deleted. This control allows the determination of the relative rates of carboxykinase-catalyzed phosphoenolpyruvate and pyruvate formation from OAA (see Results and legend of Figure 5).

Carboxylating Activity. For the determination of the kinetic parameters for the PEPCK-catalyzed carboxylation of PEP to form OAA, stoppered cuvettes were used. The reaction mixture contained in a final volume of 3 mL 100 mM Hepes–KOH (pH 7.0), 0.25 mM NADH, 33 μg/mL MDH, and 1.6 mM $MgCl_2$ in excess over MgIDP. The concentration of PEP, $KHCO_3$, EDTA, and other added divalent metal ions is indicated for each experimental condition. The pH of the bicarbonate solutions was adjusted with CO_2 immediately before use, and the solutions were freshly prepared for each experiment. CO_2 concentrations were calculated from the Henderson-Hasselbach equation. Specific activity is expressed as micromoles of OAA formed per minute per milligram of PEPCK.

Other Procedures. In the determination of the kinetic parameters for the PEP-forming and the carboxylating activities, 100 mM K^+ was always present. From the initial velocity experiments, linear double-reciprocal plots were constructed from the data by using a weighted least-squares fit (Wilkinson, 1961; Cleland, 1967).

The concentration of IDP and ITP was determined at pH 6 by the optical density at 248.5 nm by using a millimolar extinction coefficient of 12.2. The concentration of OAA and PEP was determined by measuring NADH oxidation spectrophotometrically in the presence of either malate dehydrogenase or pyruvate kinase and lactate dehydrogenase. A stock oxalacetate solution was prepared immediately before use in 50 mM Hepes, neutralized to pH 7 with KOH, and diluted in cold H_2O to the desired concentrations so that 20 μL was added to the prewarmed assay mixture.

In studying the pyruvate-forming activity, the concentrations of *free* metal ions in assays containing one added Me^{2+} and two chelators (e.g., Mn^{2+}, EDTA, and IDP in Figure 6) were determined by solving the simultaneous equations for total Me^{2+}, total EDTA, and total IDP, using literature values for the dissociation constants for the metal–chelator species. The final cubic equation in which the value of x represents the concentration of free metal and the coefficients are functions of the total concentrations of metal and chelators and their respective dissociation constants was solved by using a Hewlett

Table I: Effect of EDTA and Buffer on Pyruvate Formation from Oxalacetate Catalyzed by Purified Rat Liver Cytosol PEPCK[a]

	activity [μmol of pyruvate formed min^{-1} (mg of PEPCK)$^{-1}$]			
	100 mM Hepes-KOH (pH 7.0)		100 mM imidazole-HCl (pH 7.0)	
EDTA (μM)	no metal added	5 μM Cd^{2+} [b]	no metal added	40 μM Cd^{2+} [b]
0.5	1.11[c]	8.2	*d*	*d*
1.0	0.15	8.9	0.01	9.7
2.5	0[e]	9.3	0	10.1
5.0	0	7.4		*d*
8.0	0	2.6[c]	0	10.5
33.0	0	0	0	10.2

[a] The formation of pyruvate from OAA was determined as described under Materials and Methods in a 1-mL reaction mixture that also contained 2.1 μg of PEPCK, 0.6 mM OAA, 1 mM IDP, and the designated buffer. [b] Pyruvate formed when 5 μM Cd^{2+} (in the Hepes-buffered assays) or 40 μM Cd^{2+} (in the imidazole-buffered assays) was added after the reaction had proceeded for 2-3 min in the absence of any added divalent metal ion and with EDTA at the concentration indicated in the first column. [c] Initial rates taken from the linear portion of the recording. All other assays were linear with time. [d] Not determined. [e] A specific activity of zero indicates that the rate of pyruvate formation with PEPCK as measured by NADH oxidation was no greater than the rate of OAA decarboxylated in the absence of the enzyme.

Packard polynominal root finding program written for the 9825 A desk-top computer. When the species involved were two Me^{2+} and one chelator, the same approach was taken. In this case, the root of the cubic equation is the value of free chelator [cf. Moe & Butler (1972) for a similar case]. When two metals (Mg^{2+} and Cd^{2+}) and two chelators (EDTA and IDP) were present in the system, a simplified approach was taken. The levels of free metals were first determined with respect to the concentration of EDTA and then with respect to the concentration of IDP. This approach is valid since the affinity constant of the divalent transition metal for EDTA is several orders of magnitude greater than that for IDP.

The calculations were made by employing the following values for the formation constant (log K at pH 7.0) of the EDTA–metal complexes: Mg^{2+}, 5.4; Mn^{2+}, 10.7; Cd^{2+}, 13.1 (Sillén & Martell, 1964; O'Sullivan, 1969). The association constants (log K) for the metal–IDP complexes were 3.94 for Mn^{2+} and 3.15 for Mg^{2+} (Noce & Utter, 1975). For Cd^{2+}, a value was not found in the literature; the stability constant was assumed to be an order of magnitude lower (Walaas, 1958) than that of the ATP complex, and the value used was 4.19 (Miller & Westheimer, 1966).

Results

Effect of Reaction Components on the Decarboxylation of OAA To Form Pyruvate Catalyzed by Purified Rat Liver Cytosol PEPCK. In the absence of exogenously added Me^{2+}, and in Hepes buffer which has negligible affinity for divalent metal ions (Good et al., 1966), the pyruvate-forming activity of PEPCK is 100% inhibited by 1.5–2.5 μM EDTA in the assay mixture. In a buffer such as imidazole, with a high affinity for metals (O'Sullivan, 1969), and with 1 μM EDTA, pyruvate formation by PEPCK is only 7% of that in Hepes (Table I, second and fourth columns). The rate of pyruvate formation was greatly enhanced when Cd^{2+} in excess of EDTA was added (Table I, third and fifth columns) in either buffer system.

Requirements for the Decarboxylation of OAA To Form Pyruvate Catalyzed by Purified Rat Liver Cytosol PEPCK. The results reported in Table I indicate that the pyruvate-forming activity of PEPCK in the absence of added divalent cation is due to trace contaminants in the assay system, and the effect of EDTA is the result of removing metal ions by chelation. Because of this, EDTA at a concentration greater than 4 μM was routinely included in all assays when the requirements of the pyruvate-forming reaction were studied. For the purpose of consistency with the carboxylation reaction (Colombo et al., 1981), the decarboxylating activity of PEPCK to form pyruvate or PEP was studied in Hepes-buffered reaction mixtures.

