THE PURIFICATION AND CHARACTERIZATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

by

Huei-Che Chang, B. S.

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APPROVED:

Chairman, Dr. M. Daniel Lane

Dr. L. B. Barnett

Dr. F. A. Vingiello

Dr. K. W. King

Dr. R. R. Schmidt

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III. List of Abbreviations

PEPCK	phosphoenolpyruvate carboxkinase
PEP	phosphoenolpyruvate
AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
DPNH($NADH_2$)	dihydronicotiamide-adenine dinucleotide
EDTA	ethylene diamine tetraacetic acid
GSH	glutathione
Tris	Tris-hydroxymethylaminomethane
DEAE	diethylaminoethyl-
PCMB	p-chloromercuribenzoate
PK	pyruvate kinase
LDH	lactate dehydragenase
MDH	malate dehydragenase

IV. Literature Review and Introduction

Three distinct enzymatic carboxylation reactions have been described (1-13) in which phosphoenolpyruvate is carboxylated to form oxalacetate. These reactions differ with respect to the mechanism of disposition of the enol phosphoryl group. The least complicated reaction (Reaction 1) which is catalyzed by P-enolpyruvate carboxylase (EC 4.1.1.31) leads to the irreversible formation of oxalacetate and orthophosphate.

$$CH_2{=}C{\cdot}(OPO_3^{2-}){\cdot}CO_2^{1-} + HCO_3^{1-} \xrightarrow{Mg^{2+}} {}^{1-}O_2C{\cdot}CH_2{\cdot}CO{\cdot}CO_2^{1-} + P_i^{2-} \quad (1)$$

This enzyme, which is widely distributed in plant tissue and microorganisms, including spinach leaves (1), wheat germ (3), peanut cotyledons (4), Thiobacillus thiooxidans (5), and Escherichia coli (6), has not been found in animal tissues. The other two carboxylation reactions (Reactions 2 and 3) catalyzed by P-enolpyruvate carboxykinase (EC 4.1.1.32) (7-11) and P-enolpyruvate carboxytransphosphorylase (EC 4.1.1.3) (12, 13), respectively, appear mechanistically similar.

$$CH_2{=}C{\cdot}(OPO_3^{2-}){\cdot}CO_2^{1-} + HCO_3^{1-} + GDP^{3-}\ (\text{or IDP}) + H^{1+} \xrightleftharpoons{Mn^{2+}} \quad (2)$$

$$CH_2{=}C{\cdot}(OPO_3^{2-}){\cdot}CO_2^{1-} + HCO_3^{1-} + P_i^{2-} + H^{+} \xrightleftharpoons{Mn^{2+}\ \text{or}\ Mg^{2+}} {}^{1-}O_2C{\cdot}CH_2{\cdot}CO{\cdot}CO_2^{1-} + PP_i^{3-} + HOH \quad (3)$$

Carboxylation of P-enolpyruvate, accompanied by transfer of the enol phosphoryl group to either GDP (or IDP) or P_i, results in the formation of a new pyrophosphate bond. It is conceivable that all of the P-enolpyruvate carboxylation reactions occur by the same basic mechanism or at least have certain mechanistic features in common.

The first observation relating to P-enolpyruvate carboxykinase was made in 1943 by Evans, et al. (14). They found, with fumarate and pyruvate as substrate, that an acetone powder extract of pigeon liver fixed a substantial amount of $^{11}CO_2$. The isotope was next shown to occur exclusively in the carboxyl groups of pyruvate, lactate, fumarate, and malate.

The formation of phosphoenolpyruvate from Krebs cycle intermediates (citrate, α-ketoglutarate, succinate, fumarate, malate, and oxalacetate) and pyruvate by liver and kidney mitochondria is well documented (10, 15-18). Convincing evidence (15-17, 18) indicates that these precursors of P-enolpyruvate are metabolized via a common intermediate, oxalacetate, which serves as substrate for P-enolpyruvate carboxykinase. This enzyme, which was discovered in liver extracts and was extensively investigated in Utter's laboratory (7-9, 11, 19, 20), catalyzes the reversible GTP (or ITP) -dependent decarboxylation of oxalacetate shown in Reaction 2.

Very little is known concerning the mechanism of action of P-enolpyruvate carboxykinase. The limited number of kinetic studies that have been reported (8, 9, 21, 22) leave many critical questions unanswered. There are, however, several experimental findings (11, 23-25) which must be considered in the formulation of the reaction mechanism. Graves, et al.

(11) have shown, with the use of a deuterium tracer, that the P-enolpyruvate carboxykinase-catalyzed carboxylation reaction leads to the formation of the keto and not the enol form of oxalacetate. With the same technique, the keto form of oxalacetate was found (23) to be the product of the related reaction (nucleotide independent carboxylation of P-enolpyruvate) catalyzed by P-enolpyruvate carboxylase. An insight into the mechanism of the latter reaction was provided by Maruyama, et al. (24, 25) who found that ^{18}O from substrate $HC^{18}O_3^-$ was incorporated into product, P_i, and oxalacetate in a 1:2 ratio during the P-enolpyruvate carboxylase-catalyzed reaction. The reaction is visualized as involving two simultaneous nucleophilic displacements, one at the phosphoryl phosphorus atom and the other at the bicarbonate carbon atom. This mechanism is consistent with the formation of the keto form of oxalacetate and may, in fact, be similar in principle to the mechanism of the P-enolpyruvate-carboxykinase reaction.

While P-enolpyruvate carboxykinase has been purified from chicken liver (7, 20), lamb liver (10), guinea pig liver (18, 26), and pig liver mitochondria (this report), its intracellular distribution appears to vary greatly from species to species. Nordlie and Lardy (18) reported that in the rat, mouse, and hamster, most (90%) of the P-enolpyruvate carboxykinase is localized in the extramitochondrial (primarily soluble) cell fraction. In the pig, guinea pig, and rabbit (18), a much greater percentage (35 to nearly 100%) of the total enzyme activity is localized in the liver mitochondrial cell fraction.

P-enolpyruvate carboxykinase has been implicated in hepatic gluco-

neogenesis from pyruvate and dicarboxylic acid Krebs cycle intermediates (27-29). The "dicarboxylic acid shuttle" proposed by Utter (27) utilizes the combined catalytic activities of mitochondrial pyruvate carboxylase and P-enolpyruvate carboxykinase for the synthesis of P-enolpyruvate from pyruvate. This metabolic "bypass" has been found (27) to be both kinetically and thermodynamically feasible in certain species. It is significant in this connection that mammalian liver, kidney, and heart mitochondria possess (30-32) an active GTP (or ITP) -generating system in the form of succinic thiokinase. The nucleoside triphosphate specificity pattern of succinic thiokinase is identical with that of P-enolpyruvate carboxykinase. Evidence (16, 18) has been obtained which indicates that an effective coupling of these two enzymes is functional in liver mitochondria of some species (see Reactions 4 and 5).

$$\text{Succinyl-CoA + GDP (or IDP)+ } P_i \xrightleftharpoons{Mg^{++}} \text{succinate + CoA + GTP (or ITP)} \qquad (4)$$

$$\text{Oxalacetate + GTP (or ITP)} \xrightleftharpoons{Mn^{++}} \text{P-enolpyruvate + } HCO_3^- \text{ + GDP (or IDP)} \qquad (5)$$

Although many properties of P-enolpyruvate carboxykinase have been described (7-9, 11, 19, 20, 26), homogeneous preparations of the enzyme from animal tissues and its molecular characteristics have not been reported, nor has its reaction mechanism been elucidated. It is the pur-

pose of this thesis to report the isolation of P-enolpyruvate carboxykinase (GTP: oxalacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) in homogeneous form from pig liver mitochondria, a number of its molecular characteristics and properties, and investigation of its reaction mechanism.

V. Experimental Procedure

A. Purification of Inosine Diphosphate and Inosine Triphosphate.

IDP or ITP (sodium salts) 100 μmoles, dissolved in 10 ml of 0.02 M triethylammonium bicarbonate buffer, pH 7.5, are applied to a DEAE-cellulose column (2 x 25 cm) (HCO_3^- ionic form) previously equilibrated with the same triethylammonium bicarbonate buffer. Elution is accomplished with a linear gradient from 0.02 M to 0.40 M triethylammonium bicarbonate (pH 7.5) over a total eluate volume of 2 liters. Chromatography is carried out at 4°. IDP and ITP appear in the eluate when the triethylammonium bicarbonate concentrations reach 0.15 M and 0.21 M, respectively. The desired nucleotide fractions are pooled (usually about 100 ml), taken to dryness in a vacuum at 35-40°, water is added, and the drying process repeated. Nucleotides are then converted to their lithium salts (IDP-Li_3 or ITP-Li_4) by passage through Dowex 50-X4 in the lithium phase. After concentration to dryness as described above, the nucleotides are stored at -20°. Contamination of purified ITP with IDP was not detected in either preparation with the oxalacetate-$H^{14}CO_3^-$ exchange or $H^{14}CO_3^-$ -fixation carboxylase assays, respectively.

B. Preparation of Phosphoenolpyruvate -1-^{14}C.

Potassium pyruvate-1-^{14}C, 40 μmoles (50 μC), was incubated for 30 min at 30° in a reaction mixture containing the following components (in

micromoles unless otherwise indicated): Tris (free base), 200; ATP, 20; $MgCl_2$, 2; creatine phosphate, 10; GSH, 3; KCl, 50; crystalline pyruvate kinase, 2 mg (250 units); and creatine kinase, 1 mg (18 units) in a total volume of 1.5 ml and at a final pH of 9.0. The reaction was terminated with 10 μmoles of EDTA and chilling to 2°. After addition of 15 ml of 0.02 M triethylammonium bicarbonate buffer, pH 7.5, and 10 μmoles of unlabeled P-enolpyruvate, the solution was applied to a DEAE-cellulose column (2 x 20 cm) (HCO_3^- ionic form) previously equilibrated with the 0.02 M triethylammonium bicarbonate, pH 7.5. Elution was accomplished by using a linear gradient from 0.02 M to 0.40 M triethylammonium bicarbonate, pH 7.5, over a total eluate volume of 2 liters. Chromatography was carried out at 0-4°. Phosphoenolpyruvate-1-^{14}C (detected by enzymatic assay and radioactivity) appeared in the eluate when the triethylammonium bicarbonate concentration reached 0.11 M and was completely resolved from ^{14}C-pyruvate, ADP, and ATP which were eluted at triethylammonium bicarbonate concentrations of 0.05 M, 0.13 M, and 0.18 M, respectively. The pooled column fractions (90ml) containing P-enolpyruvate-1-^{14}C were taken to dryness at 35-40° in a vacuum, water was added and the process repeated. Phosphoenolpyruvate-1-^{14}C, 11 μmoles (2.4 μC), was recovered. The radiochemical purity of the isolated material determined paper chromatographically (R_F = 0.53; isobutyric acid-NH_4OH-water, 57:4:39,v/v, ascending solvent system (33)) was >99.5%.

