Comparative Studies of Catalytic Properties of Guinea Pig Liver Intra- and Extramitochondrial Phosphoenolpyruvate Carboxykinases*

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ABSTRACT: Guinea pig soluble-fraction and mitochondrial phosphoenolpyruvate carboxykinases were purified 7- and 50-fold, respectively, over crude tissue fractions. The latter enzyme initially was obtained in soluble form by successive freezings and thawings of mitochondria.

A comparison of catalytic properties of these two preparations was made. While, in the direction of phosphoenolpyruvate formation, the mitochondrial carboxykinase was inhibited by adenosine monophosphate and was more active (above 4 mM cation) with Mg^{2+} than with Mn^{2+} at *p*H 8.0, the soluble-fraction enzyme was not affected by the nucleotide and was more active with Mn^{2+} than with Mg^{2+} at all concentrations and *p*H values tested. However, in all other respects, i.e., *p*H optima for both forward and reverse reactions, apparent Michaelis constants assayed under a variety of conditions, synergistic effects of Mg^{2+} plus Mn^{2+} in the direction of phosphoenolpyruvate synthesis, and absolute dependence on Mn^{2+} in the direction of oxaloacetate synthesis, the two activities were catalytically indistinguishable.

On the basis of a number of criteria, including similar tissue distribution and common intramitochondrial location of the two enzymes, Utter (1963; Keech and Utter, 1963) has suggested that phosphoenolpyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase [transphosphorylating], EC 4.1.1.32) and pyruvate carboxylase (pyruvate:CO_2 ligase [ADP], EC 6.4.1.1), functioning in sequence, may catalyze reactions constituting a major pathway for phosphoenolpyruvate synthesis from pyruvate or lactate in the overall process of glucogenesis in avian liver. Nordlie and Lardy (1963) have demonstrated that the subcellular distribution pattern of mammalian liver carboxykinase activity differed, however, among various species. While the enzyme was found principally in mitochondria of rabbit liver and was absent from the soluble fraction, the guinea pig liver activity was distributed between mitochondrial and soluble portions in an approximately 2:1 ratio, and about 90% of total rat hepatic activity was present in the soluble portion. Recent studies (Shrago *et al.*, 1963; Nordlie *et al.*, 1964, 1965) have indicated that levels of phosphoenolpyruvate carboxykinase present in the soluble portion, but not mitochondria, of rat and guinea pig livers respond to alloxan diabetes, adrenalectomy, insulin or glucocorticoid administration, starvation, and other hormonal and metabolic alterations. These differences in subcellular distribution patterns and variations in response to hormonal and metabolic manipulations suggested the possibility of differences in mitochondrial and soluble[1] enzymes. It was felt that significant differences, if present, in catalytic properties of enzymes prepared from the two fractions might be suggestive of differing metabolic roles for the two carboxykinases. Reported in this paper are the results of comparative studies of catalytic properties of carboxykinases partially purified from guinea pig liver mitochondrial and soluble portions.

Experimental

Materials. Young female guinea pigs (250–400 g) were purchased from the Gopher State Caviary, St. Paul, Minn. Liver fractions prepared by the method of Schneider (1948) were suspended in 0.25 M sucrose solution. Nucleotides were purchased from Pabst Laboratories. DEAE-cellulose, reduced glutathione, oxaloacetic acid, phosphoenolpyruvate, crystalline bovine serum albumin, and malic dehydrogenase were obtained from Sigma Chemical Co. Concentrations of nucleotide solutions were determined spectrophotometrically.[2] Oxaloacetate solution concentrations were assayed

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[1] For simplicity, carboxykinase prepared from the soluble fraction of liver is referred to as the "soluble" enzyme throughout this paper. This terminology is intended to indicate the initial source of the enzyme, not its final physical state, since the mitochondrial enzyme also was obtained from the particle in soluble form following repeated freezings and thawings.

[2] Pabst circular OR-17, 1961, p. 2.

TABLE I: Purification of Mitochondrial and Soluble Phosphoenolpyruvate Carboxykinases.[a]

Carboxykinase Source	Enzyme Fraction	Total Units[b]	Total Protein (mg)	Specific Activity[c] (units/mg protein)
Mitochondria	Crude mitochondrial extract	66.	364.	0.181
	Gel extract II	17.7	1.85	8.80
	Gel extract III	8.36	1.36	6.10
Soluble fraction	Crude soluble fraction	10.5	1247.	0.0084
	Gel one supernatant solution	12.4	954.	0.0130
	Gel two supernatant solution	12.1	876.	0.0138
	DEAE-cellulose eluate	7.05	115.	0.0590

[a] Reaction mixtures contained 0.8 μmole of reduced glutathione, 4.5 μmoles of ITP, 3.35 μmoles of oxaloacetate, 11.25 μmoles of $MgSO_4$, 122 μmoles of Tris-Cl buffer, and 0.2 ml of appropriately diluted carboxykinase fraction in 0.75 ml of reaction mixture; *p*H 8.0. Incubation was for 5 minutes at 30°. [b] One unit of enzymic activity is that amount catalyzing the formation of 1 μmole of phosphoenolpyruvate per 0.75 ml of reaction mixture per minute under the defined conditions. [c] Specific activity = units of activity per mg of protein.

spectrophotometrically at *p*H 7.4 with malic dehydrogenase. Protein was determined by the method of Lowry *et al.* (1951). DEAE-cellulose was washed (Peterson and Sober, 1962), adjusted to *p*H 7.0 with HCl, and stored as a slurry. All buffers were adjusted to the desired *p*H values at room temperature (approximately 23°) and subsequently cooled to 4°.