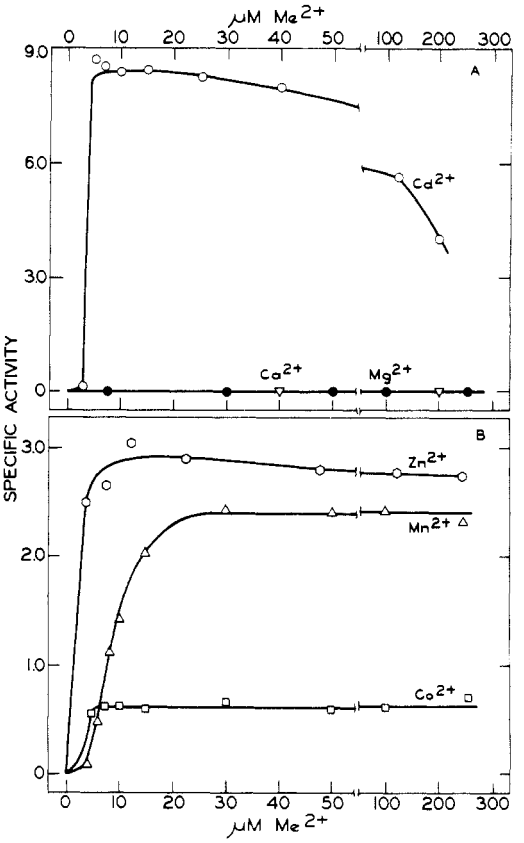

FIGURE 1: Effect of divalent metal ions on pyruvate formation catalyzed by PEPCK. The reaction mixtures contained 0.6 mM OAA and 1 mM IDP. The concentration of total added divalent cation is indicated on the abscissa. Other experimental conditions were as follows: (A) (○) Cd^{2+}, 0.81 μg of PEPCK, and 5.5 μM EDTA; (●) Mg^{2+}, 4.13 μg of PEPCK, and 5 μM EDTA; (▽) Ca^{2+}, 4.13 μg of PEPCK, and 5 μM EDTA; (B) (○) Zn^{2+}, 1.06 μg of PEPCK, and 4.5 μM EDTA; (△) Mn^{2+}, 1.38 μg of PEPCK, and 6 μM EDTA; (□) Co^{2+}, 2.75 μg of PEPCK, and 7 μM EDTA. Enzyme activity is expressed as μmol of pyruvate formed min^{-1} (mg of PEPCK)^{1}. No activity was detected in the absence of added divalent metal ions.

Figure 1 shows that the pyruvate-forming activity of PEPCK is completely dependent on the presence of added divalent metal ions. Maximum pyruvate-forming activity was achieved with each metal ion when its concentration was equal to or slightly higher than that of the EDTA present in the assay mixtures. Maximal activity with Cd^{2+} was higher than with Zn^{2+} or Mn^{2+}, and Co^{2+} was least effective. Mg^{2+} and Ca^{2+} were ineffective in activating PEPCK-catalyzed formation of pyruvate (Figure 1A). No inhibition of enzyme activity occurred with concentrations of added Mn^{2+}, Zn^{2+}, or Co^{2+} up to 250 μM, but high concentrations of Cd^{2+} (120 and 200 μM) were markedly inhibitory (Figure 1A). It is likely that this inhibition is brought about through interaction of free Cd^{2+} with the vicinal dithiol that contains the cysteine essential for PEPCK activity (Carlson et al., 1978).

The rate of pyruvate formation with rat liver cytosol PEPCK was not greater than the spontaneous rate of OAA decar-

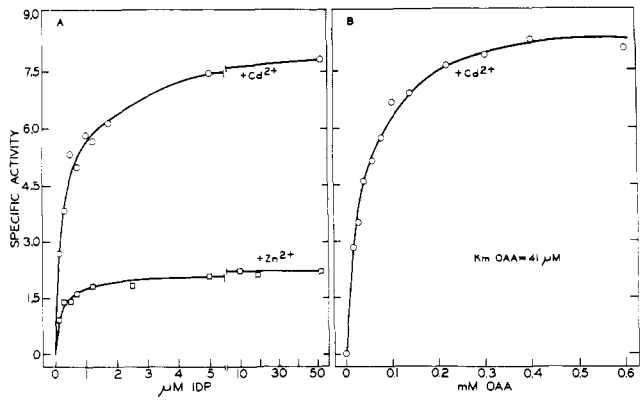

FIGURE 2: Rate of pyruvate formation as a function of the concentration of IDP or OAA with a fixed concentration of divalent metal ion. (A) Concentration of OAA was 0.6 mM; in addition, the reaction mixtures contained (○) 10 μM Cd^{2+}, 4.5 μM EDTA, and 1.04 μg of PEPCK or (□) 10 μM Zn^{2+}, 5 μM EDTA, and 2.07 μg of PEPCK. (B) IDP concentration was 0.4 mM; the reaction mixture also contained 10 μM Cd^{2+}, 5.5 μM EDTA, and 0.52 μg of PEPCK. The rates are corrected for the spontaneous decarboxylation of OAA determined in identical assay mixtures, but without PEPCK.

boxylation in the absence of PEPCK either when IDP or when any of the added divalent metal ions was omitted from the assay system as described in the legend of Figure 1.

Kinetic Parameters for the Decarboxylation of OAA To Form Pyruvate Catalyzed by Purified Rat Liver Cytosol PEPCK. The rate of pyruvate formation in the presence of added Cd^{2+} or Zn^{2+} and as a function of the concentration of IDP is shown in Figure 2A. The Lineweaver–Burk plots are linear (not shown). The apparent K_a for IDP is 0.2 and 0.25 μM with Zn^{2+} and Cd^{2+}, respectively. The maximal velocity was 3.5-fold higher with Cd^{2+} than with Zn^{2+} when the added metal salt concentration was 10 μM. Figure 2B shows an OAA saturation curve at fixed concentrations of IDP and Cd^{2+}. The reaction follows Michaelis–Menten kinetics with an apparent K_m for OAA of 41 μM.

Stoichiometry of Nucleoside Diphosphate Dependent Pyruvate Formation Catalyzed by Purified Rat Liver PEPCK. In addition to a divalent metal ion, IDP is required for the formation of pyruvate from OAA by PEPCK. The nucleoside diphosphate is not a consumable substrate but an essential activator for the reaction. The stoichiometry between IDP and PEPCK was determined by measuring the rate of pyruvate formation when the concentrations of enzyme and IDP were simultaneously varied but with the total concentration of enzyme plus IDP kept constant (Figure 3). The plot in Figure 3 is fairly symmetrical, and the maximum is obtained in the range of 0.42–0.50 and 0.5–0.58 mole fractions of IDP and PEPCK, respectively. This indicates an approximate 1:1 combining ratio under these assay conditions where Cd^{2+} was the supporting divalent cation. The plot appears to be slightly skewed to the right which could result from the presence of some inactive protein in the enzyme preparation.