C. Preparation of ^{32}P-enolpyruvate from -^{32}P-Inosine Triphosphate.

γ-^{32}P-ITP, 19 μmoles (150 μC) was incubated for 5 min at 30° with the following components (in micromoles unless otherwise specified): Tris (Cl^-) buffer, pH 7.5, 200; oxalacetate, 20; $MnCl_2$, 10; GSH, 10; and P-enolpyruvate carboxykinase (4 units per mg of protein), 1.2 mg in a total volume of 3.0 ml and a final pH of 7.5. The reaction mixture was chilled at 2°, 10 ml of 0.05 M triethylammonium bicarbonate, pH 7.5, were added, and the entire mixture was applied to a column (2 x 20 cm) of DEAE-cellulose (HCO_3^- ionic form). Elution was accomplished with a 2-liter linear triethylammonium bicarbonate gradient (0.05 M to 0.40 M, pH 7.5). The eluate, which was collected fractionally, contained two radioactive peaks and these were identified as ^{32}P-enolpyruvate (at 0.10 M triethylammonium bicarbonate) and unreacted ^{32}P-ITP (at 0.22 M triethylammonium bicarbonate). Two ultraviolet light (253 mμ) absorbing peaks corresponding to IDP (principal peak at 0.16 M triethylammonium bicarbonate) and ^{32}P-ITP (at 0.22 M triethylammonium bicarbonate), were completely resolved from ^{32}P-enolpyruvate. The fact that the IDP peak contained no ^{32}P-activity indicates that the ^{32}P-ITP was labeled exclusively in the γ position. The ^{32}P-enolpyruvate-containing fractions were pooled (92 ml) and taken to dryness in a vacuum at 35-40° and stored at -20°. The yield of ^{32}P-enolpyruvate was 76% (15 μmoles) and its radiochemical purity as judged by paper chromatography (R_F = 0.55; isobutyric acid-NH_4OH-H_2O solvent system, see preceding section) was >99.5%.

D. Sulfhydryl Group and N-Terminal Group Determination.

Sulfhydryl groups were measured by observing the increase in optical density at 412 mμ resulting from the liberation of the sulfanion of thionitrobenzoate from 5.5'-Dithiobis (2-nitrobenzoic acid) (34). The purified carboxykinase was passed through a Sephadex G 25 column in potassium phosphate buffer (pH 8.0, μ= 0.1) to remove GSH. The reaction was followed at room temperature in a Zeiss spectrophotometer with the use of cells with a 1 cm light path. Samples were read against blanks of identical reaction mixtures without added protein. An extinction coefficient of 13,600 $\underline{M}^{-1}Cm^{-1}$ (34) was employed.

The N-terminal residues of PEPCK were determined by the dinitrofluorobenzene method (35). Dinitrophenylation was carried out by reaction of 2.4 dinitrofluorobenzene (DNFB) with the protein (15.5 mg of PEPCK) in $NaHCO_3$ solution. The DNP protein was hydrolized with redistilled 5.7 $\underline{N}$ HCl under reflux for 12 hours. The DNP protein hydrolysate was then diluted 4-fold and extracted with peroxide-free ether. The ether extract, as well as the aqueous phase, was analyzed by paper chromatography. Chromatography was carried out on Whatman 3 MM filter paper which had been previously soaked in buffer solution (0.05 $\underline{M}$ phthalate buffer, pH 6) and dried at room temperature. The identity of the unknown compounds was established by comparing their R_F values with those of authentic DNP-derivatives on the same chromatogram. Two solvent systems (36), 1.5 $\underline{M}$ $NaHPO_4$ buffer and amyl alcohol (saturated with phthalic acid, 0.05 $\underline{M}$, pH 6) were used to develop (descending) the

chromatograms. After development, the chromatograms were dried at room temperature and the R_F value of each spot was determined.

E. Equilibrium Dialysis.

The equilibrium dialysis was carried out as described by Klotz, et al. (37). In each equilibrium dialysis experiment, 2 ml of 0.1 M pH 7.1 imidazole buffer solution of the enzyme (0.13 to 1.06 mg of protein; 1 to 8 x 10^{-6} M) was placed in a small dialysis bag and dialyzed against 25 ml of the same buffer solution containing radioactive substrate. The dialysis solution also contained 2mM GSH, 0.1 mM EDTA and 1mM $MnCl_2$ (when Mn^{++} was added). The substrate concentrations employed varied from 0.14 to 1.1 x 10^{-6} M. Dialyses were carried out at 0° and were stirred magnetically for 40 hrs. Substrate concentration at equilibrium was calculated from the specific activity of the substrate and the radioactivity determined on aliquots of the dialysates and the dialysis solutions. The difference between these equals the total quantity of substrate bound to enzyme.

F. Protein Determination and Definition of an Enzyme Unit.

Protein was determined spectrophotometrically at 280 mμ and 260 mμ according to the method of Warburg and Christian as described by Layne (38) unless otherwise specified.

A unit of PEPCK is defined as that amount of enzyme which catalyzes

the carboxylation of 1.0 μmole of PEP per minute under the carboxylation assay conditions described. Specific activity is expressed as units per mg of protein.

Decarboxylation rate is expressed as micromoles of OAA decarboxylated per minute.

G. Carboxylation Assay.

The IDP- and Mn^{++} -dependent carboxylation of P-enolpyruvate results in the formation of ITP and oxalacetate (Reaction 2). The reaction velocity is followed, in the presence of NADH and malate dehydrogenase, by determining either the rate of incorporation of $H^{14}CO_3^-$ into malate (acid-stable ^{14}C activity) or the rate of NADH oxidation spectrophotometrically. The $H^{14}CO_3^-$ -fixation carboxylation assay reaction mixture contains the following components (in micromoles unless specified): imidazole (Cl^-) buffer, pH 6.6, 100; $KH^{14}CO_3$ (approximately 10^5 cpm per μmole), 50; P-enolpyruvate, 1.25; IDP, 1.25; $MnCl_2$, 1.0; GSH, 2.0; NADH, 2.5; malate dehydrogenase, 1 to 5 units; and P-enolpyruvate carboxykinase, up to 0.004 unit in a total volume of 1.0 ml. The final pH is 7.0. After a 15-min incubation at 30°, the reaction is terminated by addition of 1 ml of 2 N HCl. A 0.5-ml aliquot is taken to dryness in a scintillation counting vial at 85° for 60 min in a forced draft oven. After addition of 1 ml of H_2O to the vial, followed by 10 ml of liquid scintillator, acid-stable ^{14}C activity (as ^{14}C-malate) is determined with a liquid scintillation spectrometer.

Initial velocity of bicarbonate fixation follows zero order kinetics for at least 20 min and is proportional to enzyme concentration up to a level of 0.004 unit of carboxykinase.

The more rapid spectrophotometric carboxylation assay is usually used for carboxykinase preparations carried beyond Stage 4 (cellulose phosphate chromatography) of the purification sequence. The reaction mixture and conditions for this assay are modified from those described for the $H^{14}CO_3^-$ -fixation carboxylation assay (above) to include, unlabeled, instead of ^{14}C-bicarbonate and less NADH (0.15 μmole). The initial velocity of NADH oxidation is followed for 2 min at 340 mμ (1-cm light path) after initiating the reaction with P-enolpyruvate. The carboxylation reaction follows zero order kinetics with up to 0.055 unit of carboxykinase.

H. Decarboxylation Assay.

The ITP- and Mn^{++} -dependent decarboxylation of oxalacetate catalyzed by the carboxykinase leads to the formation of IDP and P-enolpyruvate (reversal of Reaction 2). This reaction is coupled to pyruvate kinase- and lactate dehydrogenase-catalyzed reactions and the over-all reaction rate determined by following NADH oxidation spectrophotometrically. This assay can only be used for carboxykinase preparations carried beyond Stage 4 of the purification procedure because preparations from earlier steps contain NADH-oxidizing activity. The reaction mixture contains the following components

(in micromoles unless specified): Tris (Cl^-) buffer, pH 7.5, 100; oxalacetate (neutralized), 0.5; ITP, 3.0; $MnCl_2$, 1.5; $MgCl_2$, 1.0; GSH, 2.0; NADH, 0.15; pyruvate kinase, 1.25 units; lactate dehydrogenase, 2.5 units; and carboxykinase, up to 4×10^{-3} unit in a total volume of 1.0 ml. The final pH is 7.4. The rate of nonenzymatic decarboxylation of oxalacetate (to pyruvate and CO_2) is determined at 30° by following the rate of NADH oxidation at 340 mμ for 2 min in the presence of all of the components except carboxykinase. Enzymatic decarboxylation is then initiated by carboxykinase addition and the NADH oxidation rate at 30° determined again. The rate of oxalacetate decarboxylation in the presence of carboxykinase is corrected for the nonenzymatic rate (determined in the absence of carboxykinase). It is important that controls be included in which ITP is omitted to make certain that the carboxykinase preparation is not significantly contaminated with malate dehydrogenase. The rate of the enzymatic decarboxylation follows zero order kinetics for at least 2 min and is proportional to enzyme concentration up to a level of 4×10^{-3} unit (carboxylation assay) of carboxykinase.

I. Oxalacetate-$H^{14}CO_3^-$ Exchange Assay.

P-enolpyruvate carboxykinase catalyzes an ITP (or GTP)- and Mn^{++}-dependent exchange between $H^{14}CO_3^-$ and oxalacetate (7). The velocity of this exchange is considerably greater than the velocity of either the over-all carboxylation or decarboxylation reaction and is the most

sensitive assay for P-enolpyruvate carboxykinase. The reaction mixture for the $H^{14}CO_3^-$ -oxalacetate exchange assay contains the following components (in micromoles except as indicated): imidazole (Cl^-) buffer, pH 6.6, 100; oxalacetate (neutralized), 2.0; ITP, 3.0; $MnCl_2$, 2.6; $KH^{14}CO_3^-$ (approximately 10^5 cpm per μmole), 50; GSH, 2.0; and carboxykinase, up to 2 x 10^{-3} unit (carboxylation) in a total volume of 1.0 ml. The final pH is 7.0. Following a 2-min preincubation of carboxykinase with the reaction mixture (minus oxalacetate) at 30°, the reaction is initiated with oxalacetate and the mixture incubated for 4 min at 30°. The reaction is terminated and oxalacetate rapidly converted to malate by the addition of a solution (0.5 ml) containing EDTA (20 μmoles), NADH (5 μmoles), and malate dehydrogenase (15 units). After 5 min at 30°, the reaction mixture is acidified with 0.5 ml of N HCl. A 0.5-ml aliquot is taken to dryness in a scintillation counting vial at 85° for 60 min in a forced draft oven. After addition of 1 ml of H_2O to the vial, followed by 10 ml of liquid scintillator, acid-stable ^{14}C activity (as ^{14}C-malate) is determined with a liquid scintillation spectrometer. The incorporation of $H^{14}CO_3^-$ into oxalacetate (as malate) follows zero order kinetics during the 4-min assay period and is proportional to enzyme concentration when up to 2 x 10^{-3} unit of carboxykinase is assayed. The initial rate of $H^{14}CO_3^-$ -oxalacetate exchange, expressed as micromoles of HCO_3^- incorporated into oxalacetate per min (i.e. oxalacetate turnover equivalent), is calculated from ^{14}C activity incorporated (acid-stable radioactivity) and the specific activity of the $H^{14}CO_3^-$ used. While conditions of the exchange assay

permit the carboxykinase-catalyzed decarboxylation reaction to occur, loss of oxalacetate due to this during the assay could account for no more than a reduction of 5% of the initial oxalacetate concentration.

J. Isolation and Purification of Phosphoenolpyruvate Carboxykinase.

All of the operations are conducted at approximately 4° unless otherwise specified. The results of each purification step are summarized in Table 1.

Mitochondrial Acetone Powder - Fresh pig liver, 1 kg, is homogenized in 2 liters of cold 0.25 M sucrose (containing 5×10^{-4} M EDTA) for 1 min (three 20-sec periods) at top speed in a Waring Blendor (4-liter capacity). One additional liter of sucrose solution is added, and the mixture is rehomogenized for 20 sec. The homogenate is centrifuged at 1,500 x g for 15 min; the supernatant suspension is filtered through 4 layers of cheesecloth and then recentrifuged at 60,000 x g in a refrigerated Sharples centrifuge (flow rate, approximately 0.2 liter per min). The sedimented particles (principally mitochondria) are resuspended with about 100 ml of 0.25 M sucrose (5×10^{-4} M EDTA) and added slowly with rapid stirring into 20 volumes of acetone at approximately -5°. After allowing the precipitate to settle for approximately 5 min, most of the supernatant solution is decanted and the suspension is filtered under vacuum with a Buchner filter. The precipitate is washed on the filter with dry acetone followed by peroxide-free ether. After the last volume of ether has been drawn through the

filter, the precipitate is quickly transferred to a vacuum dessicator and the last traces of ether removed in a vacuum. The acetone powder (yield, approximately 50 g per kg of liver) is stable for at least four months when stored at -20°. While the procedure described deals with 1 kg of liver as starting quantity, experience in our laboratory indicates that 20 kg of liver can be processed in 1 day if an industrial size Sharples centrifuge (model 16) is used at a flow rate of 0.5 liter per min.