Assay of Enzymic Activity. Composition of reaction mixtures and conditions for individual experiments are described in legends accompanying the various tables and figures. The assay procedure for phosphoenolpyruvate carboxykinase activity for the reaction in the direction of phosphoenolpyruvate synthesis has been described previously (Nordlie and Lardy, 1963). In the presence of Mn^{2+} and in all comparative studies of metal ion specificity, $HgCl_2$ was employed for phosphoenolpyruvate determinations (Lohmann and Meyerhof, 1934; Mudge *et al.*, 1954). For measurement of the reaction in the direction of oxaloacetate synthesis, the assay mixtures routinely contained 3.2 μmoles reduced glutathione, 200 μmoles $NaHCO_3$, 2 μmoles $MnCl_2$, 500 μmoles Tris-chloride buffer, 2 μmoles phosphoenolpyruvate, 0.6 μmole DPNH, 2 μmoles IDP, and 5 units of malic dehydrogenase in a total volume of 2.5 ml; the reaction mixture *p*H was 7.4. Alterations in this basic mixture are described in the discussion of individual experiments. Oxaloacetate formed in the carboxykinase reaction was measured in the coupled system (Utter and Kurahashi, 1954) by following the decrease in DPNH absorbancy at 340 mμ with a Beckman Model DK-2 recording spectrophotometer at 30 ± 0.1°.

Purification of Mitochondrial Phosphoenolpyruvate Carboxykinase. Tissue fractionations and all steps in purification of both mitochondrial and soluble-fraction carboxykinases were carried out at 0–4° unless otherwise specifically noted. Our best purifications of both soluble fraction and mitochondrial enzymes are described in the following paragraphs and in Table I.

STEP 1. EXTRACTION OF ENZYME FROM MITOCHONDRIA. Phosphoenolpyruvate carboxykinase was obtained in soluble form from guinea pig liver mitochondria by freezing (−15°) and thawing (0°) these particles four times over a period of 36 hours (Nordlie and Lardy, 1963). The supernatant solution remaining after centrifugation of the frozen and thawed mitochondria at 34,000 × *g* for 20 minutes is termed the "crude mitochondrial extract."

STEP 2. CALCIUM PHOSPHATE GEL TREATMENT I (ADSORPTION OF CONTAMINATING PROTEINS). To 26 ml of the crude mitochondrial extract was added 11 ml of cold (0°) calcium phosphate gel (Keilin and Hartree, 1938) containing 22.8 mg solids per ml. The *p*H of the suspension was adjusted to 6.8 with 1 N acetic acid. After standing for 10 minutes the suspension was centrifuged at 5000 × *g* for 20 minutes, the active supernatant fluid was decanted, and the gel was discarded.

STEP 3. CALCIUM PHOSPHATE GEL TREATMENT II (ADSORPTION OF CARBOXYKINASE). To 33 ml of the above solution was added 18.5 ml of cold calcium phosphate gel and the *p*H was adjusted to 6.1. The resulting suspension was allowed to stand for 10 minutes and after centrifugation, as described immediately before, the supernatant solution was discarded.

STEP 4. CALCIUM PHOSPHATE GEL EXTRACTION I (ELUTION OF IMPURITIES). The gel was extracted for 10 minutes with 16.5 ml of 0.01 M Tris-chloride buffer, *p*H 7.4, and after centrifugation the supernatant solution was discarded.

STEP 5. CALCIUM PHOSPHATE GEL EXTRACTION II (ELUTION OF CARBOXYKINASE). The gel was extracted with 33 ml of 0.2 M Tris-chloride buffer, *p*H 6.8, for

15 minutes. The suspension was centrifuged and the active supernatant solution was decanted and saved.

STEP 6. CALCIUM PHOSPHATE GEL EXTRACTION III (FURTHER ELUTION OF CARBOXYKINASE). The gel resulting from the second extraction was again extracted, with 40 ml of 0.2 M Tris-chloride buffer, *p*H 7.85, for 15 minutes. After centrifugation the active supernatant was decanted and saved, and the gel was discarded.

The second and third gel extracts represented 50-fold and 30-fold enrichments, respectively, of the enzyme over crude mitochondrial extract (see Table I). The gel extracts lost activity very rapidly, either frozen or at 3°, unless stabilized by the addition of crystalline bovine serum albumin (3.6 mg/ml of extract) followed by concentration of enzyme by precipitation by slow addition of solid ammonium sulfate to a concentration of 70 g/100 ml of extract. After centrifugation at 34,000 × *g* for 20 minutes, the precipitate was taken up in 10 ml of 2 mM reduced glutathione solution, *p*H 7.0. Under these conditions the enzyme lost 37% of its activity over a period of 1 month when stored at 3°. The preparation had slightly greater stability when frozen (−15°), but lost activity very rapidly on thawing.