Antagonism between Different Divalent Metal Ions. To assess the relative ability of the different cations in supporting PEPCK activity, we examined combinations of various metal ions (Figure 4). The activity with 6 μM added Cd^{2+} (open inverted triangles) was no different from that with 12 μM added Cd^{2+} (not shown). The rate of pyruvate formation supported by 6 μM added Cd^{2+} was decreased by the simultaneous presence of Zn^{2+}, Co^{2+}, or Mn^{2+} (Figure 4, open squares in panels A, B, and C, respectively). Zn^{2+} was the most effective antagonist of the Cd^{2+}-supported PEPCK activity. The specific activity of PEPCK with 6 μM Cd^{2+} + 55 μM Zn^{2+} was essentially identical with that with 55 μM Zn^{2+}

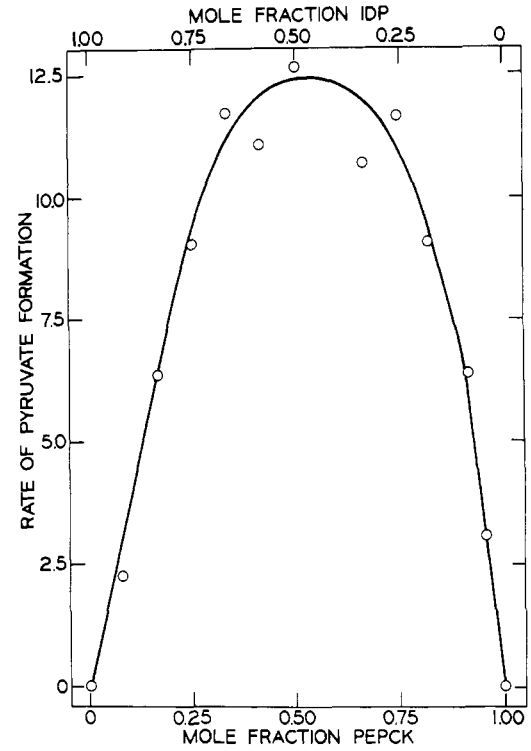

FIGURE 3: Rate of pyruvate formation as a function of varying proportions of PEPCK and IDP in the assay medium. The total concentration of PEPCK + IDP was 0.1 μM in assays containing 0.6 mM OAA, 14.4 μM Cd^{2+}, 7.4 μM EDTA, and other additions as indicated under Materials and Methods for the pyruvate-forming activity. The mole fraction of IDP and enzyme present in each assay mixture is indicated on the abscissas. The amount of pyruvate formed per minute is expressed in arbitrary units.

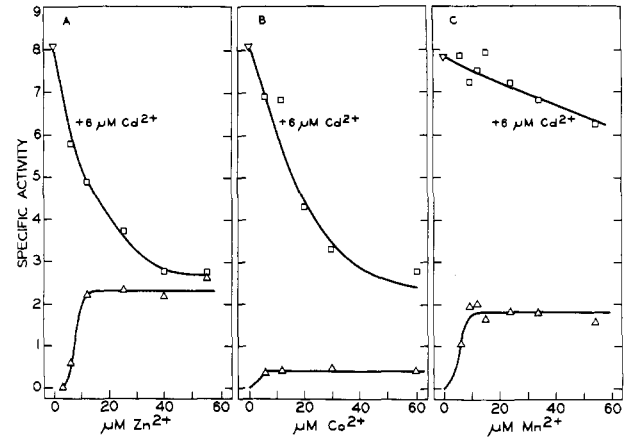

FIGURE 4: Antagonistic effect of different divalent cations on the Cd^{2+}-activated pyruvate-forming activity of PEPCK. The reaction mixture as described under Materials and Methods for the pyruvate-forming activity also contained 0.6 mM OAA, 5 μM IDP, 6 μM EDTA, and 2.1 μg of PEPCK. Additions of divalent metal ions were as follows: (▽) 6 μM Cd^{2+} alone; (□) 6 μM Cd^{2+} plus Zn^{2+} (A), Co^{2+} (B), or Mn^{2+} (C); (△) activity obtained in the absence of Cd^{2+} with either Zn^{2+} (A), Co^{2+} (B), or Mn^{2+} (C). Specific activity is expressed as μmol of pyruvate formed min^{-1} mg^{-1}.

alone. At this same concentration, Co^{2+} and Mn^{2+} were less effective in antagonizing the Cd^{2+}-stimulated pyruvate formation (Figure 4, panels B and C).

Under the assay conditions of Figure 4, no detectable pyruvate was formed with Ca^{2+} alone (up to 1 mM) or with Mg^{2+} alone (up to 6 mM). However, the combination of 2, 3, or 6 mM Mg^{2+} with 10 μM Cd^{2+} resulted, respectively, in 39%, 42%, and 52% reversal of the Cd^{2+}-supported formation of pyruvate (not shown).

Table II: Kinetic Parameters for the Initial Velocity of Phosphoenolpyruvate Formation by Purified Rat Liver Cytosol PEPCK[a]

	OAA[b]		ITP[c]	
addition	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Mg^{2+}	0.11 ± 0.01	13 ± 0.4	0.25 ± 0.03	10 ± 0.5
Mg^{2+} + Co^{2+}	0.011 ± 0.002	42 ± 1.3	0.45 ± 0.03	39 ± 0.9
Mg^{2+} + Mn^{2+}	0.035 ± 0.004	79 ± 2.5	0.15 ± 0.01	76 ± 1.9

[a] The assay mixtures as described under Materials and Methods for the PEP-forming activity also contained 20 μM EDTA, and 0.5–1 μM DTT carried over with the enzyme. The added concentration of Co^{2+} or Mn^{2+} was 40 μM. The reaction was started by the addition of PEPCK, and the initial rate was determined. [b] The apparent Michaelis constants for OAA were determined in the presence of 2 mM MgITP with 1.2 mM excess $MgCl_2$. [c] The apparent Michaelis constants for ITP were determined in the presence of 0.45 mM OAA and 1.2 mM $MgCl_2$ in excess of the amount required to form MgITP.

It is clear from Figure 4 (open inverted triangles) that IDP acts in a catalytic fashion because with 5 nmol of IDP present 17 nmol of pyruvate was formed per min in the presence of 6 μM Cd^{2+}.

Effect of Reaction Components on the Decarboxylation of OAA To Form PEP Catalyzed by Purified Rat Liver Cytosol PEPCK. The presence of an appropriate divalent cation is an absolute requirement for significant PEP formation. With the cytosolic fraction from rat liver and at high concentrations of OAA and ITP, Mn^{2+} or Mg^{2+} are effective cofactors, and an even greater rate of PEP formation is achieved with MgITP and low levels of divalent transition-metal ions (Foster et al., 1967; Snoke et al., 1971).

Preliminary experiments with highly purified rat liver cytosol PEPCK indicated that in a Hepes-buffered reaction mixture and with substrates near saturation, EDTA when included in the assay decreased the rate of product formation with Mg^{2+} as the only exogenously added cation (Colombo et al., 1981). However, the effect of EDTA was not as dramatic on PEP formation as it was on the carboxylation reaction with added Mg^{2+} (preceding paper) or on the pyruvate-forming reaction in the absence of added cations (this report).

The rate of PEP formation with 1.2 mM Mg^{2+} and 2 mM MgITP and at two concentrations of EDTA was studied with highly purified PEPCK as a function of variable concentrations of OAA. In the presence of 0.5 μM EDTA (carried over with the PEPCK) or 20.5 μM EDTA, the maximal velocities were, respectively, 15.2 and 7.5 μmol of PEP formed min^{-1} mg^{-1}, and the apparent K_m values for OAA were 21 and 107 μM, respectively. The prevailing effect of EDTA is to increase the apparent K_m for OAA by a factor of 5 while V_m decreases by a factor of 2.