Extraction and Ammonium Sulfate Fractionation - Acetone powder, 100 g, is extracted for 3 hours with 2 liters of 10^{-2} M phosphate buffer (pH 7.5) with slow stirring. The suspension is centrifuged at 30,000 x g for 10 min and the clear supernatant extract retained. This solution is brought to 45% saturation[1] with solid ammonium sulfate (0.277 g per ml of extract) introduced slowly with magnetic stirring. After standing for 30 min, the supernatant solution is recovered after centrifugation. Sufficient solid ammonium sulfate (0.099 g per ml of supernatant solution) is added to the supernatant solution to bring it to 60% saturation. The suspension is allowed to stand for 30 min, centrifuged, the precipitate dissolved in 70 ml of 0.005 M phosphate buffer, pH 7.0 (containing 5 x 10^{-4} M EDTA and 5 x 10^{-3} M GSH), and then dialyzed against 3 liters of the same buffer for 10 hours.

DEAE-cellulose Chromatography - The dialyzed enzyme from the previous step (about 7 g of protein) is applied to a DEAE-cellulose column

[1] All of the "percentage of ammonium sulfate saturation" figures obtained with solid ammonium sulfate refer to percentage of saturation at 25°.

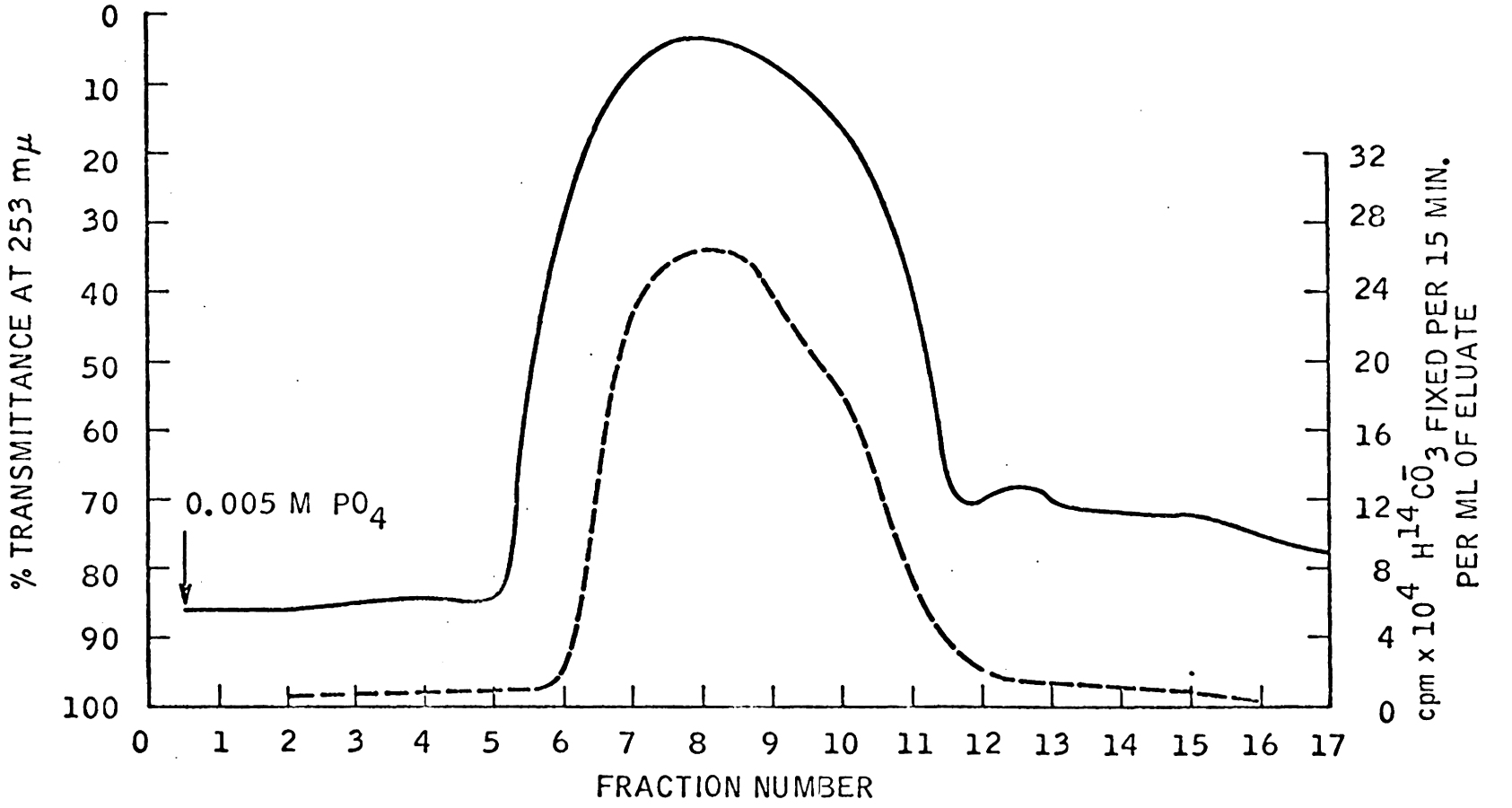
(4.5 x 36 cm packed volume) previously equilibrated with 0.5 M phosphate and then the dialysis buffer. Elution is accomplished with 0.005 M phosphate, pH 7.0 (containing 5×10^{-3} M mercaptoethanol); the column effluent is monitored continuously for ultraviolet-absorbing compounds (253 mμ, LKB Uvicord Absorptiometer) and carboxykinase activity located by the $H^{14}CO_3^-$ -fixation assay. P-enolpyruvate carboxykinase activity is eluted with the protein breakthrough peak.

Figure 1 shows a typical elution pattern for DEAE-cellulose column chromatography. Forty milliliter fractions were collected every nine minutes. In this case, fraction numbers 7 through 10 were pooled, placed in dialysis bags, then dialyzed against an ammonium sulfate solution (5×10^{-4} M EDTA and 5×10^{-4} M GSH), pH 7.5 of sufficient concentration to reach 60% saturation of equilibrium. Ninety-five percent of the carboxykinase activity and twenty-five percent of the protein applied to the column was recovered in the pooled fractions.

Cellulose Phosphate Chromatography - The protein precipitate from the pooled DEAE-cellulose column fractions is recovered by centrifugation, dissolved in about 30 ml of 0.005 M phosphate buffer, pH 7.0 (5×10^{-4} M EDTA and 5×10^{-4} M GSH), and then dialyzed against 2 liters of the same buffer for 12 hours. The dialyzed solution (about 1.8 g of protein) is applied to a column (4.5 x 40 cm) of cellulose phosphate which has been equilibrated with the dialysis buffer. Cellulose phosphate was prepared by successively washing with 0.1 N NaOH, water (until neutral), 0.1 N HCl, water (until neutral), 0.5 M potassium phosphate, pH 7.0, and then finally with 0.005 M potassium

Figure 1. Elution Pattern of PEPCK in DEAE-cellulose Column Chromatography.

A total of 7 g of protein was applied to the column (4.5 x 38 cm). Elution was as described in the text. Each fraction represents 40 ml collected during a 9 minute period. Fraction numbers 7 through 10 were pooled and prepared for further purification as described in the text. Protein in the eluate was monitored continuously by measurement of percent transmittance at 253 mμ (———) using an LKB Uvicord Absorptiometer. PEPCK activity (– – – –) was determined on an aliquot of each fraction using the standard $H^{14}CO_3^-$ fixation assay.


% TRANSMITTANCE AT 253 mμ
0
10
20
30
40
50
60
70
80
90
100
0.005 M PO4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
FRACTION NUMBER
32
28
24
20
16
12
8
4
0
cpm x 10^4 H^14CO3 FIXED PER 15 MIN. PER ML OF ELUATE

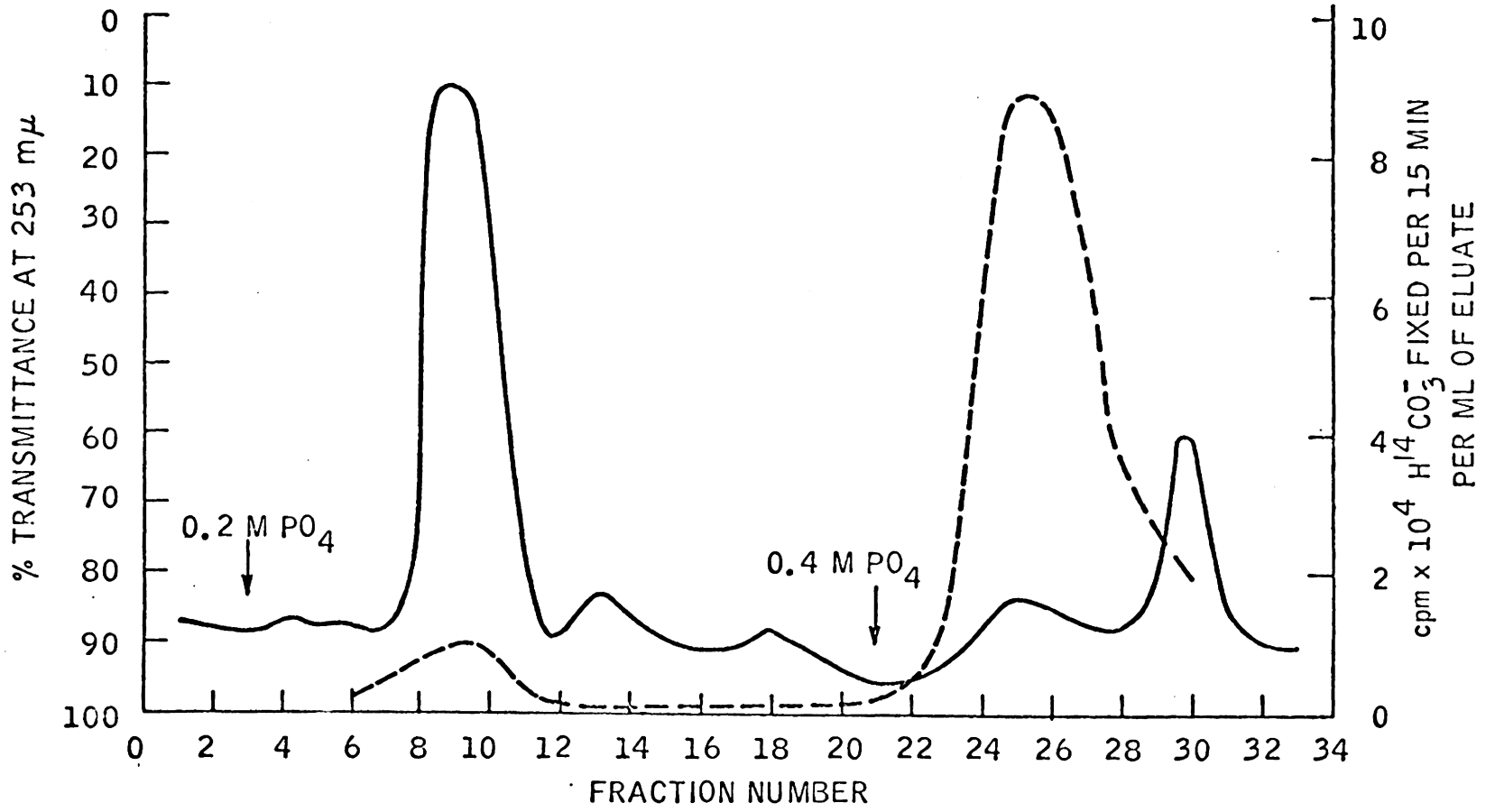
phosphate, pH 7.0. The equilibrated cellulose phosphate should not be stored longer than 2 weeks. Deviations from this equilibrium procedure usually result in failure of the column to retain the P-enolpyruvate carboxykinase. Stepwise gradient elution is accomplished by placing 800 ml of 0.005 M phosphate, pH 7.0, into the mixing chamber attached to the column and introducing the following phosphate buffers (K^+), all pH 7.0, into a separatory funnel attached to the mixing chamber: 160 ml of 0.005 M, 800 ml of 0.2 M, and 800 ml of 0.4 M. The effluent is continuously monitored for protein, collected fractionally, and fractions are assayed for carboxykinase activity as described in the previous section. P-enolpyruvate carboxykinase is eluted as a distinct protein peak after approximately 650 ml of eluate have been collected.