Purification of Soluble Fraction Phosphoenolpyruvate Carboxykinase. Attempts at purification of the carboxykinase from the soluble portion of liver by using fractional ammonium sulfate or ethanol precipitation, or high or low *p*H fractionation, resulted in extensive loss of enzymic activity. However, preparations of limited purity free of ITPase activity were obtained by the following procedure: The "soluble fraction" of guinea pig liver was obtained by differential centrifugation (DeDuve and Berthet, 1954) of homogenates prepared in 0.25 M sucrose solution. Some contaminating protein was removed by adding to 44 ml of crude soluble fraction, first, 15.7 ml calcium phosphate gel (22.8 mg solids per ml), then adjusting the *p*H to 6.8 and centrifuging, then adding an additional 6.3 ml of the gel to the supernatant fraction, adjusting the *p*H to 6.5, and again centrifuging. The carboxykinase, which was not adsorbed under these conditions, was then subjected to chromatography on DEAE-cellulose. Several glass columns (1.8 × 25 cm) were packed by gravity with DEAE-cellulose (medium mesh) to a height of 6.5 cm and washed with 0.01 M Tris-chloride buffer, *p*H 7.0. Eight ml of the second gel supernatant solution was placed on a column under slight air pressure and the column was eluted with 100 ml of 0.05 M Tris-chloride buffer, *p*H 7.0. The carboxykinase was then eluted with 0.13 M Tris-chloride buffer, *p*H 7.0, and collected in tubes containing 1.5 ml of 0.02 M reduced glutathione and 0.05 g of crystalline bovine serum albumin. The carboxykinase was collected between the tenth and twenty-seventh ml of eluate after the addition of the 0.13 M buffer. After the carboxykinase was collected from the column, it was poured immediately over solid ammonium sulfate (70 g/100 ml eluate). After several columns, run consecutively, had been collected, the ammonium sulfate suspension was centrifuged and the precipitate dissolved in 7 ml of 2 mM reduced glutathione, *p*H 7. This preparation, which lost activity slowly at 3° over a period of several days, was not stable frozen (−15°). Both the chromatography and ammonium sulfate precipitation had to be carried through as rapidly as possible to avoid low yields of the carboxykinase. This procedure resulted in a 7-fold enrichment of enzyme over the crude soluble fraction (see Table I).

The partially purified preparations of both the mitochondrial and soluble-fraction carboxykinases were shown to be free of activities removing phosphoenolpyruvate, oxaloacetate, IDP, and ITP when these substrates were incubated individually with the enzyme under conditions otherwise as employed in carboxykinase assays. A trace of DPNH oxidase (approximately 4% of the activity of either carboxykinase) was accounted for in making measurements of the oxaloacetate-producing reaction.

Results

Effects of Isolation Temperature on Subcellular Distribution Pattern. Wagle (1964) recently has reported that a mitochondrial enzyme, pyruvate carboxylase, appeared in the soluble fraction when isolation procedures were performed at room temperature rather than at 4°. To determine that the phosphoenolpyruvate carboxykinase activities present in subcellular fractions which had been separated by conventional differential centrifugation techniques (Schneider, 1948; DeDuve and Berthet, 1954) represented true mitochondrial and soluble carboxykinases, individual guinea pig livers were divided into two equal portions and the cellular fractionations were performed at 0–4° with one segment and at 23° with the other. The isolated mitochondria were then frozen and thawed four times to activate the carboxykinase (Nordlie and Lardy, 1963) and both fractions were assayed for this enzymic activity. The results (Table II) indicated that only a small amount (4%) of the mitochondrial carboxykinase appeared to enter the soluble fraction when isolation

TABLE II: Effect of Fractionation Temperature on the Distribution of Mitochondrial and Soluble Phosphoenolpyruvate Carboxykinases.[a]

Temperature of Mitochondrial Isolation (°C)	Total Units of Carboxykinase Activity		Fraction of Total Activity	
	Mitochondrial	Soluble	Mitochondrial (%)	Soluble (%)
0–4	22	7.28	75	25
23	19.8	8	71	29

[a] The assay mixture and definition of units of activity are as described in Table I.

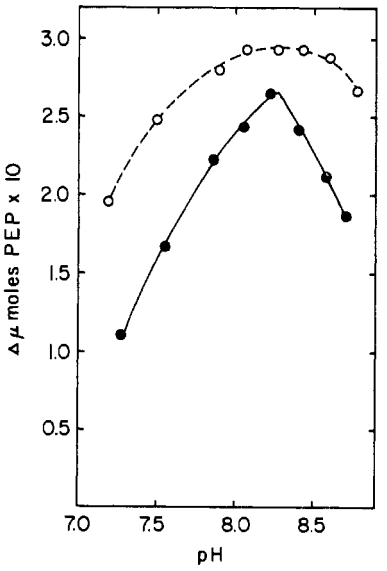

FIGURE 1: Phosphoenolpyruvate (PEP) formation as a function of *p*H. Assay mixtures contained 0.8 μmole of reduced glutathione, 3.35 μmoles of oxaloacetate, 4.5 μmoles of ITP, 11.25 μmoles of $MgSO_4$, Tris-chloride buffer of ionic strength 0.2, and 1.27 mg soluble carboxykinase (specific activity 0.018) (—●—) or 0.128 mg mitochondrial carboxykinase (specific activity 1.46) (– –○– –), in a total volume of 0.75 ml. Incubations were for 5 minutes at 30 ± 0.1°; under these conditions, phosphoenolpyruvate production was a linear function of time at all *p*H values. Further details are given in the text.