Effect of Divalent Transition Metals on the Formation of PEP by Purified PEPCK. The effect of varying the concentration of Mn^{2+}, Co^{2+}, or Cd^{2+} on the rate of PEP formation was tested under steady-state conditions at fixed levels of OAA and MgITP with EDTA included in the assay mixture (Figure 5). The rate of PEP formation was increased by approximately 5- and 10-fold, respectively, when 40 μM Co^{2+} or 40 μM Mn^{2+} was included in the reaction mixture. Maximal activation of PEP formation occurred at a concentration of added Mn^{2+} between 40 and 50 μM. With Co^{2+}, maximal activation was obtained at even lower concentrations (in fact, when only slightly higher than that of EDTA).

Controls with identical assay mixtures for PEP formation but in which pyruvate kinase was deleted and lactate dehydrogenase was present indicated no detectable PEPCK-catalyzed pyruvate formation from OAA with Mg^{2+} alone (filled hexagon) or with 1.2 mM Mg^{2+} plus variable concentrations of Co^{2+} (filled triangles). With Mg^{2+} plus variable Mn^{2+} concentrations, pyruvate formation by PEPCK (filled circles) amounted to less than 0.3% of the PEP-forming activity.

The rate of PEP formation with MgITP was enhanced ~7-fold when 20–40 μM Cd^{2+} was also included in the assay mixture. However, when the rate of NADH oxidation was determined in the absence of PK, it was apparent that part of this decrease in $A_{340\,nm}$ was due to PEPCK-catalyzed OAA decarboxylation to form pyruvate instead of PEP (filled squares, Figure 5). With 20, 30, and 40 μM added Cd^{2+}, 37.4, 36.6, and 34.7 μmol of NADH were oxidized min^{-1} (mg of PEPCK)$^{-1}$, respectively, when PK + LDH were the coupling enzymes. Under the same conditions, 0.25, 2.0, and 2.2 μmol of NADH were oxidized min^{-1} (mg of enzyme)$^{-1}$ when the PEPCK activity was coupled only to LDH. In Figure 5, the activation by increasing concentrations of Cd^{2+} (open squares) represents the total PEPCK-catalyzed decarboxylation of OAA to form PEP plus pyruvate. The actual rate of PEP formed would then be 0.4%, 5.6%, and 6.3% lower with 20, 30, or 40 μM added Cd^{2+}, respectively.

Effect of Divalent Metal Ions on the Kinetic Parameters of PEPCK. The effects of different divalent metal ions on the initial velocity of purified rat liver cytosol PEPCK were determined by varying the concentration of one substrate, at

Table III: Kinetic Parameters for the Initial Velocity of Oxalacetate Formation by Purified Rat Liver Cytosol PEPCK[a]

	CO_2[b]		PEP[c]		IDP[d]	
addition	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Mg^{2+}	0.38 ± 0.04	0.027 ± 0.0006	0.22 ± 0.04	0.026 ± 0.001	0.24 ± 0.06	0.031 ± 0.002
Mg^{2+} + Co^{2+}	5.1 ± 1	19 ± 1.5	0.10 ± 0.01	13 ± 0.5	0.40 ± 0.06	14 ± 0.6
Mg^{2+} + Mn^{2+}	0.64 ± 0.06	8.6 ± 0.2	0.20 ± 0.02	8.4 ± 0.16	0.23 ± 0.01	10.1 ± 0.15

[a] The assay mixtures described under Materials and Methods for the carboxylating activity also contained 23 μM EDTA, and 0.5–1 μM DTT carried over with the enzyme. The added concentration of Co^{2+} or Mn^{2+} was 46 μM. The reaction was started by the addition of PEPCK, and the initial rate was determined. [b] The apparent Michaelis constants for CO_2 were determined in the presence of 2.4 mM MgIDP with 1.6 mM excess $MgCl_2$; the concentration of PEP was 1.1 mM. [c] The apparent Michaelis constants for PEP were determined in the presence of 2.4 mM MgIDP with 1.6 mM excess $MgCl_2$. The concentration of $KHCO_3$ was 47 mM when the reaction was conducted with Mg^{2+} as the only added metal, and it was 60 mM when the reaction was conducted with Mg^{2+} plus Co^{2+} or Mg^{2+} plus Mn^{2+}. [d] The apparent Michaelis constants for IDP were determined with 1.6 mM $MgCl_2$ over equimolar MgIDP concentrations. The concentration of PEP was 1.1 mM. The concentration of $KHCO_3$ was 47 mM when Mg^{2+} was the only metal added, and it was 60 mM when the assay was conducted in the presence of Mg^{2+} plus Co^{2+} or Mg^{2+} plus Mn^{2+}. For the determination of the kinetic parameter with Mg^{2+} as the only added cation, PEPCK concentration was 10 μg/mL. A full-scale sensitivity of 0.1 absorbance unit and a chart speed of 0.2 in./min were used. The concentration of CO_2 in equilibrium with bicarbonate at pH 7.0 was calculated with the Henderson-Hasselbach equation by using a pK_a' of 6.34 (Umbreit et al., 1964).