Figure 2 shows a typical elution pattern for cellulose phosphate column chromatography. Forty milliliter fractions were collected every twelve minutes. In this case, fraction numbers 24 through 27 were pooled and precipitated with ammonium sulfate by the dialysis technique described under "DEAE-cellulose Chromatography." Seventy-four percent of the carboxykinase activity and seven percent of the protein applied to the column were recovered in the pooled fractions.

Hydroxylapatite Chromatography - The precipitated protein, which is recovered from the preceding step by centrifugation, is dissolved in 4 ml of 0.005 M phosphate buffer, pH 7.0 (5×10^{-4} M EDTA and 5×10^{-4} M GSH), and dialyzed against the same buffer for 10 hours. The dialyzed enzyme solution (about 100 mg of protein) is applied to a

Figure 2. Elution Pattern of PEPCK in Cellulose Phosphate Column Chromatography.

A total of 1.8 g of protein was applied to the column (4.5 x 40 cm) and eluted as described in the text. Each fraction represents 40 ml collected during a 12 minute period. The enzyme was eluted from the column by gradient elution. The first arrow on the figure indicates the point at which 0.2 M phosphate buffer was added to the reservoir; the second arrow indicates the point at which 0.4 M phosphate buffer was added to the reservoir. Fraction numbers 24 through 27 were pooled and prepared for further purification as described in the text. Protein in the eluate was monitored continuously by measurement of percent transmittance as 253 mμ (———) using an LKB Uvicord Absorptiometer. PEPCK activity (– – – –) was determined on an aliquot of each fraction using the standard $H^{14}CO_3^-$ fixation assay.


% TRANSMITTANCE AT 253 mμ
0
10
20
30
40
50
60
70
80
90
100
0.2 M PO4
0.4 M PO4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
FRACTION NUMBER
10
8
6
4
2
0
cpm x 10^4 H^14CO3^- FIXED PER 15 MIN PER ML OF ELUATE

column (2.0 x 12 cm) of hydroxylapatite already equilibrated with the dialysis buffer (EDTA omitted). Stepwise elution is carried out under pressure (2 p.s.i. from nitrogen gas cylinder) with 40 ml of 0.005 M, 150 ml of 0.05 M, and 100 to 200 ml of 0.1 M phosphate buffer (K^+), pH 7.0, containing 5 x 10^{-3} M mercaptoethanol. P-enolpyruvate carboxykinase activity and protein in the eluted fractions (3 ml) are determined as described under "DEAE-cellulose Chromatography." Elution of carboxykinase usually occurs during the application of the 0.05 M phosphate buffer to the column after approximately 150 ml of eluate have been collected.

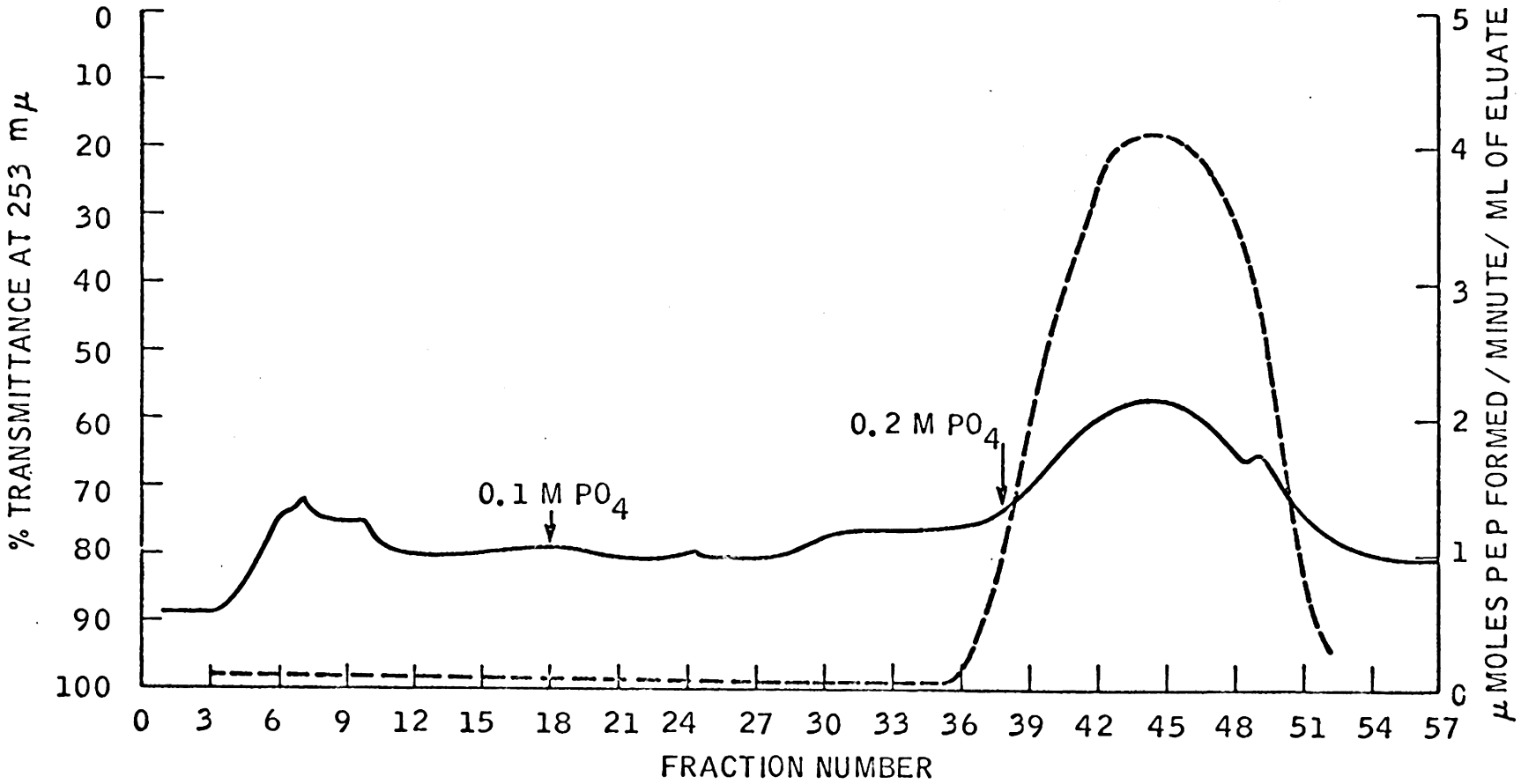
Figure 3 is a typical elution pattern for hydroxylapatite column chromatography. Three milliliter fractions were collected every nine minutes. In this run, fraction numbers 40 through 47 were pooled and precipitated at 60% ammonium sulfate saturation (pH 6.5) by the dialysis technique described earlier. Ninety percent of the carboxykinase activity and sixty-five percent of the protein applied to the column were recovered in the pooled fractions.

The enzyme, stored under 60% ammonium sulfate, pH 6.5 (5 x 10^{-4} M EDTA and 5 x 10^{-4} M GSH), loses activity at a relatively slow rate (about 10% per month).

As indicated in Table 1, a 210-fold purification of P-enolpyruvate carboxykinase from the initial liver mitochondrial acetone powder extract is achieved in excellent yield (48%) by the procedure outlined. The degree of purification from the whole liver homogenate would probably be higher since preparation of the mitochondrial acetone powder

Figure 3. Elution Pattern of PEPCK in Hydroxylapatite Column Chromatography.

A total of 100 mg of protein were applied to the column (2.5 x 12 cm) and eluted as described in the text. Each fraction represents 3 ml collected during a 9 minute period. The enzyme was eluted from the column in a batchwise manner. The first arrow on the figure indicates the point at which 0.1 M phosphate buffer was applied to the column; the second arrow indicates the point at which 0.2 M phosphate buffer was applied. Fraction numbers 40 through 47 were pooled and precipitated at 60% ammonium sulfate saturation (pH 6.5) by the dialysis technique described in the text. Protein in the eluate was monitored continuously by measurement of percent transmittance at 253 mμ (———) using an LKB Uvicord Absorptiometer. PEPCK activity (– – – –) was determined on an aliquot of each fraction using the standard decarboxylation assay.


% TRANSMITTANCE AT 253 mμ
0
10
20
30
40
50
60
70
80
90
100
0.1 M PO4
0.2 M PO4
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57
FRACTION NUMBER
μ MOLES PEP FORMED / MINUTE / ML OF ELUATE
0
1
2
3
4
5

constitutes some purification. Carboxykinase assays conducted on the whole homogenate have been found to be unreliable. If the P-enolpyruvate carboxykinase content (672 units) of the mitochondrial acetone powder extract from 2 kg of liver is taken as a minimal value, the capacity of liver to synthesize P-enolpyruvate from oxalacetate (decarboxylation rate, approximately 7 μ moles per min per unit of carboxykinase activity) can be calculated. Under the assay conditions employed, the mitochondrial cell fraction from 1.0 g of pig liver can catalyze the formation of 2.3 μ moles of P-enolpyruvate per min from oxalacetate.

Table 1. Purification of Phosphoenolpyruvate Carboxykinase.

Stage of purification	Total protein[a]	Total activity (carboxylation)	Specific activity		Yield
			Carboxylation	Decarboxylation[b]	
	g	units	units/mg protein	μmoles/min/mg protein	%
1. Acetone powder extract[c]	28.0	672	0.024		100
2. 45 to 60% saturated $(NH_4)_2SO_4$ fraction	7.2	535	0.074		80
3. DEAE-cellulose eluate	1.83	481	0.263		71
4. Cellulose phosphate chromatographic fraction	0.112	358	3.2	22	53
5. Hydroxylapatite chromatographic fraction	0.064	320	5.0[d]	35[d]	48

[a] Protein determined spectrophotometrically by the Warburg and Christian method as described by Layne (38).

[b] Decarboxylation rate determined with the decarboxylation assay described under "Experimental Procedure."

[c] Extract of 100 g of mitochondrial acetone powder which can be obtained from 2 kg of liver (wet weight).

[d] Specific activity, expressed in terms of refractometrically determined protein, is 9.1 units per mg of protein for carboxylation and 63.4 μmoles per min per mg of protein for decarboxylation.

VI. Results and Discussion

A. Ultracentrifugation Studies.

Ten preparations of the P-enolpyruvate carboxykinase, purified according to the procedures described (Steps 1 to 5 of Table 1), have been examined for purity in the analytical ultracentrifuge. In every case, sedimentation patterns revealed a single component with no evidence of inhomogeneity. A typical sedimentation pattern is shown in Figure 4. Sedimentation velocity experiments were conducted at 5-10° in 0.02 N Tris (Cl^-) buffer, pH 7.5, 0.05 M NaCl, 5×10^{-4} M GSH, and 10^{-4} M EDTA. The sedimentation coefficient, based on results from five experiments at varying protein concentrations[2] (1.5 to 4.2 g per liter) involving three enzyme preparations was calculated to be, $s^0_{20,w} = 5.21$ S (extrapolated to zero protein concentration). The equation $s_{20,w} = s^0_{20,w} - kc$, in which c = protein concentration in grams per liter and $k = 0.195$ g^{-1} liter, fits the results of these experiments.

