Alteration of Rat Liver Phosphoenolpyruvate Carboxykinase Activity by L-Tryptophan *in Vivo* and Metals *in Vitro**

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ABSTRACT: The administration of L-tryptophan to rats causes a biphasic increase in the activity of the phosphoenolpyruvate (PEP) carboxykinase measured in the 105,000*g* supernatant fraction of the liver, *i.e.*, activity doubles in the first hour, then the rate of increase declines sharply. The initial enhancement is insensitive to inhibition by puromycin or acetoxycycloheximide and presumably results from activation of preexisting enzyme. Since the slower rise after 1 hr is blocked by the above-mentioned inhibitors of protein synthesis, this phase of the increase apparently depends on the synthesis of new enzyme. Liver supernatant fractions from control rats may be stored at $-10°$ for several months without loss of PEP carboxykinase activity. Comparable preparations from rats treated with tryptophan for 1 or more hours lose nearly one-half of their original activity in a few days, but thereafter exhibit the stability of the controls. Dithiothreitol prevents this loss of activity, but does not enhance the activity of supernatant fractions from control rats. Regardless of the relative contributions of activation of preexisting enzyme and synthesis of new enzyme to the total increase in activity following treatment of rats with tryptophan, the fraction of activity lost during storage is constant. The above findings are interpreted as signifying that the administration of L-tryptophan to rats results in an approximate doubling of the intrinsic activity not only of the enzyme existing in the liver at the time of treatment, but also of enzyme synthesized after treatment. In the presence of substrate levels of Mg^{2+}, low concentrations of Mn^{2+} cause an apparent activation of PEP carboxykinase which mimics in several ways the activation of the enzyme following administration of tryptophan. It is proposed that Mg^{2+} best serves the function of complexing the nucleotide to form an active substrate and that Mn^{2+} ($K_m = 10^{-6}$ M) and certain other transition metals activate the enzyme. The activity of PEP carboxykinase in supernatant fractions of livers from fed, fasted, hydrocortisone-treated, or alloxan diabetic rats, but not from tryptophan-treated rats, is approximately doubled by including $5–10 \times 10^{-5}$ M Mn^{2+} in the assay mixture. Sulfate, which by itself has no effect on the PEP carboxykinase of normal rats, increases the activity in the presence of Mn^{2+} by an additional 20%. SO_4^{2-} increases the activity of the enzyme from tryptophan-treated rats in the absence of added Mn^{2+}. Activation of PEP carboxykinase *in vivo* following administration of tryptophan is, however, not the result of an increase in the concentration of Mn^{2+} in the cytosol of the liver. Measurements of metal concentrations in liver supernatant fractions reveal no significant difference between control and tryptophan-treated rats. In addition, the mixing of preparations from control and treated rats produces only additive activity.

In previous publications from this laboratory (Foster *et al.*, 1966b; Ray *et al.*, 1966) we described apparently paradoxical effects of administered L-tryptophan on the PEP[1] carboxykinase of rat liver. Despite a rapid increase in the assayable activity of the enzyme in rats given tryptophan, *in vivo* catalysis by PEP carboxykinase is inhibited. The latter conclusion was reached when it was found that in tryptophan-treated rats there was a rapid accumulation in the liver of lactate, pyruvate, citrate, aspartate, malate, and OAA to concentrations 5–35 times greater than normal, whereas PEP and all succeeding intermediates toward glycogen decreased to one-half of normal concentrations or less. Injection of this amino acid blocks the glycogenic effect of hydrocortisone and prevents glycogenesis from administered pyruvate, malate, and aspartate, but not from glucose or glycerol. However, when present at concentrations as high as 1.7 mM, L-tryptophan had no effect on the activity of PEP carboxykinase *in vitro*.

The rapid doubling of activity (within 1 hr) after administration of tryptophan suggested that the increase resulted primarily from an activation process such as conversion of an inactive form of the enzyme to an ac-

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[1] Abbreviations used in this work: PEP, phosphoenolpyruvate; OAA, oxalacetic acid; GSH, reduced glutathione; ITP, inosine triphosphate; acetyl-CoA, acetyl coenzyme A; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides.

tive form or alteration of the enzyme to a form with greater intrinsic activity. In the present communication we present evidence for the latter of these alternatives.

In our attempts to achieve activation of PEP carboxykinase *in vitro* we found that Mn^{2+} and Fe^{2+} at concentrations of 5–10 $\times$ 10^{-5} M approximately doubled the activity of the enzyme. The possibility that this apparent activation by Mn^{2+} was related to the *in vivo* activation following the administration of tryptophan led to a detailed study of the influence of Mn^{2+} and several other ions on activity. Present results indicate, however, that Mn^{2+} is not responsible for the *in vivo* activation.

Experimental Procedure

Male rats weighing 160–200 g (Badger Research Corp., Madison, Wis.) were maintained on water and, except during periods of fasting, Rockland chow *ad libitum*.