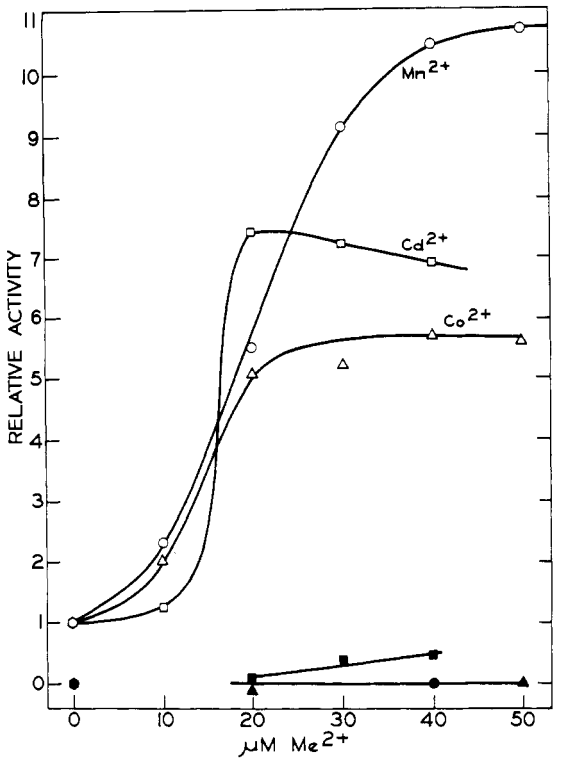

FIGURE 5: Effect of divalent metal ions on the PEP-forming activity of rat liver cytosol PEPCK when present in the assay together with millimolar levels of Mg^{2+}. The assay mixture as described under Materials and Methods for the PEP-forming activity also contained 0.6 mM OAA, 2 mM MgITP, 1.2 mM $MgCl_2$, 70 mM potassium acetate, 21 μM EDTA, and Mn^{2+} (○), Cd^{2+} (□), or Co^{2+} (△) added at the concentration indicated. The ordinate represents the stimulatory effect of Mn^{2+}, Co^{2+}, or Cd^{2+} relative to the activity obtained when PEPCK was assayed with Mg^{2+} (○) as the only added divalent cation in the assay mixture containing PK + LDH (5.1 μmol of PEP formed min^{-1} mg^{-1}). The values for PEP formation have been corrected for the nonenzymatic rate of OAA decarboxylation determined in the absence of PEPCK. The filled symbols represent the rate of pyruvate formation determined in assay mixtures of identical composition as described above for PEP formation but in which PK had been deleted: (●) 1.2 mM Mg^{2+}, Cd^{2+} (■), Co^{2+} (▲), and Mn^{2+} (●) present with 1.2 mM Mg^{2+}. The rate of pyruvate formation is corrected for the nonenzymatic rate of OAA decarboxylation determined in the absence of PEPCK.

saturating or near saturating levels of the other substrate(s). The results are summarized in Table II (OAA decarboxylation to form PEP) and Table III (carboxylation of PEP to form OAA). Experimental details are given in each table legend.

The basal Mg^{2+}-supported formation of PEP by purified PEPCK was stimulated by 40 μM added Mn^{2+} or Co^{2+}. With either OAA or ITP as the variable substrate, V_m was increased 3–4-fold by added Co^{2+} and 6–7-fold by added Mn^{2+} (Table II, second and fourth columns). The apparent K_m for OAA in the presence of Mg^{2+} alone decreased to one-tenth and one-third when Co^{2+} or Mn^{2+}, respectively, was also present (Table II, first column). Under similar experimental conditions, the apparent K_m for ITP increased almost 2-fold with Co^{2+} and decreased by 40% with Mn^{2+} (Table II, third column).

Table III shows the kinetic parameters when the enzyme was assayed in the reverse direction under metal ion conditions similar to those for the forward direction. Either Co^{2+} or Mn^{2+} greatly increased the V_m when added to the assay mixture together with millimolar Mg^{2+} levels. The apparent K_m for CO_2 and IDP increased ~14-fold and 2-fold, respectively, when Co^{2+} was present in combination with Mg^{2+} while the apparent K_m for PEP decreased by a factor of about 2. With Mg^{2+} + Mn^{2+}, the apparent K_m for CO_2 increased by a factor of 2, and no change in the apparent K_m values for PEP and IDP was observed. The lower values for V_m with Co^{2+} plus Mg^{2+} when PEP or IDP was the variable substrate are probably due to the fact that CO_2 was not saturating, but it was present at 3 times its K_m value.

Ferrous ion has been postulated to be involved in the in vivo activation of PEPCK in gluconeogenesis (Bentle et al., 1976; Bentle & Lardy, 1976, 1977). However, the use of Fe^{2+} in assays is troublesome. The "Fe–ferroactivator" stimulation of PEP formation catalyzed by PEPCK from rat liver depended on the assay used to determine enzyme activity. Bentle (1975) found that the activation was observed with the Seubert & Huth (1965) assay, but it could not be detected with assays where OAA is generated from a large pool of malate (Ballard, 1970). With PEPCK from monkey liver, Hammond & Balinsky (1978) reported support of PEP carboxylation by Fe^{2+}, but its effect on PEP formation could not be determined because of problems in the assays containing the Fe^{2+} salts (strong $A_{340\,nm}$, precipitation at high Fe^{2+} concentrations). We attempted to determine the effect of low Fe^{2+} concentration on the kinetic parameters of PEPCK. With 40 μM $FeCl_2$ and other assay conditions as in Table II, the apparent K_m for OAA was 27 μM. In the reverse direction, the apparent K_m for CO_2 was 2.7 mM. The latter value was, however, obtained by adding 30 μM $FeCl_2$ to the assays that had proceeded 10–15 min in the presence of Mg^{2+} alone and under the conditions in Table III. The K_m value for OAA is only tentative because we also encountered some problems with the Fe^{2+}-containing assays. A small increase in the $A_{340\,nm}$ occurred when 40 μM Fe^{2+} and OAA were added to the temperature-equilibrated reaction mixture. It took 2–3 min for the $A_{340\,nm}$ to stabilize. After this period, buffer or enzyme was added, and the nonenzymatic and enzymatic rates of OAA decarboxylation were determined as indicated under Materials and Methods. The rates of PEPCK-catalyzed OAA decarboxylation to form PEP were determined within the first minute of the reaction because the assay was not linear with time. No further studies with Fe^{2+} in combination with Mg^{2+} were attempted.

A comparison of the absolute values for the apparent K_m among different literature reports is difficult due to the fact that the results obtained are dependent on the source and state of purity of the PEPCK, the type of assay employed, and the assay conditions, particularly with regard to the nature of the divalent cation used. The variability of the kinetic data has been summarized by Pogson & Smith (1975), and by Hammond & Balinsky (1978).

Discussion

The inhibitory effect of EDTA, and the influence of the nature of the buffer, on the decarboxylation of OAA to form pyruvate clearly shows that a divalent transition-metal ion is an essential cofactor in this PEPCK-catalyzed reaction (Table I). The observed activity in the absence of added transition-metal ions is presumably due to stimulation by metal ion contaminants of the assay reagents rather than to the presence of a relatively firmly bound metal to the enzyme. No significant amounts of bound metals have been found associated with PEPCK from various sources (Johnston, 1970; Cannata & de Flombaum, 1974; Noce & Utter, 1975; Jomain-Baum et al., 1976).

The divalent metal requirement in the decarboxylation of OAA to pyruvate (eq 2) can be fulfilled by Cd^{2+}, Mn^{2+}, Zn^{2+}, or Co^{2+}. At a concentration of 30 μM their effectiveness varies as indicated: Cd^{2+}, 100%, Zn^{2+}, 34%; Mn^{2+}, 29%; Co^{2+}, 8%. Mg^{2+} and Ca^{2+} were completely ineffective (Figure 1). The order of effectiveness of divalent metal ions in supporting