In addition to the components added in the experiments described above, other sedimentation experiments were conducted in the presence of 2×10^{-3} M $MnCl_2$, 10^{-3} M P-enolpyruvate, both 10^{-3} ITP and 3×10^{-3} M oxalacetate, or a combination of $MnCl_2$, ITP, and oxalacetate. The presence of these substances produced no significant change in the sedimentation velocity of the carboxykinase. It is apparent, therefore, that

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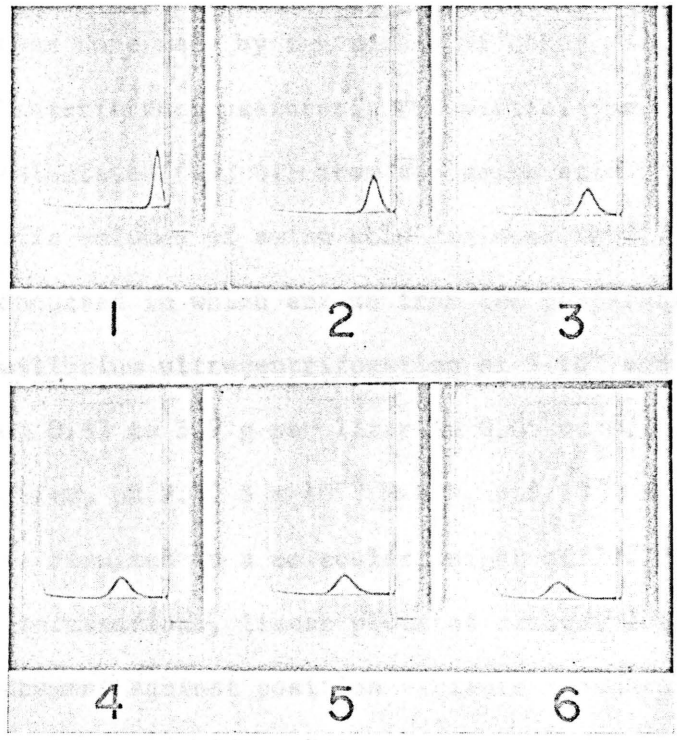
[2] Protein concentration, milligrams per ml = 0.613 (A^{1cm}_{280}).

Figure 4. Schlieren Patterns of Purified PEPCK.

Schlieren patterns of purified P-enolpyruvate carboxykinase obtained in the Spinco model E ultracentrifuge. The enzyme (Stage 5 of Table 1) was centrifuged at 56,100 rpm at 8°. Photographs 1 to 6 were taken at 18, 38, 62, 86, 94, and 118 min after initiating the run. The protein concentration[2] was 4.19 mg per ml. Prior to centrifugation, the enzyme was dialyzed against 0.02 M Tris (Cl^-) buffer, pH 7.5, containing 0.05 M NaCl, 5×10^{-4} M EDTA.


1
2
3
4
5
6

these substrates and cofactors do not materially affect the conformation of the enzyme molecule or its state of aggregation under these conditions.

B. Molecular Weight Determination.

The molecular weight was determined by sedimentation equilibrium and calculations were made by the method of LaBar and Baldwin (39) from Raleigh interference patterns. The partial specific volume of the enzyme, calculated (40, 41) from its amino acid composition and partial specific volumes of amino acid residues is 0.736. Five experiments were conducted in which enzyme from two preparations was subjected to equilibrium ultracentrifugation at 5-10° and protein concentrations[2] from 0.63 to 3.9 g per liter in 0.05 or 0.1 M NaCl, 0.02 M Tris (Cl^-) buffer, pH 7.5, 5×10^{-4} M GSH, and 10^{-4} M EDTA. These determinations resulted in a molecular weight of 73,300 ± 2,600.[3] In all of the determinations, linear plots of natural logarithm of Raleigh fringe displacement against position variable (comparator x coordinate) were obtained. This provides additional evidence that the purified P-enolpyruvate carboxykinase preparations are homogeneous. Protein concentration was determined by refractometric determination in a synthetic boundary cell with 1.862×10^{-4} as the specific refractive index increment per mg per ml. This figure is the average for several

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[3] Standard deviation.

proteins (42) corrected to a wave length of 546 mμ and 20°. The relation between absorbance at 280 mμ and refractometrically determined protein concentration is given by the equation, $\underline{c} = 0.613(\underline{A}_{280}^{1\,cm})$, where $\underline{c}$ is protein concentration in milligrams per ml and $\underline{A}$ is absorbance at 280 mμ (1-cm light path). This value is very close to that (0.609) determined from direct amino acid analysis of the enzyme. The absorbance ratio $\underline{A}_{280}:\underline{A}_{260}$ of the pure enzyme, determined on numerous occasions, is 1.75 ± 0.02.[3] To convert protein determined by the Warburg and Christian method described by Layne (38) to refractometrically determined protein, the former should be multiplied by a factor of 0.552. Ultraviolet and visible absorption spectra reveal no characteristic chromophoric groups which would distinguish this enzyme from most other proteins.

C. Amino Acid Composition.

The sample of purified carboxykinase for analysis was first freed of small molecule contaminants by gel filtration on a Sephadex G-25 column. For amino acid analysis, two 1.1-mg samples in ampules were dissolved in 2.0 ml of glass-distilled 5.7 $\underline{N}$ HCl, gassed with N_2, frozen, and the ampules sealed under vacuum. After evaporation in a vacuum at 45°, excess acid was removed by repeated evaporation after water addition. Samples were hydrolyzed at 121° for 22 and 41 hours, respectively, after which complete amino acid analyses (except tryptophan and cysteine + cystine) were conducted with a Beckman/Spinco model 120B amino acid analyzer.

Destruction of proline, threonine, and serine was corrected for by extrapolation to zero hydrolysis time. With the exception of these three amino acids, agreement between the two analyses (22- and 41-hour hydrolysis) was within 2%. Cystine (half-cystine) analysis was conducted by determination of cysteic acid after performic acid oxidation and hydrolysis of the protein. Performic acid oxidation of 3.2 mg of gel-filtered (Sephadex G-25) enzyme protein was accomplished by using the method of Moore (43). After a 22-hour hydrolysis with 6 N HCl and concentration to dryness as described above, cysteic acid analysis was conducted with the Beckman/Spinco amino acid analyzer previously calibrated with authentic cysteic acid. Tryptophan was determined from the ultraviolet absorption spectrum of the enzyme in 0.1 N NaOH by the method of Goodwin and Morton (44). Table 2 summarizes the amino acid composition of P-enolpyruvate carboxykinase; the amino acid distribution reveals no particular atypical features of the protein. The sum of amino acyl residue weight (0.99 mg per mg of protein[2]) agrees very closely with spectrophotometrically determined protein calculated from the equation relating A_{280} to refractometrically determined protein.

Table 2. Amino Acid Composition of Liver Mitochondrial Phosphoenolpyruvate Carboxykinase.

Amino acid	Concentration[a]	Residues per molecule of enzyme[b]
	μmole per mg protein	
Lysine	0.358	26
Arginine	0.659	48
Histidine	0.158	12
Tyrosine	0.157	12
Tryptophan[d]	0.178	13
Half-cystine[e]	0.198	15
Methionine	0.271	20
Aspartic acid	0.720	53
Glutamic acid	0.953	70
Threonine[c]	0.420	31
Serine[c]	0.420	31
Proline[c]	0.766	56
Glycine	0.876	64
Alanine	0.755	55
Valine	0.606	44
Leucine	0.797	58
Isoleucine	0.387	28
Phenylalanine	0.362	27

[a] Protein concentration determined from milligrams of protein per ml = 0.613 (A_{280}^{1cm}).
[b] Molecular weight = 73,300.
[c] Corrected for destruction during hydrolysis.
[d] Determined spectrophotometrically (44).
[e] Determined as cysteic acid after performic acid oxidation and hydrolysis(43).

D. Sulfhydryl and N-Terminal Groups.

Fifteen sulfhydryl groups per molecule of PEPCK were detected by Ellman's reagent titration (34) which account for the total half cystine content of the enzyme indicated by amino acid analysis. When the activity of the enzyme was followed spectrophotometrically during the course of titration, it was found that titration of two -SH groups per molecule results in complete inactivation of the enzyme. This inactivation occurs almost instantaneously. It is apparent that free sulfhydryl groups are essential for the catalytic activity of the enzyme.

Analysis of N-terminal residues by dinitrophenylation was performed on 0.2 μmoles (15.5 mg) of PEPCK. In both solvent systems (1.5 M $NaHPO_4$ buffer or amyl alcohol saturated with 0.05 M phthalic acid, pH 6), two DNP-amino acids were observed on the chromatograms in addition to dinitroaniline and dinitrophenol. These had R_F values identical with DNP-glutamate and DNP-isoleucine in both solvent systems. It is tentatively concluded that PEPCK has two N-terminal amino acid groups, namely DNP-glutamate and DNP-isoleucine.

E. Inhibition by Sulfhydryl Reagents and Fluoride.

Amino acid analysis of pig liver mitochondrial P-enolpyruvate carboxykinase was found (Table 2) to contain 15 half-cysteine residues per molecule, hence potentially 15 cysteinyl residues per molecule. There

have also been indications that the enzyme was stabilized in the presence of sulfhydryl compounds. Purified P-enolpyruvate carboxykinase (2.2×10^{-4} μmole; 0.016 mg) was allowed to react for several minutes at 30° with graded levels (10^{-4} to 2×10^{-3} μmole) of p-chloromercuribenzoate. As shown in Table 3, at a p-chloromercuribenzoate level (10^{-3} μmole) 5 times the enzyme level, 50% inhibition of the oxalacetate decarboxylation rate was observed. The inhibition (100%) at the 2×10^{-3} μmole level of p-chloromercuribenzoate addition was reversed by subsequent addition of GSH to an extent dependent upon the level of GSH addition. It is apparent that free sulfhydryl groups are essential for the catalytic activity of the enzyme. N-Ethylmaleimide, while inhibitory, is effective only at a much higher level of addition that p-chloromercuribenzoate. Fluoride was found to be a good inhibitor of the decarboxylation reaction, whereas arsenate was ineffective. Neutralized hydroxylamine (0.05 M) has no effect on the rate of IDP-dependent carboxylation of P-enolpyruvate with the $H^{14}CO_3^-$ -fixation assay (malate dehydrogenase deleted).

Table 3. Inhibition of Phosphoenolpyruvate Carboxykinase-catalyzed Oxalacetate Decarboxylation by Sulfhydryl Reagents, Fluoride, and Arsenate.

Inhibitor	Oxalacetate decarboxylation rate	Inhibition
	mole/min	%
None	0.0276	
p-Chloromercuribenzoate, 10^{-4} μmole	0.0274	0
p-Chloromercuribenzoate, 2.0×10^{-4} μmole	0.0248	10
p-Chloromercuribenzoate, 5.0×10^{-4} μmole	0.0188	33
p-Chloromercuribenzoate, 10^{-3} μmole	0.0137	50
p-Chloromercuribenzoate, 1.5×10^{-3} μmole	0.0060	78
p-Chloromercuribenzoate, 2.0×10^{-3} μmole	0.000	100
p-Chloromercuribenzoate, 2.0×10^{-3} μmole; then GSH, 10 μmoles	0.0124	55
p-Chloromercuribenzoate, 2.0×10^{-3} μmole; then GSH, 25 μmoles	0.0212	23
p-Chloromercuribenzoate, 2.0×10^{-3} μmole; then GSH, 50 μmoles	0.0215	22
N-Ethylmaleimide, 1.0 μmole	0.0232	16
Potassium fluoride, 10^{-3} M	0.0130	53
Sodium arsenate, 2×10^{-3} M	0.0277	0

In a final volume of 0.4 ml (0.01 M Tris (Cl^-), pH 7.5), a constant quantity of enzyme (2.2×10^{-4} μmole; 0.016 mg) was allowed to react at 30° for 2 min with p-chloromercuribenzoate or N-ethylmaleimide after which time, aliquots were assayed with the decarboxylation assay with GSH added. Where indicated, GSH was added to the enzyme-inhibitor mixture, incubated for 1 min, and then assayed. Fluoride and arsenate were tested as additions to the standard decarboxylation assay medium.