PEP carboxykinase was assayed in the supernatant fraction of rat liver homogenized at 1–2° in 9 ml of 0.25 M sucrose/g of tissue and centrifuged at 105,000*g* for 1 hr at 0–1°. Our standard reaction mixture for the assay of this enzyme is a modification of that described by Nordlie and Lardy (1963) and contains in a final volume of 1.5 ml: 1.6 μmoles of GSH (Sigma Chemical Co.), 6.7 μmoles of OAA (Calbiochem, A grade), 9.0 μmoles of Na_3HITP (P-L Laboratories), 22.5 μmoles of $MgCl_2$, 20 μmoles of KF, 94 μmoles of Tris (Cl), and up to 0.20 ml of the liver supernatant solution. The final pH is 8.0. Incubation is at 30° and the reaction is initiated by addition of enzyme. Any modification of this standard reaction mixture is noted in the appropriate table or figure. Unless otherwise stated, the influences of various ions and compounds on the activity of the enzyme were studied with supernatant fractions of livers obtained from rats fasted for 24 hr before death. PEP, the product of the enzymatic reaction, was cleaved with mercuric ion (Lohmann and Meyerhof, 1934). Inorganic phosphate was estimated by the method of Sumner (1944) before and after the cleavage with mercury.

Protein in the supernatant fraction of liver was determined by the biuret method (Layne, 1957). Bovine serum albumin was used as a standard. Doses of the various compounds given the rats, the route of their administration, and the solvents are indicated in the tables and figures. Control rats received doses of the appropriate solvent. Acetoxycycloheximide was a generous gift from Dr. J. Oleson of Chas. Pfizer and Co. The sources of other chemicals used in this work are listed in a previous publication (Foster *et al.*, 1966b).

Results and Discussion

Activation of Liver PEP Carboxykinase by L-*Tryptophan in Vivo.* The increase in assayable activity of hepatic PEP carboxykinase following the administration of L-tryptophan to rats seems to occur in two phases—an initial rapid increase which in 1 hr results in a doubling of activity, and a slower rate of increase after 1 hr

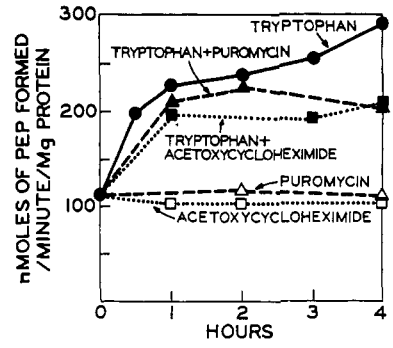

FIGURE 1: The influence of puromycin and acetoxycycloheximide on the tryptophan-induced elevation of PEP carboxykinase activity *in vivo*. L-Tryptophan (75 mg/100 of g rat) was administered intraperitoneally as a suspension in 0.9% NaCl. Puromycin dihydrochloride was dissolved in water, adjusted to pH 6.5 with NaOH, and injected intraperitoneally in doses of 8.7 mg/100 g at hourly intervals beginning 0.5 hr before tryptophan. Acetoxycycloheximide (100 μg/100 g) was injected intraperitoneally in 0.9% NaCl for 0.5 hr before tryptophan. Each point represents the average activity in three or more animals.

(Foster *et al.*, 1966b). The rapidity of the early response suggested that an activation process was the basis for the initial doubling of activity. This hypothesis was tested by following the time course of the tryptophan-induced elevation of PEP carboxykinase activity in rats given doses of puromycin (Gorski *et al.*, 1961) or acetoxycycloheximide (Young *et al.*, 1963) sufficient to block protein synthesis. Figure 1 shows that these inhibitors did not prevent the doubling of activity during the first hour after injection of tryptophan. This result supports the conclusion that the initial, rapid increase in activity is the result of an activation process in which preexisting enzyme is converted either from an inactive to an active form or from a form with lesser to one with greater intrinsic activity. The ability of puromycin or acetoxycycloheximide to inhibit completely the slower rate of increase in activity occurring between 1 and 4 hr after treatment indicates that the basis for the increase during this period is *de novo* synthesis of PEP carboxykinase. It will be shown below that during the second phase of the increase a change in the intrinsic activity of the newly synthesized enzyme also contributes to the overall rise of activity.

Properties of PEP Carboxykinase. Since the activation of PEP carboxykinase *in vivo* may involve a change in the intrinsic activity of the enzyme, several experiments were designed to compare properties of the enzyme from control and tryptophan-treated rats. The results are summarized in Table I. Activity was measured in three different buffer systems over the pH ranges 5.5–8.2 (Tris-maleate), 7.2–8.5 (Tris-Cl), and 8.6–9.7 (glycine buffer). pH measurements at 22° were made on duplicate reaction mixtures. The combined results of these experiments showed that PEP carboxykinase from both con-

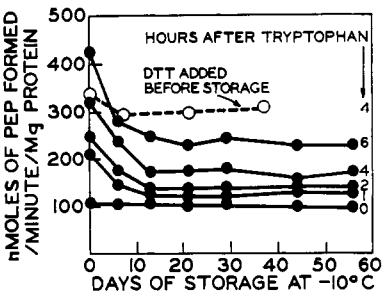

FIGURE 2: Effect of storage at −10° on the PEP carboxykinase activity of liver superantant fractions from a control rat and from rats treated with L-tryptophan (75 mg/100 g) for 1–6 hr before death. In one sample, dithiothreitol (DTT) was added to a final concentration of 10 mM. Each line contains data points from a supernatant fraction of a single rat's liver.