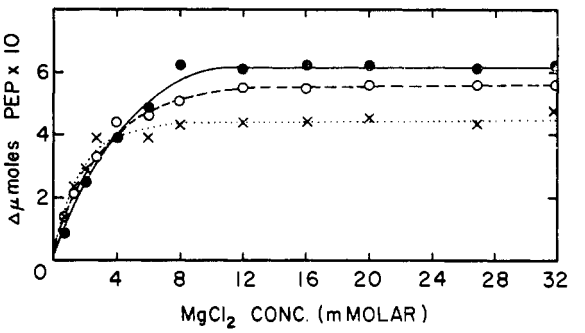

FIGURE 2: Effect of Mg^{2+}/ITP ratio on phosphoenolpyruvate (PEP) formation with the mitochondrial carboxykinase. Reaction mixtures contained 0.8 μmole of reduced glutathione, 122 μmoles of Tris-chloride, 3.35 μmoles of oxaloacetate, 0.02 mg of mitochondrial carboxykinase (specific activity 4.2), 2 (· ·×· ·), 4 (– –○– –), or 6 (—●—) mM ITP, and indicated amounts of $MgCl_2$ per 0.75 ml reaction mixture; *p*H 8.0. Incubations were for 5 minutes at 30°.

was carried out at 23° rather than 0–4°. These results illustrate that the mitochondrial carboxykinase was not easily released from the mitochondria under these conditions and minimize the possibility that the soluble carboxykinase was simply a manifestation of the rupture of mitochondria during isolation. This conclusion is supported by results of recent studies (Shrago *et al.*, 1963; Nordlie *et al.*, 1964, 1965) which indicate that soluble, but not mitochondrial, carboxykinase is responsive to various hormonal and metabolic alterations.

Effects of pH on Carboxykinase Activities. Figure 1 depicts the activity of mitochondrial and soluble carboxykinases, assayed in the direction of phosphoenolpyruvate synthesis, as a function of *p*H. Substrate concentrations were in all instances those previously (Nordlie and Lardy, 1963) found to be saturating, but not inhibitory, with the mitochondrial enzyme at *p*H 8.0. The limits of linearity of activity–protein concentration and activity–incubation time relationships were ascertained in supplementary experiments carried out at 0.4 *p*H-unit intervals throughout the range indicated in Figure 1. Using protein concentrations and incubation periods (5 minutes) found to be within the limits of linearity, we then redetermined activities simultaneously, in duplicate, in individual experiments each covering the entire *p*H range studied. The *p*H measurements were carried out, with a Beckman expanded-scale meter, on reaction mixtures prepared simultaneously with, and duplicating, enzymic-activity assay mixtures. These measurements were carried out at 30° also; readings were taken immediately after addition of enzyme, and at intervals over a period of 5 minutes. No change in *p*H from that initially observed was noted in any instance. The *p*H-activity curves obtained with both preparations (Figure 1) were very similar, differing only slightly in the somewhat broader optimal range (*p*H 8.0–8.4) for the mitochondrial preparation than with the soluble activity (*p*H 8.2). In the direction of oxaloacetate formation, maximal activity was observed at *p*H 7.4 with both soluble and mitochondrial enzymes.

Metal Ion Activation. Since the carboxykinase system involved nucleotide substrates and required metal ion activation, it was necessary to determine the effects of concentrations of those materials on enzymic activity under a variety of experimental conditions. The effect of Mg^{2+} concentration on activity was tested at three levels of ITP (2 mM, 4 mM, and 6 mM) with both the mitochondrial and soluble-fraction carboxykinases. The mitochondrial enzyme showed maximal activity with a Mg^{2+}/ITP ratio of between 2:1 and 3:1 with no inhibition by excess metal (Figure 2). In contrast, the soluble carboxykinase was inhibited by metal ion concentrations which exceeded a Mg^{2+}/ITP ratio of 1 (see Figure 3).

Preliminary experiments carried out at *p*H 8.0 indicated that while Mg^{2+}, Mn^{2+}, and to a lesser extent Fe^{2+} and Co^{2+} were all able to activate both carboxykinases in the direction of phosphoenolpyruvate synthesis, the soluble carboxykinase was most active with Mn^{2+} while the mitochondrial enzyme was more active with Mg^{2+} than with Mn^{2+}. In the direction of

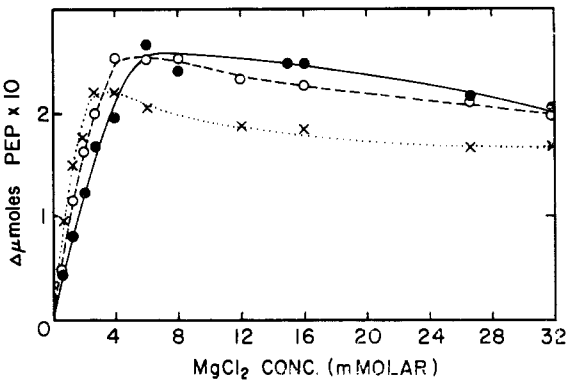

FIGURE 3: Effect of Mg^{2+}/ITP ratio on phosphoenolpyruvate (PEP) formation with the soluble carboxykinase. Reaction mixtures were as in Figure 2, except that 1.6 mg soluble enzyme (specific activity 0.03) was employed.