F. Requirements for Phosphoenolpyruvate Carboxykinase-catalyzed Reaction.

The requirements for the P-enolpyruvate carboxykinase-catalyzed carboxylation reaction are summarized in Table 4. An absolute requirement for P-enolpyruvate, nucleoside diphosphate, bicarbonate, and divalent cation are indicated. Neither ADP, IMP, GMP, nor ITP (chromatographically pure) can replace IDP (or GDP). Purified carboxykinase preparations exhibit no pyruvate kinase activity when tested at a level of 2 enzyme units (approximately 40 times the usual assay level). Since most commercial ITP preparations exhibit some activity due to an IDP impurity, chromatographically purified ITP was employed in this experiment. While Mg^{++} shows essentially no activity under these conditions (0.002 M $MgCl_2$ and pH 7.0), at pH 7.5 it exhibits a maximum velocity 16% that for Mn^{++} and a K_m (total Mg^{++}) = 1.9×10^{-3} M. Neutralized hydroxylamine (0.05 M) has no effect on the carboxylation rate.

Table 4. Requirements for Phosphoenolpyruvate Carboxykinase-catalyzed Carboxylation Reaction.

Additions to and deletions from complete reaction mixture[a]	P-enolpyruvate carboxylation rate
	μmole/min
None (complete)	0.042
Phosphoenolpyruvate carboxykinase deleted	0.000
Phosphoenolpyruvate deleted	0.000
IDP deleted	0.000
$KHCO_3$ deleted	0.001
$MnCl_2$ deleted	0.000
$MnCl_2$ deleted, $MgCl_2$ added, 2 μmoles	0.001
IDP deleted, GDP added, 1.25 μmoles	0.046
IDP deleted, ADP added, 1.25 μmoles	0.000
IDP deleted, IMP or GMP added, 1.25 μmoles	0.000
IDP deleted, purified ITP[b] added, 1.25 μmoles	0.000

The complete reaction mixture and assay conditions are those described in the text for the standard spectrophotometric carboxylation assay.

[a] Complete reaction mixture contained 0.01 mg of purified P-enolpyruvate carboxykinase.

[b] ITP was purified by DEAE-cellulose chromatography as described under "Experimental Procedure."

G. Requirements for Phosphoenolpyruvate Carboxykinase-catalyzed Oxalacetate Decarboxylation.

The requirements for the P-enolpyruvate carboxykinase-catalyzed reaction are shown in Table 5. It is clear that oxalacetate, nucleoside triphosphate (GTP and ITP preferentially), and divalent cation are required for carboxykinase-catalyzed oxalacetate decarboxylation. In agreement with the results of Cannata and Stoppani (22) with ADP and ATP and a similar enzyme from bakers' yeast, nucleoside diphosphate (IDP) can replace nucleoside triphosphate (ITP) for the decarboxylation reaction with purified pig liver mitochondrial carboxykinase (Table 5, Experiments 1 and 3). The reaction product is pyruvate, however, rather than P-enolpyruvate, the product of the ITP-dependent decarboxylation. The IDP-dependent decarboxylation reaction is dependent upon Mn^{++}; neither ADP nor IMP can replace IDP and P-enolpyruvate strongly inhibits the reaction. P-enolpyruvate (and not pyruvate) and IDP are the sole reaction products (besides HCO_3^-) of ITP-dependent oxalacetate decarboxylation. This is indicated both by the dependence of pyruvate formation upon the presence of pyruvic kinase in the reaction mixture (Table 5) and the stoichiometry between ITP and oxalacetate disappearance and P-enolpyruvate and IDP formation. Of the nucleoside triphosphates tested (Table 5, Experiment 2), GDP and ITP are most active, UTP to a lesser extent, and CTP and ATP are inactive. This is in general agreement with the findings of Utter, Kurahashi, and Rose (8).

H. Requirements for Phosphoenolpyruvate Carboxykinase-catalyzed Oxalacetate - $H^{14}CO_3^-$ Exchange Reaction.

The requirements for the oxalacetate-$H^{14}CO_3^-$ exchange reaction, summarized in Table 5, are the same as those for the over-all decarboxylation reaction except for an additional requirement for HCO_3^- and the inability of IDP to replace ITP. Absolutely no exchange was supported by chromatographically pure IDP or IMP as nucleoside triphosphates even at extremely high carboxykinase concentrations. Acetoacetate, α-ketoglutarate, and β-ketoglutarate were each tested at 2, 5, and 10 x 10^{-3} M as substitutions for oxalacetate at high carboxykinase levels (0.5 unit). These exchange assays were modified to the extent that the reactions were terminated with neutralized hydroxylamine (100 μ moles) which replaced malate dehydrogenase and NADH since keto acids other than oxalacetate were involved. Both termination procedures were equally effective in preventing nonenzymatic decarboxylation of the β-keto acids at low pH. Acetoacetate, α-ketoglutarate, and β-ketoglutarate were completely inactive as oxalacetate replacements in the exchange reaction, which indicates a relatively high degree of specificity for this reaction.

Table 5. Requirements for phosphoenolpyruvate carboxykinase-catalyzed oxalacetate decarboxylation and oxalacetate-$H^{14}CO_3^-$ exchange reactions.

The complete reaction mixtures and assay conditions are those described in the text for the standard decarboxylation and oxalacetate -$H^{14}CO_3^-$ exchange assays.

Additions to and deletions from complete reaction mixture	Oxalacetate decarboxylation rate	Oxalacetate-$H^{14}CO_3^-$ exchange rate
	μmole/min	cpm incorporated into oxalacetate/min
Experiment 1[a]		
None (complete)	0.039	2445
Phosphoenolpyruvate carboxykinase deleted	0.000	10
Oxalacetate deleted	0.000	12
$MnCl_2$ deleted	0.002	68
ITP deleted	0.000	4
ITP deleted, 3.0 μmoles of IDP added[b]	0.023	5
ITP deleted, 3.0 μmoles of IMP added	0.000	13
Pyruvate kinase deleted[c]	0.000	
Lactate dehydrogenase deleted	0.000	
$MgCl_2$ deleted	0.034	
Experiment 2[d]		
None (complete)	0.048 (79%)	6560 (75%)
ITP deleted, 3.0 μmoles of GTP added	0.061 (100%)	4910 (100%)
ITP deleted, 3.0 μmoles of UTP added	0.014 (23%)	1060 (16%)
ITP deleted, 3.0 μmoles of ATP added	0.000 (0)	100 (1.5%)
ITP deleted, 3.0 μmoles of CTP added	0.000 (0)	79 (0.9%)
Experiment 3[e]		
None (complete, 0.5 μmole of ITP added)	0.018	
Pyruvate kinase deleted	0.001	
ITP deleted, 0.5 μmole of IDP[b] added	0.011	
ITP, pyruvate kinase deleted; 0.5 μmole of IDP[b] added	0.010	
ITP, pyruvate kinase, $MnCl_2$ deleted; 0.5 μmole of IDP[b] added	0.001	
ITP, pyruvate kinase deleted; 0.5 μmole of ADP added	0.001	
ITP, Pyruvate kinase deleted; 0.5 μmole of IMP added	0.000	
ITP, pyruvate kinase deleted; 0.5 μmole of IDP[b] and 0.25 μmole of P-enolpyruvate added	0.001	

Table 5. Requirements for phosphoenolpyruvate carboxykinase-catalyzed oxalacetate decarboxylation and oxalacetate-$H^{14}CO_3^-$ exchange reactions.

Notes:

[a] Reaction mixtures for decarboxylation and exchange contained 4.9×10^{-3} and 10^{-3} unit of purified P-enolpyruvate carboxykinase, respectively.
[b] IDP was chromatographically purified by DEAE-cellulose chromatography as described under "Experimental Procedure."
[c] Same result with 1.0 unit of enzyme.
[d] Reaction mixtures for decarboxylation and exchange contained 6.1×10^{-3}, 3×10^{-3} unit of purified P-enolpyruvate carboxykinase.
[e] Complete reaction mixture was modified as follows. $MgCl_2$ was omitted, 0.2 μmole instead of 1.5 μmoles of $MnCl_2$ was used to minimize nonenzymatic decarboxylation, and ITP concentrations were as indicated in table. P-enolpyruvate carboxykinase level was 7.0×10^{-3} unit.

I. Relative Rates of Phosphoenolpyruvate Carboxykinase-catalyzed Carboxylation, Decarboxylation, and Oxalacetate-$H^{14}CO_3^-$ Exchange Reactions.

It is clear from previous investigations (7, 8, 10) and this thesis that mitochondrial P-enolpyruvate carboxykinase catalyzes the reversible IDP (or GDP)- and Mn^{++} -dependent carboxylation of P-enolpyruvate as well as an ITP (or GTP)- and Mn^{++} -dependent exchange between oxalacetate and $H^{14}CO_3^-$. In order to determine whether the oxalacetate-$H^{14}CO_3^-$ exchange represents a true partial reaction in the over-all carboxylation process, careful comparative kinetic studies were conducted. A comparison of the relative rates of the carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange reactions at pH 6.8, 7.3, and 8.0 is made in Table 6. The carboxykinase-catalyzed decarboxylation rate is from 8- to 18-times faster than the carboxylation rate over the pH range of 6.8 to 8.0. It is particularly significant that the oxalacetate-$H^{14}CO_3^-$ exchange reaction catalyzed by carboxykinase is considerably more rapid than either the carboxylation or decarboxylation processes. At pH 6.8 the exchange rate is 30- and 3.6-fold greater, respectively, than the carboxylation and decarboxylation rates. It is clear that the process by which the β-carboxyl group of oxalacetate exchanges with $H^{14}CO_3^-$ cannot be accounted for by the over-all decarboxylation reaction ($\longrightarrow$ P-enolpyruvate, IDP, and HCO_3^-) followed by the over-all carboxylation reaction. Instead, these results indicate that involvement of a partial reaction in the over-all decarb-

oxylation (and carboxylation) process. Cleavage of the β-carboxylate carbon—carbon bond of oxalacetate yielding HCO_3^- (or CO_2) and a 3 carbon intermediate of some sort appears necessary for exchange to occur. As shown earlier, oxalacetate-$H^{14}CO_3^-$ exchange is dependent upon ITP (or GTP) and Mn^{++} as is the over-all decarboxylation reaction. Corresponding nucleoside diphosphates will not substitute. This ITP (or GTP) requirement indicates participation of ITP in C—C bond cleavage in an initial rapid step (permitting rapid exchange) which precedes a second (and slower) step in which IDP and P-enolpyruvate are formed completing the over-all decarboxylation reaction. While the enzymatic reaction sequence represented in Reactions 6 and 7 is consistent with the experimental facts presented thus far, other formulations are possible. These alternative formulations together with experimental evidence rendering them invalid will be described later in this report.

$$\text{Enzyme} + \text{P-enolpyruvate} + \text{IDP} \underset{\underline{k}_{-1}}{\overset{\underline{k}_1}{\rightleftharpoons}} \text{enzyme-}\underline{X} \qquad (6)$$

$$\text{Enzyme-}\underline{X} + HCO_3^- \text{ (or } CO_2) \underset{\underline{k}_{-2}}{\overset{\underline{k}_2}{\rightleftharpoons}} \text{oxalacetate} + \text{ITP} \qquad (7)$$

Reversal of Reaction 7 is visualized as leading to the formation of HCO_3^- and a transient intermediate ($\underline{X}$, presumably enzyme-bound) composed of the elements ITP and a 3 carbon moiety (originating from carbon atoms 1, 2, and 3 of oxalacetate). The more rapid rate of

oxalacetate-$H^{14}CO_3^-$ exchange than either the rate of the over-all carboxylation or decarboxylation reaction may be ascribed to k_2 and k_{-2} being greater than k_1 and k_{-1}.