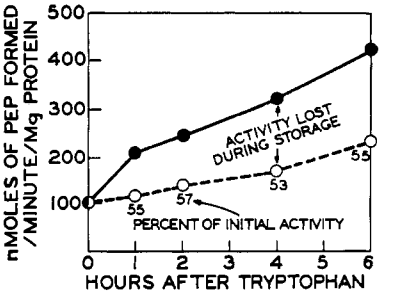

FIGURE 3: PEP carboxykinase activity before (—●—) and after (- - -○- - -) storage at −10° for periods exceeding 20 days of liver supernatant fractions from rats treated with L-tryptophan for 0–6 hr. The figure is a replot of some of the data of Figure 2.

trol and tryptophan-treated rats had the same optimum pH range for activity. The apparent Michaelis constants for OAA and GTP determined at pH 8.0 seem not to be significantly different for the PEP carboxykinases from the two sources. Since our assay procedure is not suitable for determining initial reaction velocities, the K_M values reported in Table I are probably greater than the true values and are presented only for purposes of comparison of the enzymes from control and tryptophan-treated rats.

To determine whether the apparent activation of PEP carboxykinase was associated with a change in quaternary structure, the sedimentation coefficients of the enzymes from control and treated rats were determined by the method of Martin and Ames (1961). We used as a standard crystalline rhodanese which according to Westley and Green (1959) has an $s_{20,w}$ value of 3.2 S. The sedimentation coefficients for the PEP carboxykinases from the two sources were not significantly different. From the sedimentation coefficients, the approximate molecular weight of PEP carboxykinase from the cytosol of rat liver was calculated to be 56,000. This is considerably smaller than the PEP carboxykinase of pig liver mitochondria reported by Chang and Lane (1966) to have a molecular weight of 73,300.

An apparently significant difference between the PEP carboxykinases from control and tryptophan-treated rats was revealed upon storage of the enzyme preparations at −10°. The activity of preparations from control rats remained constant during storage for several months (a typical experiment is shown in Figure 2), but preparations from treated rats lost about one-half of their initial activity in 10–12 days after which time there was no further loss. The activity of preparations from rats given tryptophan for only 1 hr before death declined on storage almost to the level of activity found in preparations from control rats. However, with supernatant fractions from animals treated with tryptophan for longer periods, activity declined during storage to levels significantly above that of control animals. In general, it appears that the enzyme from tryptophan-treated rats changes on storage to the stable enzyme typical of the control animal. The ability of dithiothreitol (Cleland, 1964) to prevent loss of activity in supernatant fractions from treated rats suggests that the loss of activity is due to oxidation of certain thiol groups that are either not present or not susceptible to oxidation in the enzyme from control animals. The presence of dithiothreitol during prolonged storage of control preparations did not alter their activity. These differences between preparations of PEP carboxykinase from the two sources may indicate that activation of the enzyme *in vivo* involves a subtle change in enzyme structure which results in an increase in the intrinsic activity.

Hypothesis. Correlation and interpretation of the re-

TABLE I: Properties[a] of PEP Carboxykinase from Control and Tryptophan-Treated Rats.

Property	Control	Treated[b]
Activity plus and minus standard deviation[c]	108 ± 5	296 ± 27
Optimum pH for activity	7.3–8.1	7.3–8.2
Apparent K_M for OAA (M)	13×10^{-5}	9×10^{-5}
Apparent K_M for GTP (M)	6×10^{-5}	8×10^{-5}
Sedimentation coefficient (S)	4.1	4.3
Approximate molecular weight	54,000	59,000

[a] Experimental procedures used in studying the various properties are given in the text. [b] L-Tryptophan (75 mg/100 g of rat) was given intraperitoneally as a suspension in 0.9% NaCl for 4 hr before death. [c] Given in nanomoles of PEP formed per minute per milligram of protein.

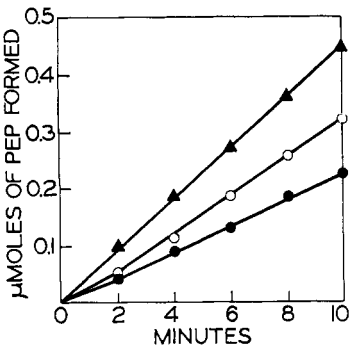

FIGURE 4: The influence of Mn^{2+} on the rate of formation of PEP by rat liver PEP carboxykinase. In addition to the standard components listed in the Experimental Procedure, the reaction mixtures contained zero (●), 5×10^{-6} (○) or 1×10^{-4} M (▲) $MnCl_2$, and 0.21 mg of liver-soluble fraction protein. Incubated at 30°.