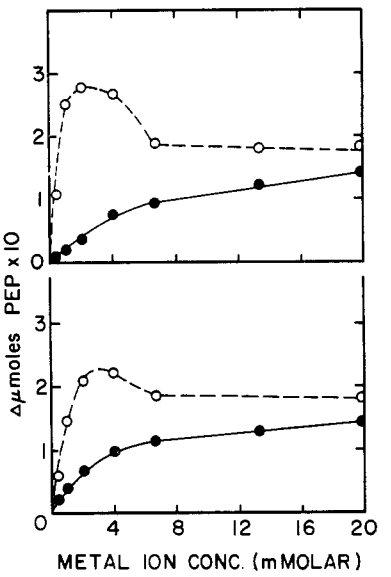

FIGURE 4: Metal ion specificity of the soluble (upper) and mitochondrial (lower) carboxykinases at *p*H 7.3. Reaction mixtures contained 0.8 μmole of reduced glutathione, 122 μmoles of Tris-chloride buffer, 3.35 μmoles of oxaloacetate, 4.5 μmoles of ITP, the indicated amount of Mg^{2+} (—●—) or Mn^{2+} (– –○– –), and 2.3 mg of soluble-fraction carboxykinase protein (specific activity 0.034) or 0.054 mg of mitochondrial carboxykinase protein (specific activity 2.7) per 0.75 ml reaction mixture. Incubations were for 5 minutes at 30°; *p*H 7.3. PEP = phosphoenolpyruvate.

oxaloacetate synthesis both the mitochondrial and soluble enzymes, assayed under conditions described under Experimental, exhibited an absolute requirement for Mn^{2+} which could not be replaced by Mg^{2+}, Ca^{2+}, or Zn^{2+}.

To investigate further divalent cation activation of the two carboxykinases catalyzing the reaction in the direction of phosphoenolpyruvate synthesis, we determined activities as functions of metal ion concentrations at three different *p*H values. At *p*H 7.3 (Figure 4) the soluble and mitochondrial enzymes both showed a greater activity with Mn^{2+} than with an equal concentration of Mg^{2+}. However, at *p*H 8.0 (Figure 5), while the soluble carboxykinase still was more active with Mn^{2+} than with Mg^{2+} at all concentrations tested, the mitochondrial enzyme exhibited greater activity with Mg^{2+} than Mn^{2+} above 4 mM divalent cation concentrations. These studies were repeated at both *p*H values with the crude preparations (specific activity 0.20 and 0.009 for the mitochondrial and soluble enzymes, respectively) and the results were qualitatively the same as those obtained with partially purified preparations. Studies with the crude preparations were extended to *p*H 8.9 and results similar to those at *p*H 8 were obtained.

Metal activation studies performed with impure enzyme preparations, such as those reported here, are subject to the limitation that results may be influenced by nonspecific binding of metal ions by contaminating proteins. To overcome this limitation the experimental approach described in Figure 5 was employed and results were interpreted as follows: With either partially purified enzymes or crude tissue fractions, activities were measured as functions of divalent cation concentrations over a rather wide range. Both Mn^{2+}- and Mg^{2+}-activated enzymic activities passed through optima and then decreased either markedly or slightly as metal ion concentrations were progressively increased beyond optimal concentrations (see Figure 5). With this experimental approach, while no absolute conclusions may be reached regarding the actual concentration requirement for divalent cation, it is valid to conclude that saturating levels of divalent cations were achieved. With saturating levels of Mn^{2+} or Mg^{2+}, i.e., those concentrations giving maximal activities in the experiments described in Figure 5, the ratio, maximal Mn^{2+}-stimulated activity/maximal Mg^{2+}-stimulated activity, was quite different for purified soluble and mitochondrial carboxykinases. Values of 1.83 for the former and 0.88 for the latter preparations were determined from the data in Figure 5.

To substantiate further the fact that variations in metal ion activation were due to differences in catalytic properties of the two carboxykinases rather than resulting from nonspecific metal ion binding by proteins other than carboxykinases, the experiments described in Table III were performed. Phosphoenolpyruvate formation was measured, in the presence of 8 mM Mg^{2+} or 3 mM Mn^{2+}, with partially purified mitochondrial or soluble carboxykinases alone and combined in various proportions. The ratios, Mn^{2+}-stimulated activity/Mg^{2+}-stimulated activity, are recorded in the right-hand portion of the table. Values based on direct observations of phosphoenolpyruvate production with various proportions of total activity contributed by mitochondrial and soluble enzymes combined in individual reaction mixtures

TABLE III: Effects of Various Combinations of Mitochondrial and Soluble Carboxykinase Preparations on Ratios of Mn^{2+}-stimulated Activity/Mg^{2+}-stimulated Activity.[a]