Table 6. Relative Rates of Phosphoenolpyruvate Carboxykinase-catalyzed Carboxylation, Decarboxylation, and Oxalacetate-$H^{14}CO_3^-$ exchange Reactions.

	Reaction rate at[a]					
	pH 6.8		pH 7.3		pH 8.0	
	Amount	Relative	Amount	Relative	Amount	Relative
	μmole/min		μmole/min		μmole/min	
Carboxylation	0.0075	1.0	0.0052	1.0	0.0027	1.0
Decarboxylation	0.0625	8.3	0.0587	11.3	0.0500	18.5
Oxalacetate-$H^{14}CO_3^-$ exchange	0.225	30	0.177	34	0.0745	28

The initial rates of carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange were determined by using the basic assay methods described earlier. Imidazole (Cl^-) buffer was used at pH 6.8 and 7.3 and Tris (Cl^-) buffer at pH 8.0.

[a] Reaction rates are in terms of micromoles per min per 5×10^{-3} unit of purified P-enolpyruvate carboxykinase.

J. GTP-^{14}C-GDP Exchange Reaction.

While differences in the rates of carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange (Table 6) catalyzed by carboxykinase led to the formulation of the two-step reaction sequence shown in Reactions 6 and 7, other, seemingly less likely, interpretations are possible. In order to narrow these possibilities, the kinetics of several other potential exchange reactions were investigated. The first of these exchange reactions, i.e., GTP-^{14}C-GDP exchange, would be expected to proceed at a rate equal to or greater than the oxalacetate-$H^{14}CO_3^-$ exchange were either of the following two reaction sequences valid.

$$\underline{E} + \text{P-enolpyruvate} \underset{\text{(rate-limiting)}}{\rightleftharpoons} \underline{E}\text{-}\underline{X} \qquad (8)$$

$$\underline{E}\text{-}\underline{X} + HCO_3^- \text{ (or } CO_2) \rightleftharpoons \underline{E}\text{-P} + \text{oxalacetate} \qquad (9)$$

$$\underline{E}\text{-P} + \text{GDP (or IDP)} \rightleftharpoons \underline{E} + \text{GTP (or ITP)} \qquad (10)$$

or

$$\underline{E} + \text{P-enolpyruvate} \underset{\text{(rate-limiting)}}{\rightleftharpoons} \underline{E}\text{-P-enolpyruvate} \qquad (11)$$

$$\underline{E}\text{-P-enolpyruvate} + \text{GDP (or IDP)} + HCO_3^- \text{ (or } CO_2) \rightleftharpoons \underline{E} + \text{GTP (or ITP} + \text{oxalacetate} \qquad (12)$$

The rate-limiting steps in either direction for both reaction sequences

are indicated.

The rates of GTP-GDP-8-^{14}C exchange and oxalacetate-$H^{14}CO_3^-$ exchange were compared under identical conditions and at carboxykinase levels sufficient to support essentially zero-order oxalacetate-$H^{14}CO_3^-$ exchange kinetics. As shown in Table 7, GTP-^{14}C-GDP exchange does not occur under conditions which permit rapid oxalacetate-$H^{14}CO_3^-$ exchange. This clearly eliminates both reaction sequences (Reactions 8 to 10 and 11 and 12) as candidates for the over-all enzymatic reaction mechanism. As shown in Table 8, at higher enzyme levels (160-fold higher) an extremely slow oxalacetate-dependent GTP-^{14}C-GDP exchange can be detected. This probably results from the over-all decarboxylation and concomitant carboxylation reactions which permit incorporation of ^{14}C-activity into GTP from ^{14}C-GDP. This exchange rate is approximately 3% of the carboxylation rate which could be catalyzed (under standard carboxylation assay conditions) by the quantity of carboxykinase employed.

Table 7. Comparison of Rate of GTP-^{14}C-GDP Exchange and Oxalacetate-H$^{14}CO_3$ Exchange.

Incubation time	GTP-^{14}C-GDP exchange (total ^{14}C-GDP incorporated into GTP)		Oxalacetate-H$^{14}CO_3^-$ exchange (total H$^{14}CO_3^-$ incorporated into oxalacetate)	
min	cpm	μmole	cpm	μmole
0	11,200		28	
2	7,950	0.00	37,480	0.47
4	9,700	0.00	65, 480	0.83

Reaction mixtures included (in micromoles): imidazole (Cl^-) buffer, pH 6.6, 100; $KHCO_3$ (^{14}C-labeled bicarbonate in oxalacetate-H$^{14}CO_3^-$ exchange experiment only, 7.9 x 10^4 cpm per mole), 50; GTP, 3.0; GDP (GDP-8-^{14}C in GTP-GDP exchange experiments only, 5.43 x 10^5 cpm per mole), 1.5; $MnCl_2$, 2.0; oxalacetate, 2.0; GSH, 2.0; and purified carboxykinase, 8.0 x 10^{-3} unit in a total volume of 1.0 ml. Final pH was 7.0. Following incubation at 30°, oxalacetate-H$^{14}CO_3^-$ exchange rate was determined as described under "Experimental Procedure." GTP-^{14}C-GDP exchange was determined after stopping the reaction with 0.1 ml of 50% isobutyric acid by paper chromatographic isolation of GTP followed by specific activity determination. Aliquots of the reaction mixture were applied to sheets of Whatman No. 3MM paper and chromatographed ascending by using the isobutyric acid-NH_4OH-H_2O (57:4:39,v/v) solvent system (33). Elution was accomplished with 0.02 M phosphate buffer, pH 7.0.

Table 8. Factors Affecting Phosphoenolpyruvate Carboxykinase-catalyzed GTP-^{14}C-GDP Exchange.

Additions to or deletions from basic reaction mixture	Specific activity of isolated GTP		GTP-^{14}C-GDP exchange rate[a]
	Uncorrected	Corrected for enzyme deleted control	
	cpm/μmole	cpm/μmole	μmoles/min
Enzyme deleted	7,900		
$MnCl_2$ deleted	8,200	300	10^{-3}
None	9,050	1,250	10^{-3}
Oxalacetate added	68,420	60,520	0.041

The basic reaction mixture included (in micromoles except as indicated): imidazole (Cl^-) buffer, pH 7.0, 25; GTP, 2.0; GDP-8-^{14}C (1.13×10^6 cpm per mole), 1.44; $MnCl_2$, 1.6; GSH, 2; and purified P-enolpyruvate carboxykinase, 1.3 units in a final volume of 1.0 ml. Final pH was 7.0. Oxalacetate was added at a level of 0.5μ mole. All tubes were incubated for 3 min at 30°. The incorporation of ^{14}C-activity into GTP was determined as described in Table 7.

[a] See Table 7.

K. Oxalacetate-P-enolpyruvate-1-^{14}C Exchange Reaction.

The results of the GTP-^{14}C-GDP exchange experiments exclude the two sequences represented by Reactions 8 to 10, and 11 and 12 but leave open the following sequence (Reactions 13 and 14) as an alternative to the mechanism proposed in Reactions 6 and 7.

$$\underline{E} + \text{IDP (or GDP)} \underset{\text{(rate-limiting)}}{\rightleftharpoons} \underline{E}\text{-IDP (or -GDP)} \qquad (13)$$

$$\underline{E}\text{-IDP (or -GDP)} + \text{P-enolpyruvate} + HCO_3^- \text{ (or } CO_2) \rightleftharpoons \underline{E} + \text{ITP (or GTP)} + \text{oxalacetate} \qquad (14)$$

In order to test this sequence, the rates of oxalacetate-^{14}C-or ^{32}P-labeled P-enolpyruvate exchange were compared with the rate of oxalacetate-$H^{14}CO_3^-$ exchange under identical conditions. The oxalacetate-P-enolpyruvate-1-^{14}C experiments are summarized in Tables 9 and 10. Under conditions which permit rapid oxalacetate-$H^{14}CO_3^-$ exchange, essentially no oxalacetate-P-enolpyruvate-1-^{14}C exchange could be detected, hence the sequence represented by Reactions 13 (rate-limiting step) and 14 is invalid. Deletion of ITP or HCO_3^- individually or together did not promote exchange. Under conditions which permitted carboxylation to occur (oxalacetate and ITP deleted, IDP added), ^{14}C-P-enolpyruvate was converted to oxalacetate.

In summary, the investigations reported in this thesis support the minimal two-step sequence shown in Reactions 6 and 7 for the carboxy-

kinase-catalyzed carboxylation of P-enolpyruvate. Comparative kinetic studies (Table 6) show that the rate of oxalacetate-$H^{14}CO_3^-$ exchange is 30- and 4-times faster than the over-all carboxylation and decarboxylation rates, respectively, at pH 6.8. This and the fact that there is an ITP (or GTP) requirement for oxalacetate-$H^{14}CO_3^-$ exchange indicates that cleavage of the β-carboxylate C—C bond occurs concomitantly with or following a prior reaction of ITP. These results are compatible with the partial reactions proposed (Reactions 6 and 7); moreover, $\underline{k}_1$ and $\underline{k}_{-1}$ are implicated as rate-limiting steps in the over-all carboxylation and decarboxylation processes, respectively. Since the more rapid exchange process involves only $\underline{k}_2$ and $\underline{k}_{-2}$, $\underline{k}_2$ and $\underline{k}_{-2}$ must be greater than $\underline{k}_1$ and $\underline{k}_{-1}$.

Several alternative reaction mechanisms (depicted in Reactions 8 to 14) to Reactions 6 and 7 have been considered and rejected on the basis of appropriate isotope exchange experiments. Initial reaction of GTP (or ITP) with enzyme to form phosphoryl-enzyme and GDP (or IDP) as shown in Reactions 8 to 10 and 11 and 12 has been ruled out (Tables 7 and 8) by the finding that essentially no GTP-^{14}C-GDP exchange occurs under conditions which permit rapid GTP-dependent oxalacetate-$H^{14}CO_3^-$ exchange. One other exchange reaction, namely, oxalacetate-P-enolpyruvate-1-^{14}C exchange did not occur at carboxykinase levels and under a variety of conditions which permitted rapid oxalacetate-$H^{14}CO_3^-$ exchange. These experiments rule out Reactions 13 and 14 as a valid mechanism for the carboxykinase-catalyzed reaction.

A mechanism compatible with these results and other known characteristics of the reaction is presented as follows.

$$\text{Enzyme} + \text{IDP (or GDP)} + \text{P-enolpyruvate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \begin{matrix} & & \text{IDP (or GDP)} \\ & \diagup & \\ \text{enzyme} & & \\ & \diagdown & \\ & & \text{P-enolpyruvate} \end{matrix} \qquad (15)$$

$$\begin{matrix} & & \text{IDP (or GDP)} \\ & \diagup & \\ \text{Enzyme} & & \\ & \diagdown & \\ & & \text{P-enolpyruvate} \end{matrix} + HCO_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{enzyme} + \text{ITP (or GTP)} + \text{oxalacetate} \qquad (16)$$

Table 9. Comparison of Rates of Oxalacetate-Phosphoenolpyruvate-1-^{14}C Exchange and Oxalacetate-$H^{14}CO_3^-$ Exchange.