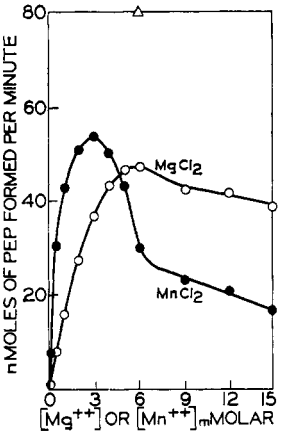

FIGURE 5: The activity of rat liver PEP carboxykinase as a function of the concentration of added Mg^{2+} or Mn^{2+}. Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 20 μmoles of KF, 94 μmoles of Tris (Cl), the indicated concentrations of $MgCl_2$ or $MnCl_2$, and 0.43 mg of protein from rat liver soluble fraction. Reaction time 5 min at 30°. The single point (Δ) represents the activity when $MgCl_2$ was 6 mM and $MnCl_2$ was 0.1 mM.

sults presented in Figures 1 and 2 lead to the following hypothesis for explaining the biphasic increase in PEP carboxykinase activity following the administration of L-tryptophan to rats. The initial doubling of activity, which was not blocked by inhibitors of protein synthesis, is due almost entirely to an increase in the intrinsic activity of the enzyme existing in the liver at the time of treatment with tryptophan. The slower rate of increase after the initial doubling is a result not only of synthesis of new enzyme, but also of an approximate doubling of the intrinsic activity of the newly synthesized enzyme. During storage the enzyme reverts to its less active form. The purpose of Figure 3, which is a replot of some of the data of Figure 2, is to illustrate support for the above hypothesis. Accordingly, the lower curve in Figure 3 describes the increase in activity due to synthesis of new enzyme. The difference in activity between the upper and lower curves is a measure of the enhancement of activity resulting from a change in the intrinsic activity of the enzyme.

Search for Regulators. Since the administration of L-tryptophan to rats results in dramatic changes in the concentrations of many liver metabolites (Ray *et al.*, 1966), it seemed possible that the tryptophan-induced increase in the assayable activity of PEP carboxykinase was a result of a change in the concentration of a hepatic metabolite. The following compounds were tested *in vitro* at concentrations of at least 2×10^{-3} M for an effect on the activity of the enzyme in supernatant fractions from control rats: citrate, L-aspartate, L-malate, pyruvate, DL-lactate, fumarate, succinate, α-ketoglutarate, L-glutamate, DL-isocitrate, *cis*-aconitate, L-glutamine, acetyl-CoA, coenzyme A, glucose 6-phosphate, fructose 1,6-diphosphate, glucose 1-phosphate, 6-phosphogluconate, fructose 6-phosphate, DL-α-glycerophosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, glucose, DPN^+, DPNH, TPN^+, and TPNH. Of these compounds only DL-α-glycerophosphate affected activity. It inhibited 23% at a concentration of 2.7×10^{-3} M. However, since the concentration of L-α-glycerophosphate in the liver of a fasted rat is only about 0.2 μmole/g (Ray *et al.*, 1966), it is highly unlikely that the 50% decrease in this metabolite in the tryptophan-treated rat is responsible for the alteration in the activity of the enzyme. The possibility that some of the above compounds would have affected activity if the concentrations of OAA and ITP in the assay mixture were lower will be tested when a suitable assay for determining initial reaction velocities is devised.

Activation by Mn^{2+} in Vitro. The standard reaction mixture which has been used in this laboratory for the assay of PEP carboxykinase does not contain added Mn^{2+}. Since Mn^{2+} has been shown to be an essential cofactor for the PEP carboxykinase of pig liver mitochondria (Chang and Lane, 1966), and since Mn^{2+} and Mg^{2+} have a synergistic effect on the activity of PEP carboxykinase from both the mitochondrial and soluble fractions of guinea pig liver (Holten and Nordlie, 1965), we investigated the effect of Mn^{2+} on the activity of PEP carboxykinase from the cytosol of rat liver. Figure 4 shows the influence on the rate of PEP formation of adding Mn^{2+} at two different concentrations to the standard reaction mixture. At 5×10^{-6} M Mn^{2+} the rate of the reaction was about 1.5 times that when no Mn^{2+} was added, but there was a lag period of about 2 min before this rate was established. Increasing the Mn^{2+} concentration to 1×10^{-4} M eliminated the lag period and increased the rate to twice that occurring in the absence of added Mn^{2+}. The lag period observed at the lower concentration of Mn^{2+} may indicate (Malmström and Rosenberg, 1959) that the Mn^{2+} reacts with the en-

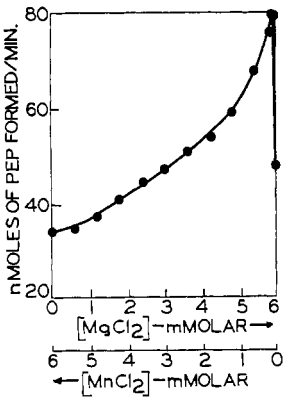

FIGURE 6: The activity of rat liver PEP carboxykinase as a function of the proportions of Mg^{2+} and Mn^{2+} in the assay mixture. Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 20 μmoles of KF, 94 μmoles of Tris (Cl), 9.0 μmoles of $MgCl_2$ plus $MnCl_2$, and 0.43 mg of protein from rat liver supernatant fraction. Reaction time was 5 min at 30°.