Carboxykinase Preparation		Carboxykinase Activity		Mn^{2+}-stimulated Activity/ Mg^{2+}-stimulated Activity	
Mitochondrial (%)	Soluble (%)	Mg^{2+} ($10 \times \Delta$ μmoles phosphoenolpyruvate/5 minutes)	Mn^{2+}	Observed	Theoretical
100	0	2.04	1.84	0.90	
0	100	1.42	2.88	2.00	
20.3	79.7	1.51	2.43	1.61	1.77
45.0	55.0	1.77	2.64	1.49	1.50
71.1	28.9	2.13	2.81	1.32	1.22

[a] Assay mixtures and other details were as described in the legend to Figure 5, except that 6.0 μmoles $MnCl_2$ or 2.25 μmoles $MgCl_2$ were included in assay mixtures. Partially purified soluble or mitochondrial carboxykinases (specific activities 0.046 and 1.46, respectively) were employed. Units of activity were determined with optimal concentrations of Mg^{2+} for each enzyme. Proportions of activity contributed to various reaction mixtures by each preparation are indicated in the table.

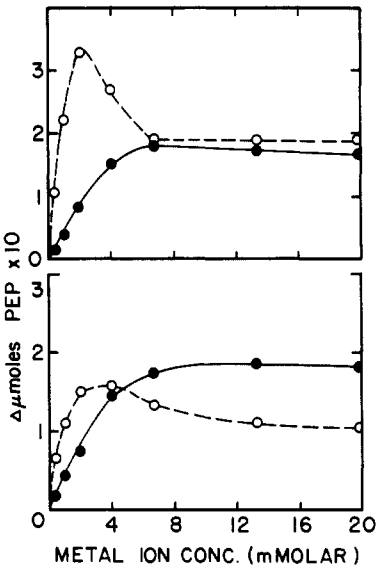

FIGURE 5: Metal ion specificity of the soluble (upper) and mitochondrial (lower) carboxykinases at *p*H 8.0. Reaction mixtures contained 0.8 μmole of reduced glutathione, 122 μmoles of Tris-chloride buffer, 3.35 μmoles of oxaloacetate, 4.5 μmoles of ITP, the indicated amounts of Mg^{2+} (—●—) or Mn^{2+} (- -○- -), and 2.3 mg of soluble carboxykinase protein (specific activity 0.034) or 0.37 mg of mitochondrial carboxykinase protein (specific activity 2.0) per 0.75 ml reaction mixture; *p*H 8.0. Incubations were for 5 minutes at 30°. PEP = phosphoenolpyruvate.

agreed well with those theoretical values (listed in the column on far right in Table III) calculated on the basis of activities determined with either fraction alone. Supplementary experiments, in which 0.8 μmole of phosphoenolpyruvate replaced ITP plus oxaloacetate in reaction mixtures otherwise identical with those described in Table III, indicated that the partially purified enzymes used in these studies were free of phosphoenolpyruvate-removing activities. Hence, the observed effects of metal ions were on the carboxykinase reaction itself. In other experiments of this same design, in which crude tissue mitochondrial and soluble fractions were employed, similar agreement between observed and theoretical activity ratios were obtained. Results of these experiments further substantiate the conclusion that observed differences in activities with Mn^{2+} and Mg^{2+} were true reflections of differences in catalytic properties of the two carboxykinases.

At *p*H 8.0 the effects of Mn^{2+} and Mg^{2+} included in reaction mixtures individually and together were studied; results of these experiments are presented in Table IV. With both the soluble and mitochondrial carboxykinases there was a synergistic effect when Mg^{2+} (13 mM) and Mn^{2+} (3 mM) were present together. The effects of the two ions were nearly additive and may reflect the role of a Mg-ITP complex in the phosphoryl transfer reaction (Nordlie and Lardy, 1963) as well as the role of Mn^{2+} in facilitating the decarboxylation of oxaloacetate (Steinberger and Westheimer, 1951; Utter *et al.*, 1954). In the direction of oxaloacetate formation, with Mn^{2+} at saturating levels (0.80 mM), the addition of Mg^{2+} (0.80 mM) caused no stimulation with the mitochondrial carboxykinase (specific activity 7.2); this effect was not tested with soluble fraction enzyme. The role of metal ions will be considered further under Discussion.

Michaelis Constants for Forward and Reverse Reactions. Apparent Michaelis constants determined for various substrates involved in both forward and reverse reactions are compiled in Table V. Each figure represents an average of values determined in at least

two individual experiments in which all activity measurements were performed in duplicate. Because of the complex nature of the reactions studied (three substrates were involved in one reaction and two in the reverse direction, and nucleotide–metal ion binding appeared to be involved [Nordlie and Lardy, 1963]), it was necessary to evaluate apparent Michaelis constants for certain substrates under a variety of experimental conditions. Specific experimental details are given in Table V. Enzymic activity was linear with protein concentration and incubation time in all instances. When K_m values were determined for an individual substrate, concentrations of other substrates were held at saturating or near saturating levels. Apparent Michaelis constants were calculated in the conventional manner from double-reciprocal plots (Lineweaver and Burk, 1934). In general, apparent Michaelis constants for the various substrates determined for mitochondrial and soluble enzymes agreed relatively closely (Table V); a differentiation between enzymes prepared from the two tissue fractions cannot be made on this basis.