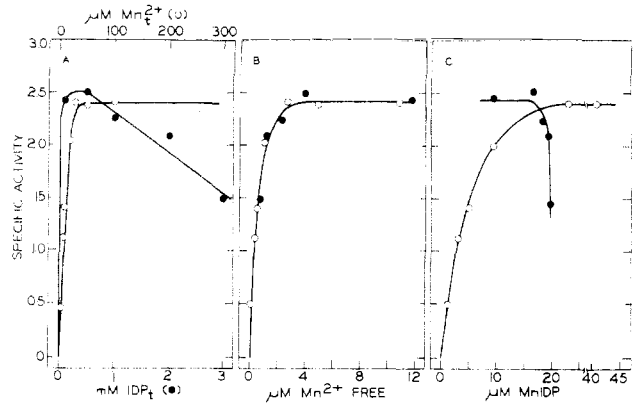

FIGURE 6: Free Mn^{2+} is required for the pyruvate-forming activity of rat liver cytosol PEPCK. The reaction mixtures contained 6 μM EDTA, 0.6 mM OAA, and either 1 mM added IDP with variable Mn^{2+} concentration (○) or 25 μM Mn^{2+} with variable IDP concentration (●) in panel A. The same data are expressed as a function of the calculated concentration of free Mn^{2+} in panel B or as a function of the calculated concentration of MnIDP in panel C. The calculation of the free species in solution was done as indicated under Materials and Methods. Specific activity is expressed as μmol of pyruvate formed min^{-1} mg^{-1}.

reaction 2 and the actual V_m was the same whether the nonconsumable substrate IDP was present at 1 mM (Figure 1) or at 5 μM (Figure 4). The rate of pyruvate formation in assays containing 0.6 mM OAA, 5 μM IDP, 6 μM EDTA, and 29 nM PEPCK was maximal with 6 and 12 μM added Cd^{2+} or 12 μM added Zn^{2+}, Mn^{2+}, or Co^{2+}. With the latter three metals added at 6 μM, the enzyme activity was ~26%, 58%, and 70% of the maximal, respectively (Figure 4).

Since the activity in the absence of added cation was usually 100% inhibited between 1.5 and 2 μM EDTA, we can assume for the sake of discussion and calculations that the concentration of contaminant metal ions present in the assay mixture is of the order of 1.5 μM. Inasmuch as EDTA has a very high affinity for heavy metals (log K > 10 for Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and others), we can further assume that the EDTA present in the uncomplexed form is the difference between total added EDTA and 1.5 μM (O'Sullivan, 1969). This EDTA concentration and the concentration of total divalent metal(s) and IDP added to the assay mixtures were used in order to calculate the amount of free metal, free IDP, and Me^{2+}-IDP complex as indicated under Materials and Methods.

Although the simultaneous presence of a divalent metal and IDP is an absolute requirement for PEPCK to catalyze pyruvate formation, the question arises as to what is the actual nature of the activating species. We analyzed reaction 2 in more detail by using Mn^{2+} as the activating metal. With IDP concentration fixed at 1 mM, increasing Mn^{2+} concentration up to 30 μM stimulated pyruvate formation, and from there on the activity was maximal (Figure 6A, open circles). Conversely, with Mn^{2+} concentration fixed at 25 μM, varying the concentration of IDP increased sharply the rate of the reaction, and with IDP concentration greater than 0.5 mM less pyruvate was formed (Figure 6A, closed circles). A replot of these data as a function of the free Mn^{2+} concentration or as a function of MnIDP concentration is shown in Figure 6B,C. Two points can be made concerning the effect of the metal in the reaction. First, free Mn^{2+} is required as an activator inasmuch as almost the same relationship between specific activity vs. free Mn^{2+} is obtained in the ascending portion of the curve with fixed IDP and variable Mn^{2+} concentrations (open circles in Figure 6A,B) as in the descending portion of the curve with fixed Mn^{2+} and variable IDP concentrations (closed circles in Figure 6A,B). Second, MnIDP per se is not inhibitory. Rather, the decline in activity is due to the removal of free Mn^{2+} since with 3 mM total IDP and 25 μM Mn^{2+} (Figure 6A, closed circles) free Mn^{2+} is 0.76 μM and MnIDP is ~20 μM (closed circles, first point in Figure 6B and last point in Figure 6C, respectively) while with 1 mM total IDP and 30 μM Mn^{2+} (Figure 6A, open circles) free Mn^{2+} is 2.7 μM and MnIDP is ~23 μM (open circles, fifth point in Figure 6B,C, respectively). In other words, in the region between 17 and 23 μM MnIDP (Figure 6C), the difference between the two curves is in the level of free Mn^{2+} which decreases from ~4 to ~0.8 μM for the curve with the closed circles, while it is ~3 μM for the open circles. The level of free IDP is greater than 0.9 and 0.09 mM (open and closed circles, respectively in Figure 6C). The apparent K_a for the activation by free Mn^{2+} is ~0.6 μM (Figure 6B).

The question concerning the nature of the nucleotide-activating species is less clear. With Mn^{2+} concentration fixed at 25 μM and with variation of the concentration of added IDP between 0.25 and 15 μM (not shown in Figure 6A, initial portion of curve with closed circles), a saturation curve for the activation is obtained. The apparent K_a is ~0.13 μM for free IDP and ~24 nM for MnIDP, which is in the range of the PEPCK concentration of the assay. The calculated concentration of free Mn^{2+} ranged from 21 to 19 μM (>30K_a). The apparent K_a values for total IDP were 0.2 and 0.25 μM, respectively, with Zn^{2+} and Cd^{2+} as the supporting cations (Figure 2). With the latter ion, the Job plot in Figure 3 indicated a 1:1 stoichiometry of interaction between enzyme and nucleotide diphosphate.

The effects of combinations of active divalent metal ions at or near maximal concentrations were antagonistic rather than additive. An increase in the concentration of either Zn^{2+}, Co^{2+}, or Mn^{2+} in combination with 6 μM Cd^{2+} resulted in a reduction of PEPCK activity supported by Cd^{2+} alone (Figure 4). The order of effectiveness in reversing the Cd^{2+}-supported formation of pyruvate was Zn^{2+} > Co^{2+} > Mn^{2+} since the concentration of added Zn^{2+}, Co^{2+}, and Mn^{2+} to achieve 50% reversal of the Cd^{2+} activation was ~8, 22, and >60 μM, respectively. Although these values are only approximate, they suggest that the relative affinity of PEPCK for Cd^{2+} is greater than that for Zn^{2+}, Co^{2+}, or Mn^{2+}. In spite of the fact that as judged by the antagonistic effects on activity the interaction of the metals appears to some extent competitive, the results do not establish that the two metals (in any of the pair combinations) are actually competing for the same site of the enzyme. However, there is a preliminary report by Jomain-Baum et al. (1977) which indicates that the rat liver cytosol PEPCK Co^{2+} binding is abolished by high levels of Mn^{2+}. This at least suggests that Co^{2+} and Mn^{2+} may interact at the same site.