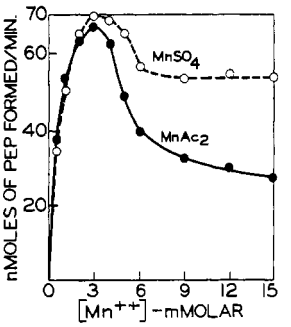

FIGURE 7: Influence of the concentration of two different manganese salts on the activity of rat liver PEP carboxykinase. Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 20 μmoles of KF, 94 μmoles of Tris (Cl), the indicated concentrations of $MnAc_2$ or $MnSO_4$, and 0.41 mg of liver supernatant fraction protein. Reaction time was 5 min at 30°.

zyme to form the active metal–enzyme complex. However, since other factors including the purity of the protein (Smith and Spackmann, 1955) affect the lag phenomenon, the presence of a lag period is not conclusive evidence for the formation of a metal–enzyme complex. A study of the kinetics and mechanism of the activation awaits the purification of PEP carboxykinase from the cytosol of rat liver. This work is currently in progress in this laboratory.

Figure 5 shows the activity of rat liver PEP carboxykinase as a function of the concentration of either $MgCl_2$ or $MnCl_2$. Maximum activity was obtained in reaction mixtures containing 3 mM $MnCl_2$. Activity was 12% lower at the optimum $MgCl_2$ concentration of 5–6 mM. Since the concentration of ITP in these experiments was 6 mM, maximum activity in the magnesium system was reached at nearly equal proportions of Mg^{2+} and ITP, while in the manganese system the optimum ratio of Mn^{2+} to ITP was 1:2. As is shown in a later figure, the inhibition at concentrations of $MgCl_2$ above 6 mM is due largely to Cl^+, since very little inhibition is observed if the anion accompanying the Mg^{2+} is Ac^- or SO_4^{2-}. The sharp decline in activity in the manganese system between 3 and 6 mM $MnCl_2$ probably indicates inhibition by free Mn^{2+} since the concentration of free ITP, a presumed inhibitor of the reaction, would be decreasing as the concentration of Mn^{2+} is increased from 3 to 6 mM. In other experiments in which the concentration of ITP was either 3 or 8 mM, the optimum ratio of $MnCl_2$ to ITP was also 1:2 and a similar sharp decline in activity was observed as the ratio was increased from 1:2 to 1:1. A striking synergistic effect of Mg^{2+} and Mn^{2+} is illustrated by the single point at the top of Figure 5.

The synergism of Mg^{2+} and Mn^{2+} is shown in more detail in Figure 6. In this experiment the total concentration of $MgCl_2$ plus $MnCl_2$ was held constant at 6 mM while the proportions of each were varied. Activity increased with increasing proportion of $MgCl_2$ until at 5.8–5.95 mM $MgCl_2$ and 0.2–0.05 mM $MnCl_2$, respectively, optimum proportions of the two metal ions were achieved. When the concentration of $MnCl_2$ was reduced from 0.05 to 0 mM, activity declined by 40%. In a similar experiment in which the total of $MgCl_2$ plus $MnCl_2$ was fixed at 3 mM, maximum activity was obtained when the $MgCl_2$ concentration was 2.7–2.95 mM and the $MnCl_2$ concentration 0.3–0.05 mM; activity was about 7% higher than when the total of $MgCl_2$ plus $MnCl_2$ was 6 mM.

Since in Figure 6 maximum activity was obtained at a high ratio of Mg^{2+}:Mn^{2+}, it seems likely that the function of Mg^{2+} is to form a MgITP complex which acts as a substrate in the reaction. The low concentration of Mn^{2+} required for maximum activity when substrate concentrations of MgITP are present suggests that Mn^{2+} combines with the enzyme rather than with one of the substrates or products of the reaction. This concept for the function of Mn^{2+} is in agreement with the data of Figure 4 showing a lag phase for activation of the enzyme by low (5×10^{-6} M) concentrations of Mn^{2+}.

Influence of SO_4^{2-} on Activity. Figure 7 shows PEP carboxykinase activity as a function of the concentration of $MnAc_2$ or $MnSO_4$ in reaction mixtures not containing Mg^{2+}. The general shape of these curves may be compared to that of the curve obtained with $MnCl_2$ (Figure 5). As in the case of $MnCl_2$, the optimum concentration of either $MnAc_2$ or $MnSO_4$ was 3 mM. However, with SO_4^{2-} as the anion the decline in activity above the optimum concentration of Mn^{2+} was much less than when the anion was Cl^- or Ac^-.

When the influence of the concentration of $MgCl_2$, $MgAc_2$, or $MgSO_4$ on PEP carboxykinase activity was studied, the results depicted by the lower three curves in Figure 8 were obtained. The figure also shows how these

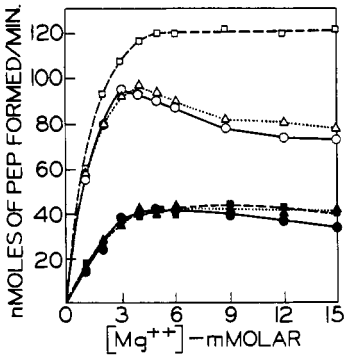

FIGURE 8: Activity of rat liver PEP carboxykinase as a function of the concentration of three different magnesium salts in the absence of Mn^{2+} (closed symbols) or in the presence of 1×10^{-4} M Mn^{2+} (open symbols). Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 20 μmoles of KF, 94 μmoles of Tris (Cl), the indicated concentrations of $MgCl_2$ (● and ○), $MgAc_2$ (▲ and △), or $MgSO_4$ (■ and □), 1×10^{-4} M $MnCl_2$ (open symbols only), and 0.41 mg of supernatant fraction protein. Reaction time was 5 min at 30°.