AMP Inhibition. A number of nucleotides previously were found to inhibit the ITP-activated carboxykinase of crude guinea pig liver mitochondrial extracts (Nordlie and Lardy, 1962). Since AMP was one of the most effective of these inhibitors but binds metal ions to a much lesser extent than nucleoside di- and triphosphates (see Bock, 1960), and since adenine nucleotides were shown in supplementary experiments to be inactive as substrates in the carboxykinase reactions,[3] this nucleotide was chosen for comparative inhibition studies of purified mitochondrial and soluble carboxykinases. Under the conditions described in Table VI, the mitochondrial but not the soluble activity was inhibited by 1.33 mM AMP. These results were obtained with either ITP or GTP as phosphoryl donor. ATP at this same concentration was without effect on either activity. Inhibition by AMP was competitive with respect to phosphoryl donor (see Figure 6).

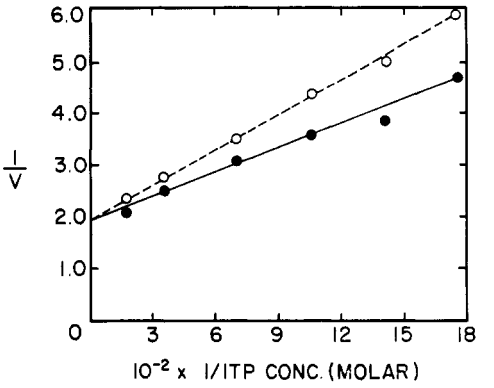

FIGURE 6: Inhibition of mitochondrial carboxykinase by AMP. Reaction mixtures contained 0.8 μmole of reduced glutathione, 22.5 μmoles of $MgSO_4$, 3.35 μmoles of oxaloacetate, 122 μmoles of Tris-chloride buffer, no AMP (—●—) or 4.51 μmoles of AMP (- -○- -), indicated concentrations of ITP, and 0.028 mg mitochondrial carboxykinase protein (specific activity 4.4) in a volume of 0.75 ml; *p*H 8. ITP concentrations are expressed as molarity. Incubation was for 5 minutes at 30°.

TABLE IV: Synergistic Effects of Magnesium and Manganous Ions.[a]

Metal Salt	Carboxykinase Activity: Soluble Carboxykinase (Δ μmoles phosphoenolpyruvate × 10)	Carboxykinase Activity: Mitochondrial Carboxykinase (Δ μmoles phosphoenolpyruvate × 10)
$MgCl_2$	1.59	1.59
$MnCl_2$	4.5	1.42
$MgCl_2 + MnCl_2$	5.2	2.83

[a] Reaction mixtures contained 0.8 μmole of reduced glutathione, 4.5 μmoles of ITP, 3.35 μmoles of oxaloacetate, 122 μmoles of Tris buffer, 10 μmoles of $MgCl_2$ or 2 μmoles of $MnCl_2$ or both, 0.04 mg of mitochondrial carboxykinase protein (specific activity 1.04) or 2.08 mg of soluble carboxykinase protein (specific activity 0.029) in a volume of 0.75 ml; *p*H 8.0. Incubation was for 5 minutes at 30°.

Discussion

The original objective of the studies reported in this paper was to elucidate differences, if present, in catalytic properties of phosphoenolpyruvate carboxykinases obtained from mitochondrial and soluble fractions of guinea pig liver. Two such differences became apparent: (a) inhibition of mitochondrial but not soluble activity by AMP, and (b) variation in the relative levels of stimulation by Mn^{2+} and Mg^{2+} of phosphoenolpyruvate production catalyzed by mitochondrial and soluble preparations.

However, perhaps more significantly, catalytic properties of carboxykinases prepared from the two sources were found in many respects to be nearly identical. It was impossible to distinguish between

[3] Both partially purified carboxykinases exhibited a substrate specificity identical with that reported for the purified avian liver enzyme by Kurahashi *et al.* (1957); i.e., they were specific for inosine or guanosine nucleotides. The observation of a slight activity (approximately 8–15% that obtained with equimolar ITP at *p*H 8.0) with UTP (but not with UDP in the reverse reaction) was consistent with the findings of Kurahashi *et al.* (1957), who attributed the relatively slight UTP activity observed with avian liver enzyme to contamination of this nucleoside triphosphate preparation with ITP or GTP, which they could not separate chromatographically from UTP.

TABLE V: Apparent Michaelis Constants for Mitochondrial and Soluble-Fraction Phosphoenolpyruvate Carboxykinases.[a]

Metal Salt Concentration		Apparent Michaelis Constants: Carboxykinase Preparation Mitochondrial (mM)	Soluble (mM)
$MgCl_2$/ITP ratio = 1	K_m ITP		1.2
$MgCl_2$/ITP ratio = 3		1.2	0.6
$MgCl_2 = 3.3 \times 10^{-2}$ M		0.9	0.6
$MgCl_2 = 1.5 \times 10^{-2}$ M, $MnCl_2 = 2.7 \times 10^{-3}$ M		1.8	1.5
$MgCl_2$/ITP ratio = 1	K_m OAA		1.9
$MgCl_2$/ITP ratio = 3		1.1	3.0
$MgCl_2 = 1.5 \times 10^{-2}$ M, $MnCl_2 = 2.7 \times 10^{-3}$ M		2.5	0.9
$MnCl_2 = 1.2 \times 10^{-3}$ M	K_m IDP	0.07	0.16
$MnCl_2$/IDP ratio = 2		0.19	0.62
$MnCl_2$/IDP ratio = 1	K_m PEP	0.13	0.14
$MgCl_2 = 1.5 \times 10^{-2}$ M, $MnCl_2 = 2.7 \times 10^{-3}$ M	K_m GTP	1.4	1.4