One finding of interest is that the Cd^{2+}-supported formation of pyruvate (6 μM EDTA, 10 μM Cd^{2+}, and 5 μM IDP) was not affected by raising the concentration of Mg^{2+} up to 1 mM but became progressively inhibited at higher levels of Mg^{2+}. Without Cd^{2+}, the same levels of Mg^{2+} did not support pyruvate formation. This is consistent with the notion of Mg^{2+} being able to antagonize Cd^{2+}-supported activity in at least three ways: (a) displacing Cd^{2+} from the enzyme, (b) decreasing the concentration of free IDP or of CdIDP by formation of MgIDP, or (c) displacing Cd^{2+} and also forming MgIDP. It can be calculated that out of the 5 μM added IDP ~2.8 μM is MgIDP, 2.0 μM is free IDP, and 0.27 μM is CdIDP when 1 mM Mg^{2+} is present in combination with Cd^{2+} whereas there is ~4.4 μM MgIDP, ~0.5 μM free IDP, and

52 nM CdIDP when the Mg^{2+} concentration is raised to 6 mM. With Cd^{2+} as the supporting cation, the IDP activation follows Michaelis-Menten kinetics (Figure 2) with apparent K_a values of 0.23 and 0.026 μM, respectively, if free IDP or CdIDP is considered. Therefore, the rate of pyruvate formation should be 68% of the maximal with 0.5 μM free IDP; instead, it was 48% (52% reversal of Cd^{2+}-supported activity, see Results). Therefore, it is conceivable that high Mg^{2+} levels inhibited not only by decreasing the level of free IDP (or CdIDP) but also by displacing Cd^{2+} from the enzyme.

Some of the results presented here could conceivably provide a rationale with regard to the reported requirements of different PEPCK's in catalyzing the decarboxylation of OAA to form pyruvate. There is agreement that nucleoside diphosphate is required as activator (Cannata & Stoppani, 1963; Cannata & de Flombaum, 1974; Chang & Lane, 1966; Noce & Utter, 1975; Jomain-Baum & Schramm, 1978; Colombo et al., 1978). However, in these same reports, the effects of divalent cations differ widely (activation, inhibition, or no effect). Metal-complexing agents have been found to inhibit pyruvate formation in the absence of added divalent cations (Noce & Utter, 1975; Cannata & Stoppani, 1963; this report). The observed effects of added cations might depend critically on the concentration and nature of the trace metal contaminant(s), and on the effectiveness and relative affinity of the added cation for the enzyme. For instance, with rat liver PEPCK, we find that the rate of pyruvate formation (1.9 μmol min^{-1} mg^{-1}, with 0.1 mM IDP and no EDTA) appears to be inhibited by Co^{2+}, stimulated by Cd^{2+}, and not affected by Mn^{2+} or Zn^{2+} when the metals were present at 40 μM. However, with 3 μM EDTA in the assay, each of these metals at 40 μM activated pyruvate formation. An absolute requirement for a divalent transition-metal ion has been demonstrated only for pig liver mitochondria PEPCK by Chang & Lane (1966), who used chromatographically pure IDP and imidazole buffer in their assay. A similar effect has been reported with imidazole buffer for the carboxylation of PEP catalyzed by bullfrog liver PEPCK (Goto et al., 1979).

In view of these results regarding the effect of metal ions in reaction 2 and the effect of EDTA on the values of the apparent K_m for OAA in reaction 1 when Mg^{2+} was the only metal added to the assay (see Results), it becomes apparent that studies involving the effect of cations on PEPCK activity must be carried out under well-defined conditions and should be interpreted with care. A further example of these kinds of problems was found in the Cd^{2+} activation of PEPCK-catalyzed reaction 1 or 2. No activation by 20–30 μM Cd^{2+} was apparent if 1 mM DTT was also present in the assay mixture. This can be explained by the well-known affinity of Cd^{2+} for dithiol compounds (Garber & Fluharty, 1968; Yoshida et al., 1979) so that no free metal is available for the activation. The lack of Cd^{2+} activation of PEP formation reported by Bentle & Lardy (1976) was probably due to the high DTT level present in their incubations.

The effects that micromolar amounts of divalent transition-metal ions have on the kinetic parameters of PEPCK (Tables II and III) may be of physiological importance. In the direction of PEP synthesis (considered to be the physiological direction), the more dramatic effect of micromolar Co^{2+} and Mn^{2+} concentrations in combination with millimolar amounts of Mg^{2+} is on the K_m for OAA, which is decreased to one-tenth and one-third, respectively, as compared with that for Mg^{2+} alone. The concentration of OAA in the cytosol is in the range of 1–10 μM (Ray et al., 1966; Williamson et al., 1968, 1969); at pH 7.0, with 100 mM K^+, and Mg^{2+} as the only added divalent cation, the rate of PEP formation would be, respectively, ~2–8% of the V_{max} since the K_m for OAA is 110 μM (100–10-fold higher than the physiological concentrations). In contrast, when micromolar levels of Co^{2+} or Mn^{2+} were present in combination with high levels of Mg^{2+}, the K_m for OAA is decreased to 11 and 35 μM, respectively (Table II), i.e., approximately equal to and 3.5-fold higher than the physiological upper limit of the reported OAA concentration in liver, or 10- and 35-fold higher than the lower limit. Therefore, for the lower and upper limits of liver OAA concentration, the rate of PEP formation increases respectively from ~2% to 15% of the V_m and from ~8% to 48% of the V_m when Co^{2+} is present in combination with Mg^{2+} and from ~2% to 5% of the V_m and from ~8% to 22% of the V_m when Mn^{2+} was present in combination with Mg^{2+}. It should also be noted that, in addition to increasing the affinity of the enzyme for OAA, Co^{2+} and Mn^{2+} stimulate the V_m of PEPCK-catalyzed formation of PEP by 3.4- and 6.3-fold, respectively (Table II). The K_m for ITP was only doubled and halved when Co^{2+} or Mn^{2+}, respectively, was present together with Mg^{2+}. This effect is probably not of physiological importance because the K_m for the physiological nucleotide (GTP) is ~10-fold lower than that with ITP (Colombo et al., 1978; Jomain-Baum et al., 1976). Therefore, PEPCK is likely to be saturated at the concentration of GTP in liver (0.1–0.6 mM, Chance et al., 1965; Clifford et al., 1972).

If it is assumed that the rate of PEP synthesis by PEPCK is limited by the cellular levels of OAA, then the combined K_m and V_m effect of either Co^{2+} or Mn^{2+} in combination with Mg^{2+} results in an ~20-fold increase, relative to the rate with Mg^{2+} alone, of the velocity of the enzyme in the direction of PEP synthesis. However, the decarboxylation of OAA to form PEP is reversible (Utter & Kurahashi, 1954; Jomain-Baum & Schramm, 1978). The net flux toward PEP formation is evidently influenced by the extent of the reverse reaction. In this case, it is less clear which of the substrates could be limiting under physiological conditions. The concentration of PEP in rat liver is in the range of 0.04–0.15 mM (Hornbrook et al., 1965; Ray et al., 1966), and the concentration of bicarbonate is 20 mM (Long, 1961) (at pH 7.0, CO_2 = 4.3 mM). Inspection of the kinetic parameters in Table III indicates that the K_m for CO_2 with Mg^{2+} + Co^{2+} is slightly higher than its physiological concentration while the K_m for PEP is higher than the physiological range when the assay was performed with Mg^{2+} or Mg^{2+} + Mn^{2+}. With an OAA concentration of 10 μM, a PEP concentration of 100 μM, and a CO_2 level of 4.3 mM, it can be calculated that the net flow toward PEP formation increases from 0.94 μmol min^{-1} mg^{-1} with Mg^{2+} alone to 9.3 and 14.2 μmol min^{-1} mg^{-1} with Mg^{2+} + Co^{2+} and with Mg^{2+} + Mn^{2+}, respectively, if PEP is assumed to be the limiting substrate for the reverse reaction and from 0.9 to 10.5 and 5.2 μmol min^{-1} mg^{-1} if CO_2 is taken as the limiting substrate. The activating effect of micromolar levels of Co^{2+} or Mn^{2+} when included in the assay together with Mg^{2+} results then not only in an increased V_{max} but also, and most importantly, in a decreased K_m for OAA, which is decreased to, or near, physiological levels of OAA. The combined effects of transition-metal ions on these two parameters effect a 5–14-fold increase in the net rate of PEP formation (foward minus reverse rates). Evidently, and this is not a small point, the in vivo availability (most likely as a free metal) and the nature of the divalent metal itself are limiting factors for PEPCK activation and activity. In rat liver cytosol, Mg is present at 1.3 mM (Thiers & Vallee, 1957), a concentration sufficient to account for the Mg–nucleotide