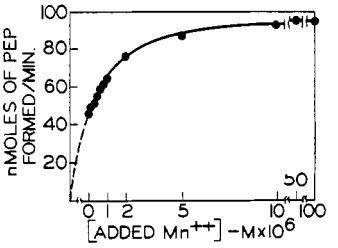

FIGURE 9: Activity of rat liver PEP carboxykinase as a function of the concentration of Mn^{2+}. Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 9.0 μmoles of $MgAc_2$, 20 μmoles of KF, 94 μmoles of Tris (Cl), the indicated concentrations of added $MnCl_2$, and 0.41 mg of protein from rat liver soluble fraction. The enzyme was incubated with the different concentrations of $MnCl_2$ before addition to reaction mixtures also containing $MnCl_2$. Reaction time was 5 min at 30°. Each point represents the average of two or three assays.

results were influenced by the presence in the reaction mixtures of $MnCl_2$ at a concentration of 0.1 mM. In the absence of Mn^{2+} (lower three curves) the relation between activity and concentration of Mg^{2+} was, with the exception of some slight inhibition at the higher concentrations of $MgCl_2$, identical for all three magnesium salts. In the presence of 0.1 mM Mn^{2+}, $MgCl_2$, or $MgAc_2$ did not activate as much as $MgSO_4$ and the former salts inhibited significantly at the higher concentrations. Although this may indicate a competition between Mg^{2+} and Mn^{2+} with respect to activation of the enzyme, it is difficult to understand how SO_4^{2-} could be affecting such a competition. With $MgSO_4$, maximum activity was attained at Mg^{2+} concentrations of 5 mM or greater regardless of the presence or absence of Mn^{2+}. Perhaps the most significant feature of Figure 8 is that in the presence of Mn^{2+} the enzyme exhibits greater activity if the anion of the magnesium salt is SO_4^{2-} rather than Ac^- or Cl^-.

In the experiment depicted in Figure 8 the concentration of SO_4^{2-} always equalled the concentration of Mg^{2+}. We, therefore, examined the effect on activity of different concentrations of SO_4^{2-} at a fixed concentration of Mg^{2+}. In reaction mixtures containing Mn^{2+}, additions of Na_2SO_4 increased activity linearly up to a maximum stimulation of 20% at 6 mM. Further increases of Na_2SO_4 up to 15 mM did not alter this maximum response. This stimulation of activity could not be attributed to an effect of Na^+, because NaAc at concentrations as high as 30 mM did not affect activity measured either in the presence or absence of Mn^{2+}. The mode of action of SO_4^{2-} is being investigated.

Apparent K_M for Mn^{2+}. When the activity of PEP carboxykinase in assay mixtures containing 6 mM Mg^{2+} was measured as a function of the concentration of Mn^{2+}, the results depicted in Figure 9 were obtained. To eliminate the lag period previously observed at low concentrations of Mn^{2+}, the enzyme was preincubated with $MnCl_2$ at the same concentrations as present in the final reaction mixtures. One question arising from Figure 9 is whether the activity measured in the absence of added Mn^{2+} is due to activation of the enzyme by Mg^{2+} or to activation by Mn^{2+} endogenous to the enzyme preparation or some other reagent. If it is assumed that activity in the absence of added Mn^{2+} is due to endogenous Mn^{2+}, and if it is further assumed that the relationship between reaction velocity and concentration of Mn^{2+} follows first-order kinetics, an extension (broken line) of the experimental curve (solid line) produces what appears to be a rectangular hyperbola in which the distance on the abscissa between the point of origin of the hyperbola and zero added Mn^{2+} is an estimate of this endogenous Mn^{2+}. If activity in the absence of added Mn^{2+} is due to activation of the enzyme by Mg^{2+}, then the above distance would represent the theoretical concentration of Mn^{2+} which, in the absence of activation by Mg^{2+}, would be required to obtain the reaction velocity recorded in Figure 9 at zero added Mn^{2+}.

An estimate of the concentration of Mn^{2+} that would account for the activity in the absence of added Mn^{2+} was obtained by replotting the data of Figure 9 as $v/(V-v)$ *vs.* the concentration of added Mn^{2+}. V, the maximum velocity, was estimated from Figure 9. From the slope and the intercept on the ordinate of this curve, the concentration of "apparent" endogenous Mn^{2+} was calculated to be 8.1×10^{-7} M. When this value was added to the various values for the concentration of added Mn^{2+} and the data were replotted in the double-reciprocal manner, Figure 10 was obtained. The equation of the line was obtained by the method of least squares and an

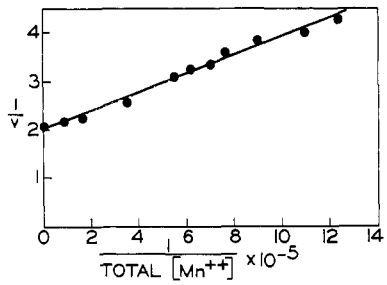

FIGURE 10: A Lineweaver–Burk plot of reciprocal velocity of PEP formation (μmoles/5 min) *vs.* reciprocal total Mn^{2+} concentration (molar). Total Mn^{2+} is the sum of Mn^{2+} added to the reaction mixtures and "endogenous" Mn^{2+}. The calculation of the latter is described in the text. Data were taken from Figure 9.