[a] The composition of the reaction mixtures in the direction of phosphoenolpyruvate (PEP) synthesis is the same as in Table I. In each instance, the appropriate substrate concentration was varied within a range giving straight lines in a Lineweaver-Burk plot. Assay mixtures for reactions in the direction of oxaloacetate (OAA) synthesis are given under Methods. Metal concentrations for each experiment are listed in this table.

activities prepared from the two sources on the basis of *p*H optima determined in both directions, or on the basis of apparent Michaelis constants evaluated under a variety of conditions. These marked similarities in fundamental catalytic properties, and rather subtle differences in metal ion activation and nucleotide inhibition, suggest the possibility that the mitochondrial and soluble-fraction carboxykinases are quite possibly variants of the same enzyme protein. The observed qualitative differences in metal ion activation could be reflections of rather subtle differences in secondary and tertiary structure of the enzyme, which in turn could be influenced by variations in composition of the original subcellular environment of the enzyme. For example, the essentially irreversible binding of metal ions or other materials at locations other than the active enzymic site of the carboxykinase molecules could be involved in the determination of the final spatial configuration of the molecules. Variations in the chemical composition of mitochondrial and soluble portions of the cell would then be reflected, finally, in slight differences in catalytic properties of carboxykinases originating in these two cellular compartments. The nature and concentrations of subcellular constituents also conceivably could effect the state of polymerization (or depolymerization) of the enzyme molecule, also resulting in moderate changes in catalytic properties.

In theory, at least, such hypotheses are compatible with the observed differences in inhibition by the nucleotide AMP. It has been pointed out (Dixon and Webb, 1964) that the fact that proteins are synthesized on nucleic acid templates may result in stereochemical relationships optimal for protein-nucleotide interactions. One such possible interaction (that of AMP with carboxykinase protein) may be modified by, or may modify, the structure of the protein such that the catalytic properties of the enzyme are (mitochondria

TABLE VI: AMP Inhibition of Mitochondrial and Soluble Phosphoenolpyruvate Carboxykinase Activities.[a]

Carboxykinase Preparation	Addition	Carboxykinase Activity: 13. mM $MgCl_2$ PEP[b] (Δ μmoles $\times$ 10)	13. mM $MgCl_2$ Inhibition (%)	2.7 mM $MnCl_2$ PEP[b] (Δ μmoles $\times$ 10)	2.7 mM $MnCl_2$ Inhibition (%)
Soluble	None	1.59		4.11	
	AMP	1.59	0	4.03	2
Mitochondrial	None	3.88		3.46	
	AMP	3.19	17.8	2.41	30

[a] The reaction mixture contained 0.8 μmole of reduced glutathione, 3.35 μmoles of oxaloacetate, 1.5 μmoles of ITP, the indicated amount of metal, 122 μmoles of Tris-Cl buffer, and 2.08 mg soluble carboxykinase protein (specific activity 0.087) or 0.034 mg mitochondrial carboxykinase protein (specific activity 2.3) in 0.75 ml; *p*H 8.0. Incubation was for 5 minutes at 30°. AMP (1.0 μmole) was included when indicated. [b] PEP = phosphoenolpyruvate.

carboxykinase) or are not (soluble carboxykinase) inhibited by inclusion of this nucleotide in reaction mixtures. Attempts to develop, in rabbits, antibodies for the mitochondrial carboxykinase were unsuccessful; such studies, which might provide additional information on the protein nature of carboxykinases present in soluble and mitochondrial portions of the cell, must await the availability of purified enzymes in larger amounts than have been obtained thus far.

While the observed variations in metal ion activation appear to be real, as discussed under Results, a more fundamental interpretation of the significance of these variations is not deemed feasible at the present time. Metal ions may be involved in the carboxykinase-catalyzed reactions in at least five ways: (a) by formation of Mg-ITP chelates (Nordlie and Lardy, 1963); (b) by complexing with oxaloacetate (Steinberger and Westheimer, 1951; Utter *et al.*, 1954); (c) by formation of Mn^{2+} chelate of CO_2 (Stiles, 1960; Cannata and Stoppani, 1963); (d) by participation in enzyme-substrate binding of the foregoing complexes (a) to (c), or in other ways; and (e) by promoting retention of configuration of the active enzyme by binding at other than active sites. Obviously, each of these processes would be affected by metal ion concentrations, *p*H, concentration of substrates, and, perhaps, the nature and concentration of protein present. Since "little is known of the intimate details of the [carboxykinase] reaction . . ." (Utter, 1961), further consideration of the meaning of the results must await elucidation of this rather complicated reaction mechanism.

The marked similarities of the two activities preclude the assignment of different metabolic functions to the mitochondrial and soluble carboxykinases solely on the basis of catalytic properties. The involvement of the enzyme present in the soluble portion of the rat liver cell in a new glucogenic pathway recently has been suggested, however (Lardy *et al.*, 1964).

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