triphosphate requirement of the basal PEP-forming activity.

The data in Table II indicate that Mn^{2+} and Co^{2+} increase the V/K ratio by ~22- and 37-fold, respectively. At saturating levels of substrate (Figure 5), maximal activation was achieved with a lower concentration of Co^{2+} than with Mn^{2+}. Since the total concentratijon of Co in the liver is 1 μM (Bowen, 1966) and the cytosol concentration of Mn is 1.8 μM (Thiers & Vallee, 1957), their involvement under physiological conditions is unlikely. Snoke et al. (1971) also did not favor an activating role for Co^{2+} or for Mn^{2+} because these ions did not render the enzyme susceptible to quinolinate inhibition. Because the Fe^{2+}-activated enzyme is inhibited by quinolinate and because the cytosol concentration of Fe is 185 μM (Thiers & Vallee, 1957), a role for Fe^{2+} as activator with the enzyme in hepatic cytosol was favored (Snoke et al., 1971). In vitro activation studied at fixed saturating concentration of substrates, and with preincubation conditions, led to the postulate that in vivo activation of PEPCK activity occurred through its interaction with Fe^{2+} and ferroactivator protein (Bentle et al., 1976; Bentle & Lardy, 1977).

One might ask whether the combination of ferroactivator and Fe^{2+} lowers the K_m for OAA more effectively than does Fe^{2+} alone or whether the ferroactivator functions only to enhance the effectiveness of the extremely low concentrations of Fe^{2+} that can be expected in living cells (Christopher et al., 1974; Davis et al., 1962).

Any conclusion concerning the contribution of divalent transition metals per se to the control of PEPCK activity is only speculative inasmuch as the problem in deciding whether any individual metal plays a significant role in the in vivo regulation is dependent on whether the concentration of such metal ion actually changes during various metabolic states. The kinetic effects are in the right direction; the metal-activated enzyme has more activity at low and physiological OAA concentrations.

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Purification and Characterization of Carboxypeptidase A from Rat Skeletal Muscle†

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ABSTRACT: Carboxypeptidase A (EC 3.4.17.1) has been purified 44 000-fold in 33% yield from rat skeletal muscle by a four-step procedure. Purification in the presence of dichlorovinyl dimethyl phosphate conveniently inactivates an accompanying chymotrypsin-like enzyme and other serine protease(s) to ensure isolation of pure carboxypeptidase A free of polypeptide contaminants. The enzyme preparation consists of two components with molecular weights of approximately 39 300 and 37 800. The rat muscle carboxypeptidase is very similar to bovine pancreatic carboxypeptidase A in terms of (1) substrate specificity, (2) kinetics and molecular activity, (3) influence of metal ions on catalysis, (4) interaction with inhibitors, (5) effects of ionic strength on activity, and (6) stability and activity as functions of pH. Both muscle and pancreatic carboxypeptidases exhibit enhanced esterolytic activity when assayed in the presence of a variety of indoles and imidazoles or after incubation at relatively high concentrations of $MnSO_4$. The muscle enzyme is substantially less stable than its pancreatic homologue, and in impure preparations is very much less soluble. The latter property is attributable to a binding substance present in such preparations which renders muscle but not pancreatic carboxypeptidase A insoluble until ionic strength is increased to values near 2 M.

Elevation in the activity of a cytosolic aromatic amino acid esterase in skeletal muscle from mice, hamsters, and chickens with genetic muscular dystrophy has been under study for some time in our laboratory. Besides being elevated in dystrophic muscle, the esterase is increased after denervation of rat muscle (Meyer et al., 1972; Nwizu et al., 1974) and is increased 20-fold in muscle from vitamin E deficient rats (Meyer et al., 1972) and quokkas (W. L. Meyer and B. A. Kakulas, unpublished experiments). Variation in the level of the esterase has been described as a function of muscle development and in preliminary surveys of human neuromuscular disorders (Meyer et al., 1972, 1973, 1974). Esterase activity rapidly decreases in muscle when rats are fasted (Meyer et al., 1972), and its level is strongly influenced by dietary protein (J. R. Feussner and W. L. Meyer, unpublished experiments). In attempting to determine the relationship between levels of the cytosolic aromatic amino acid esterase and tissue and blood amino acid pools, it was observed that free phenylalanine and tyrosine were particularly elevated in the blood and muscle of dystrophic mice and chickens (Meyer et al., 1973). A powerful system for releasing phenylalanine was detected in the insoluble fraction of skeletal muscle (Reed & Meyer, 1974). The spectrum of amino acids released by this system was determined (Reed, 1975), which led to the hypothesis that the system was composed of chymotrypsin-like[1] and carboxypeptidase A-like activities for which evidence was reported (Meyer & Reed, 1975). These two enzymatic components of the phenylalanine releasing enzyme system appear to be located in mast cells scattered through the muscle, and the chymotrypsin-like insoluble aromatic amino acid esterase is entirely distinct from the previously mentioned cytosolic aromatic amino acid esterase for which no proteolytic activity has been detected (W. L. Meyer, M. L. Douglas, J. P. Reed, and D. N. Weinberg, unpublished experiments). We here report on the purification and characterization of the carboxypeptidase A component from rat skeletal muscle. Reports on carboxypeptidase from rat liver (Haas & Heinrich, 1979) and rat peritoneal mast cells (Everitt & Neurath, 1980) have

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‡ During part of this work, J.E.B. was a recipient of a summer scholarship from the Muscular Dystrophy Association. Present address: Physiology Department, Dartmouth Medical School, Hanover, NH 03755.

[1] Enzymes discussed in this paper are classified (International Union of Biochemistry, 1979) as follows: chymotrypsin, EC 3.4.21.1; carboxypeptidase A, EC 3.4.17.1; lysozyme, EC 3.2.1.17; trypsin, EC 3.4.21.4; aldolase, EC 4.1.2.13; carboxypeptidase B, EC 3.4.17.2.

0006-2960/81/0420-2767$01.25/0