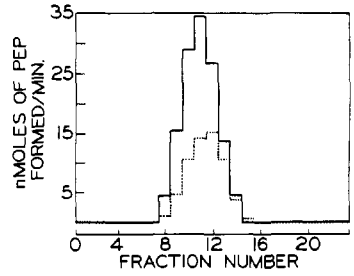

FIGURE 11: The association of PEP carboxykinase activities measured in the presence or absence of Mn^{2+} with a single species of the enzyme as evidenced by density gradient centrifugation. Aliquots of a supernatant fraction of rat liver were layered on each of two sucrose density gradients. After centrifugation in a Spinco SW 39L rotor at 39,000 rpm and 3° for 21.1 hr, the gradients were fractionated and activity in each fraction was measured. Reaction mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of GSH, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 20 μmoles of KF, 9.0 μmoles of $MgAc_2$, 94 μmoles of Tris (Cl), no Mn^{2+} (broken line) or 1×10^{-4} M $MnCl_2$ (solid line), and the total volume of each fraction. Reaction time at 30°: 10 min if the reaction mixture contained Mn^{2+} or 20 min if no Mn^{2+} was added.

apparent K_M for Mn^{2+} (free plus bound) of 9.6×10^{-7} M was calculated. Calculations based on measurements of manganese in the soluble fraction of livers (see below) indicate that the manganese in the enzyme preparations is about 0.02 that required to account for the activity recorded in Figure 9 at zero added Mn^{2+}. The activity observed in the absence of added Mn^{2+} may reflect an intrinsic activation by the Mg^{2+} present or it could be the result of a bound metal.

An absolute specificity for Mn^{2+} as an activator of PEP carboxykinase from the cytosol of rat liver seems unlikely on theoretical grounds (Malmström and Rosenberg, 1959). Preliminary studies with other metal cations have shown that the activity of PEP carboxykinase is increased by adding Fe^{2+} or Co^{2+}, is not altered by Cu^{+} or Cr^{2+}, and is strongly inhibited by 1 mM Cu^{2+} or Ni^{2+}.

Evidence for a Single Enzyme Species. As seen in Table I, the sedimentation coefficient and molecular weight of PEP carboxykinase from the cytosol of rat liver are about 4.2 S and 56,000, respectively. It was of interest to determine whether the increase in activity caused by the addition of Mn^{2+} to our reaction mixtures could be ascribed to activation of the 4.2S enzyme or to activation of some polymer or subunit of the enzyme possibly present in the supernatant fractions of liver. After simultaneous centrifugation of equal aliquots of the supernatant fraction of liver in each of two identical sucrose gradients (Martin and Ames, 1961) and fractionation of the gradients, each fraction was assayed for PEP carboxykinase activity. For one series of fractions, 0.1 mM $MnCl_2$ was included in the reaction mixtures. The results given in Figure 11 show that Mn^{2+} increases activity only in those fractions which exhibit activity in the absence of added Mn^{2+}. This presumably means that it is only the 4.2S enzyme which is activated by Mn^{2+}. The total activity recovered when Mn^{2+} was added to the reaction mixtures was 2.1 times that recovered when no Mn^{2+} was added. Prior to centrifugation of the liver supernatant fraction, its activity in the presence of 0.1 mM Mn^{2+} was increased 2.0-fold.

Relation of Mn^{2+} and SO_4^{2-} to Metabolic and Hormonal Regulation of PEP Carboxykinase. Previous papers from this laboratory (Shrago *et al.*, 1963; Young *et al.*, 1964; Foster *et al*, 1966a,b) have reported the activity of PEP carboxykinase in livers of rats treated in a variety of ways to study the influence of metabolic and hormonal factors on the activity of this enzyme. It appears, however, from the present results that in not including Mn^{2+} and SO_4^{2-} in our standard assay mixture we have assayed PEP carboxykinase under conditions conducive to less than maximal activity. Some of the alterations in the activity of the enzyme reported in our previous publications could have resulted from changes in the concentration of Mn^{2+} in the liver cytosol. Accordingly, the hepatic activity of PEP carboxykinase was altered by three types of hormonal or metabolic manipulation of rats and the influence of Mn^{2+} and/or SO_4^{2-} on the assayable activity of the enzyme is given in Table II. In all rats except those given tryptophan, addition of Mn^{2+} to the assay system increased activity about 100%, addition of Mn^{2+} plus SO_4^{2-} increased activity about 140%, and addition of SO_4^{2-} had no effect unless Mn^{2+} was also added. The activity of PEP carboxykinase from tryptophan-treated rats was not increased by addition of Mn^{2+} to the assay system, but was increased slightly by SO_4^{2-} even in the absence of added Mn^{2+}. Application of Fisher's t test disclosed this increase to be highly significant ($P = \ll 0.01$). Although this study included only a few of the metabolic and hormonal factors influencing the *in vivo* activity of PEP carboxykinase, we have drawn the tentative conclusions, again with an exception for the tryptophan-treated rat, that the previously reported alterations in the activity of the enzyme

TABLE II: Effects of Mn^{2+} and SO_4^{2-} *in Vitro* on the Activity of Hepatic PEP Carboxykinase from Rats Subjected to Various Hormonal and Metabolic Alterations.