Incubation time	Enzyme level	Oxalacetate-^{14}C-P-enolpyruvate exchange (total ^{14}C-P-enolpyruvate incorporated into oxalacetate)		Oxalacetate-$H^{14}CO_3^-$ exchange (total $H^{14}CO_3^-$ incorporated into oxalacetate)	
min	unit	dpm	μmole	dpm	μmole
0	5×10^{-3}	1,056		30	
2	5×10^{-3}	993	0	15,960	0.20
4	5×10^{-3}	1,290	0.003	30,550	0.38
4	2.5×10^{-3}			14,450	0.18

The basic reaction mixture contained (in micromoles except as indicated): imidazole (Cl^-) buffer, pH 6.6, 100; ITP, 3.0; P-enolpyruvate (P-enolpyruvate-1-^{14}C in oxalacetate-^{14}C-P-enolpyruvate exchange experiments only, 8.1×10^5 dpm per μmole), o.54; $KHCO_3$ ($KH^{14}CO_3$ in oxalacetate-$H^{14}CO_3^-$ exchange experiments only, 8.1×10^4 dpm per μmole) 50; oxalacetate, 2; $MnCl_2$, 2.6; GSH, 2.0; and purified P-enolpyruvate carboxykinase, units as indicated in a final volume of 1.0 ml. Final pH was 7.0. Following incubation at 30° for the time indicated, 1.0 ml of a saturated solution of 2,4-dinitrophenylhydrazine HCl in 1 N HCl was added allowing 10 min for complete reaction at 30°. After gassing with N_2, the hydrazones were extracted with ethylacetate, the ethylacetate extract was washed with H_2O and counted as described earlier by Maruyama and Lane (4). No detectable hydrolysis of ^{14}C-P-enolpyruvate occurred under these conditions.

Table 10. Failure of Phosphoenolpyruvate Carboxykinase to Catalyze Oxalacetate-P-enolpyruvate -1-^{14}C Exchange under Varied Conditons.

Additions to or deletions from basic reaction mixture	Enzyme level	Total ^{14}C-P-enolpyruvate incorporated into oxalacetate	
	unit	dpm	μmole
None	5×10^{-3}	993	0.003
Enzyme deleted		745	
$KHCO_3$ deleted	5×10^{-3}	715	0
ITP deleted	5×10^{-3}	787	0.001
$KHCO_3$ and ITP deleted	5×10^{-3}	898	0.002
$KHCO_3$ and ITP deleted; 3.0 μmoles of IDP added	5×10^{-3}	808	0.001
ITP and oxalacetate deleted; 1.5 μmoles of IDP added (carboxylation)	5×10^{-2}	6600	0.072

The basic reaction mixture and procedure for measuring ^{14}C-P-enolpyruvate incorporation into oxalacetate are the same as those described in Table 9. Reaction mixtures were adjusted to a final pH of 7.0 and incubated for 4 min at 30°.

L. Substrate Binding and K_s Values for Substrates.

Table 11 summarizes the K_s values of substrates determined by equilibrium dialysis (37), each value being obtained from an average of 4 to 8 experiments.

Substrate binding investigated by equilibrium dialysis (37) indicates that PEP binding by PEPCK absolutely requires divalent cation Mn^{++}, and the K_s value is 2.3×10^{-6} M for PEP. Both GDP and GTP are tightly bound by PEPCK (K_s values are 3.6×10^{-6}, without Mn^{++}; 7.2×10^{-6}, with Mn^{++} for GDP and 3.4×10^{-6}, without Mn^{++}; 8.0×10^{-6}, with Mn^{++} for GTP) in the presence or absence of divalent cation. The fact that both GDP and GTP has higher K_s values in the presence of the divalent cation than in its absence may be because the free nucleotide species binds more tightly to PEPCK than the metal-nucleotide complex. Tight binding of ITP was not detected in the presence or absence of the metal ion.

The tight binding of PEP by PEPCK only in the presence of Mn^{++} detected by equilibrium dialysis suggests that the metal ion plays a role in the formation of a PEPCK-manganese-PEP complex.

Table 11. K_s Values of Substrates Detected by Equilibrium Dialysis.

Substrate	Mn^{++} (Added)	K_s
		M
^{32}P-PEP	-	-
^{32}P-PEP	+	2.3×10^{-6}
^{14}C-GDP	-	3.6×10^{-6}
^{14}C-GDP	+	7.2×10^{-6}
^{14}C-ADP	-	-
^{14}C-GTP	-	3.4×10^{-6}
^{14}C-GTP	+	8.0×10^{-6}
^{32}P-ITP	-	-
^{32}P-ITP	+	-

The experimental conditions were those described under "Experimental Procedure."

M. Kinetic Constants of Substrates in Decarboxylation Reaction.

The kinetic constants (K_m and V_m) for oxalacetate ITP and GTP in decarboxylation reaction are given in Table 12. The K_m values indicate that all of the substrates are present at saturating conditions under the standard decarboxylation assay conditions.

Table 12. Kinetic Constants for Substrates in the Decarboxylation Reaction.

Substrate	K_m	V_m
	M	moles/min
Oxalacetate	1.57×10^{-4}	0.057
ITP	3.82×10^{-4}	0.062
GTP	1.74×10^{-4}	0.074

K_m and V_m determined by Lineweaver-Burk analysis (45, 46) and initial decarboxylation velocities by using the decarboxylation assay described in text. Composition of complete reaction mixture (in micromoles unless indicated otherwise): Tris (Cl^-) buffer, pH 7.5, 50; oxalacetate, 0.5; ITP (except when K_m for GTP was determined), 1.5; $MnCl_2$, 1.5; $MgCl_2$, 2.0; GSH, 2.0; NADH, 0.225; PK, 1.2 units; LDH, 2.5 units; and carboxykinase 2 to 4 $\times 10^{-3}$ unit in a total volume of 1.0 ml. Final pH was 7.45. (Concentration range used in Lineweaver-Burk analysis: Oxalacetate, 0.05 to 1.25 mM; ITP, 0.06 to 1.70 mM; GTP, 0.05 to 1.25 mM.)

VII. Summary

Phosphoenolpyruvate carboxykinase has been isolated from pig liver mitochondria and purified 208-fold from the initial mitochondrial acetone powder extract in good yield (48%). The enzyme appears homogeneous in sedimentation velocity experiments and has a sedimentation coefficient ($s^0_{20,w}$) of 5.21 S. Its molecular weight, determined by sedimentation equilibrium, is 73,300. The ultraviolet and visible absorption spectra of the enzyme revealed no characteristic chromophores. The amino acid composition of the enzyme is reported. Fifteen sulfhydryl groups per molecule of phosphoenolpyruvate carboxykinase account for its total half-cystine content. The N-terminal groups of phosphoenolpyruvate carboxykinase were found to be glutamic acid and isoleucine by Sanger's method. Substrate binding investigated by equilibrium dialysis indicates that PEP binding by phosphoenolpyruvate carboxykinase requires Mn^{++}, while GDP or GTP binding does not require the metal ion. K_s values determined by equilibrium dialysis were 2.3×10^{-6} M for PEP, 3.6×10^{-6} M for GDP and 3.4×10^{-6} M for GTP.

At 30° mitochondrial P-enolpyruvate carboxykinase catalyzes the inosine diphosphate- and Mn^{++}- dependent carboxylation of 664 moles of phosphoenolpyruvate per min per mole of enzyme at pH 7.0 and the ITP- and Mn^{++}- dependent decarboxylation of 4650 moles of oxalacetate per min per mole of enzyme at pH 7.5. The enzyme exhibits a relatively high degree of specificity for inosine and guanosine nucleotides in the carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange reactions. While purified IDP cannot replace ITP in the oxalacetate-$H^{14}CO_3^-$

exchange reaction, IDP can replace ITP in the decarboxylation reaction. In contrast to ITP-supported decarboxylation, IDP-supported decarboxylation leads to pyruvate rather than P-enolpyruvate formation. The enzyme is inhibited by low concentrations (10^{-6} M) of p-chloromercuribenzoate which can be reversed by high concentrations of glutathione.

Comparative kinetic studies at pH 6.8, 7.3, and 8.0 reveal that the phosphoenolpyruvate carboxykinase-catalyzed inosine triphosphate-dependent oxalacetate-$H^{14}CO_3^-$ exchange is much more rapid than either the over-all decarboxylation or carboxylation reactions. At pH 6.8, the relative carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange rates are 1.0, 8.3, and 30, respectively. Under conditions which permit rapid P-enolpyruvate carboxykinase-catalyzed oxalacetate-$H^{14}CO_3^-$ exchange, neither GTP-GDP-8-^{14}C-, nor oxalacetate-P-enolpyruvate-1-^{14}C-exchange occurs at a significant rate. The inability of carboxykinase to catalyze these exchange reactions renders unlikely (a) the formation of phosphorylenzyme from GTP (or ITP) and enzyme as a step in the oxalacetate-$H^{14}CO_3^-$ exchange reaction or (b) the dissociation of either enzyme-P-enolpyruvate or enzyme-IDP (or GDP) as a rate-limiting step in the over-all decarboxylation reaction.

A mechanism compatible with these results and other known characteristics of the reaction is presented.

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THE PURIFICATION AND CHARACTERIZATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

by

Huei-Che Chang

ABSTRACT

Phosphoenolpyruvate carboxykinase has been isolated from pig liver mitochondria and purified 208-fold from the initial mitochondrial acce-ctone powder extract in good yield (48%). The enzyme appears homogeneous in sedimentation velocity experiments and has a sedimentation coefficient ($s^0_{20,w}$) of 5.21 S. Its molecular weight, determined by sedimentation equilibrium is 73,300. The ultraviolet and visible absorption spectra of the enzyme revealed no characteristic chromophores. The amino acid composition of the enzyme is reported. Fifteen sulfhydryl groups per molecule of phosphoenolpyruvate carboxykinase account for its total half-cystine content. The N-terminal groups of phosphoenol-pyruvate carboxykinase were found to be glutamic acid and isoleucine by Sanger's method. Substrate binding investigated by equilibrium dialysis indicates that PEP binding by phosphoenolpyruvate carboxykinase requires Mn^{++}, white GDP or GTP binding does not require the metal ion. K_s values determined by equilibrium dialysis were 2.3×10^{-6} M for PEP, 3.6×10^{-6} M for GDP and 3.4×10^{-6} M for GTP.

At 30° mitochondrial P-enolpyruvate carboxykinase catalyzes the inosine diphosphate- and Mn^{++} - dependent carboxylation of 664 moles of phosphoenolpyruvate per min per mole of enzyme at pH 7.0 and the ITP- and Mn^{++}- dependent decarboxylation of 4650 moles of oxalacetate per min per mole of enzyme at pH 7.5. The enzyme exhibits a relatively high degree of specificity for inosine and guanosine nucleotides in the carboxylation, decarboxylation and oxalacetate-$H^{14}CO_3^-$ exchange reactions. While purified IDP cannot replace ITP in the oxalacetate-$H^{14}CO_3^-$

exchange reaction, IDP can replace ITP in the decarboxylation reaction. In contrast to ITP-supported decarboxylation, IDP-supported decarboxylation leads to pyruvate rather than P-enolpyruvate formation. The enzyme is inhibited by low concentrations (10^{-6} M) of p-chloromercuribenzoate which can be reversed by high concentrations of glutathione.

Comparative kinetic studies at pH 6.8, 7.3, and 8.0 reveal that the phosphoenolpyruvate carboxykinase-catalyzed inosine triphosphate-dependent oxalacetate-$H^{14}CO_3^-$ exchange is much more rapid than either the over-all decarboxylation or carboxylation reactions. At pH 6.8, the relative carboxylation, decarboxylation, and oxalacetate-$H^{14}CO_3^-$ exchange rates are 1.0, 8.3, and 30, respectively. Under conditions which permit rapid P-enolpyruvate carboxykinase-catalyzed oxalacetate-$H^{14}CO_3^-$ exchange, neither GTP-GDP-8-^{14}C-, nor oxalacetate-P-enolpyruvate-1-^{14}C-exchange occurs at a significant rate. The inability of carboxykinase to catalyze these exchange reactions renders unlikely (a) the formation of phosphorylenzyme from GTP (or ITP) and enzyme as a step in the oxalacetate-$H^{14}CO_3^-$ exchange reaction or (b) the dissociation of either enzyme-P-enolpyruvate or enzyme-IDP (or GDP) as a rate-limiting step in the over-all decarboxylation reaction.

A mechanism compatible with these results and other known characteristics of the reaction is presented.