Treatment[a] (no. of animals)	Addition to Assay Mixture[b,c]			
	None	Mn^{2+}	SO_4^{2-}	Mn^{2+} + SO_4^{2-}
Fed (4)	58 ± 5	110 ± 6	59 ± 4	138 ± 5
+Hydrocortisone (5)	88 ± 9	175 ± 25	88 ± 8	204 ± 27
Fasted for 24 hr (8)	111 ± 4	214 ± 8	112 ± 5	260 ± 9
+L-Tryptophan (5)	331 ± 20	334 ± 25	385 ± 32	384 ± 32

[a] Hydrocortisone (2.5 mg/100 g of rat) was injected subcutaneously in 0.9% NaCl for 4 hr before death; L-tryptophan (75 mg/100 g) was given intraperitoneally as a suspension in 0.9% NaCl for 4 hr before death. [b] Assay mixtures (1.5 ml, pH 8.0) contained: 1.6 μmoles of reduced glutathione, 6.7 μmoles of OAA, 9.0 μmoles of Na_3HITP, 22.5 μmoles of $MgAc_2$, 20 μmoles of KF, 94 μmoles of Tris (Cl), supernatant fraction of liver, and, where indicated, 0.1 mM $MnCl_2$ and/or 10 mM Na_2SO_4. [c] Activity plus and minus standard deviation given in nanomoles of PEP formed per minute per milligram of protein.

do not have as their basis a change in the Mn^{2+} content of the liver cytosol, and that on a relative scale the activity measured after different treatments of rats is the same in the absence as in the presence of Mn^{2+} and SO_4^{2-}. In view of the very low amounts of manganese in the cytosol of rat liver (Thiers and Vallee, 1957), it may be that assays performed in the absence of added Mn^{2+} reflect more accurately the *in vivo* activity of PEP carboxykinase.

Activation in Vivo Independent of Manganese. The fact that supernatant fractions from the livers of tryptophan-treated rats do not show greater PEP carboxykinase activity when Mn^{2+} is included in the reaction mixture, but respond with increased activity to SO_4^{2-} in the absence of added Mn^{2+} could be interpreted as indicating that an increase in the concentration of Mn^{2+} in the soluble fraction of liver is the basis of the tryptophan-induced increase in activity. Therefore, the concentrations of manganese and other metals in the supernatant fractions of livers from control and tryptophan-treated rats were determined. The manganese content of the liver supernatant fraction of control and tryptophan-treated rats (Table III) is not significantly different and is in substantial agreement with values previously reported (Thiers and Vallee, 1957).[2] In addition, if preparations from control and tryptophan-treated rats are mixed, the activity of the mixture is the sum of the individual activities. Presumably, this would not be the case if excess Mn^{2+} (or some other ion or metabolite) were available in the preparations from the treated animals to activate the enzyme in the preparations from controls.[2] Although activation of PEP carboxykinase by Mn^{2+} mimics in some respects the activation of the enzyme by tryptophan *in vivo*, the latter does not seem to be a consequence of the former.

TABLE III: The Concentration of Manganese in the Soluble Fraction of Livers from Control and Tryptophan-Treated Rats.[c]

	−Tryptophan	+Tryptophan
ng of manganese/mg of soluble fraction protein	4.9[a] ± 1.6[b]	4.6[a] ± 1.1[b]

[a] Each value represents the average of duplicate determinations on supernatant fractions from 4 rats. [b] Standard deviation of the mean. [c] Rats were fasted for 24 hr before treatment. L-Tryptophan (75 mg/100 g of rat) was injected intraperitoneally as a suspension in 0.9% NaCl for 4 hr before death. Control rats received saline. Livers were homogenized in 3 ml of 0.25 M sucrose/g of tissue. Manganese had been removed from the 0.25 M sucrose by passage through a column of Dowex 50 (H^+ form) and the homogenates were centrifuged at 105,000*g* for 1 hr. Aliquots of the supernatant fractions were dried in platinum crucibles and then incinerated at 450 ± 10° for 24 hr. The ash was dissolved in redistilled 6 N HCl. Aliquots were assayed for manganese by emission spectrography.

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[2] The tryptophan treatment did not significantly alter the calcium, barium, aluminum, or molybdenum content of the supernatant fractions. Total iron present was in excess of that measurable in the control rats and in two of the rats treated with tryptophan. In two rats given tryptophan the iron content was at the measurable limit. We conclude that tryptophan treatment had not elevated total iron content. Currently studies are under way to determine whether tryptophan treatment alters the content of nonheme iron.

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