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SHEEP KIDNEY PHOSPHOENOLPYRUVATE CARBOXYLASE PURIFICATION AND PROPERTIES

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SUMMARY

Phosphoenolpyruvate carboxylase (GTP: oxaloacetate carboxy-lase (transphosphorylating), EC 4.1.1.32) has been purified and obtained in a homogeneous form from sheep kidney cortex mitochondria. The purification procedure involved extraction of the freeze-dried mitochondria, $(NH_4)_2SO_4$ fractionation, Sephadex G-100 gel filtration and ion-exchange chromatography on SE-Sephadex and DEAE-Sephadex.

Ultracentrifugal analysis and acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate gave molecular weight estimates of 71 100 and 72 000, respectively. However, the protein behaved as a molecule with a molecular weight of about 50 000 on gel filtration using Sephadex and BioGel. Amino acid analyses showed significant differences in composition between the sheep kidney and pig liver enzymes.

The specificity of the enzyme with respect to nucleotide and divalent cation requirements at varying pH values has been investigated. The enzyme was shown to catalyse a Mn^{2+}-dependent $^{14}CO_2$:oxaloacetate exchange reaction in the absence of added nucleotide. Addition of either ITP or IDP markedly stimulated this exchange activity.

INTRODUCTION

Previous studies have established a role for phosphoenolpyruvate (PEP) carboxylase (GTP:oxaloacetate carboxy-lase (transphosphorylating), EC 4.1.1.32) in both renal and hepatic gluconeogenesis especially in the case of the cytosol activity[1–10]. Although renal gluconeogenesis appears to be as active as its hepatic counterpart, the quantitative contribution of the kidney cortex to glucose production is only about 5–10% of the total glucose production because of the difference in tissue weight[9].

Abbreviation: PEP, phosphoenolpyruvate.

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Hepatic PEP carboxylases from a variety of species are remarkably similar with respect to kinetic and physical properties[11–17], while only slight differences exist between cytosolic and mitochondrial activities from the same tissue[15,17]. Therefore, an investigation of the PEP carboxylase from sheep kidney was initiated so that a detailed comparison of the hepatic and renal activities could be achieved.

MATERIALS AND METHODS

Sources of reagents were the same as those previously described[18]. In addition, oxaloacetic acid, pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) were purchased from California Corporation for Biochemical Research, GDP, ITP, ADP and NADH from Sigma Chemical Company, GTP from P. L. Biochemicals and $(NH_4)_2SO_4$ (enzyme grade) from Mann Research Laboratories. All grades of Sephadex were products of Pharmacia. All other reagents were analytical reagent grade. Radioactive nucleotides were prepared by the method of Symons[19].

Protein was measured by the method of Layne[20]. One unit of enzyme catalysed the carboxylation of 1 μmole of PEP per min at 30 °C.

PEP carboxylase assay methods

Three assay methods were used to determine PEP carboxylase activity[13]: (i) the IDP- and Mn^{2+}-dependent carboxylation of PEP; (ii) the Mn^{2+}-dependent $H^{14}CO_3^-$–oxaloacetate exchange reactions; (iii) the ITP- and Mn^{2+}-dependent decarboxylation of oxaloacetate.

(i) The carboxylation of PEP was followed by the incorporation of $H^{14}CO_3^-$ into oxaloacetate which was converted to aspartate by L-aspartate–2-oxoglutarate aminotransferase (EC 2.6.1.1) as previously described[18]. All radioactive counting was corrected for quenching by the channels ratio method[21].

This assay procedure was used routinely in preference to the decarboxylation assay method because of its greater sensitivity and convenience.

(ii) PEP carboxylase catalyses a Mn^{2+}-dependent exchange reaction between $H^{14}CO_3^-$ and oxaloacetate and this reaction is stimulated by inosine (or guanosine) di- and triphosphates. Aliquots of the enzyme (up to 0.06 unit for a 2-min incubation) were incubated in reaction mixtures (total volume, 0.5 ml) containing (in μmoles): imidazole–HCl, pH 6.5 (adjusted at 30 °C), 50; $MnCl_2$, 2; ITP, 1; oxaloacetate, 1; $NaH^{14}CO_3$ (2.5 μCi), 10; GSH, 0.8. All solutions were previously adjusted to pH 6.5. The reaction was initiated by the addition of oxaloacetate, incubated at 30 °C for 2 min and stopped by the addition of 0.05 ml of 6 M HCl saturated with 2,4-dinitrophenylhydrazine. The [^{14}C]oxaloacetate was stabilized by its conversion to the 2,4-dinitrophenylhydrazone. The assay solutions were processed in triplicate as for the carboxylation assay.

(iii) The ITP- and Mn^{2+}-dependent decarboxylation of oxaloacetate by PEP carboxylase leads to the formation of IDP and PEP. The spectrophotometric assay method of Chang and Lane[13] was found to be unsatisfactory with this enzyme since the non-enzymic rate of decarboxylation of oxaloacetate is dependent on the concentration of free Mn^{2+} or Mg^{2+} and, to minimize this reaction, excess ITP must be present. However, this level of free ITP would be inhibitory to the enzymic reaction. Therefore, the assay method used was modified from that of Seubert and Huth[22],

whereby PEP formation was measured. Aliquots of the enzyme (up to 0.03 unit) were incubated in reaction mixtures (total volume, 0.5 ml) containing (in μmoles): imidazole–HCl, pH 6.5 (adjusted at 30 °C), 50; ITP, 0.5; oxaloacetate, 1; $MnCl_2$, 1; GSH, 0.8. All solutions were previously adjusted to pH 6.5. The reaction was initiated by the addition of oxaloacetate, incubated at 30 °C for 5 min and stopped by the addition of 15 mg of $NaBH_4$. The solutions were immediately placed in an ice-bath for 2 min and 0.2 ml of 15% (w/v) $HClO_4$ was added. The solutions were neutralised with $KHCO_3$, centrifuged to remove precipitated materials and the supernatant retained for PEP estimations. PEP was estimated spectrophotometrically. Aliquots of the supernatant were added to a solution (total volume, 1 ml) containing (in μmoles): Tris–HCl, pH 7.4, 50; KCl, 10; ADP, 0.5; $MgCl_2$, 1; NADH, 0.25; pyruvate kinase, 1 unit; lactate dehydrogenase, 1 unit; and the oxidation of NADH was followed at 340 nm.

IDP (or GDP) can partially replace ITP (or GTP) in the decarboxylation reaction. However, in this case, pyruvate is the product. This observation is in agreement with observations obtained using the enzyme from pig liver[13] and yeast[23], although in the latter case, in the conversion of oxaloacetate to pyruvate, ADP replaces ATP.

RESULTS AND DISCUSSION

Purification of PEP carboxylase

All operations were conducted at 0–4 °C. The averages of the results obtained from several preparations are presented in Table I.

TABLE I

PURIFICATION OF SHEEP KIDNEY MITOCHONDRIAL PEP CARBOXYLASE

Treatment	*Protein (mg)*	$\frac{A_{280\ nm}}{A_{260\ nm}}$	*Activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Extract, pH 6.0	13565	0.88	826	0.06	100
$(NH_4)_2SO_4$ fractionation	4443	1.19	728	0.16	88.6
Sephadex G-100, pH 6.8	450	1.58	458	1.02	56.8
SE-Sephadex, pH 6.2	58.6	1.76	186	3.1	23.5
DEAE-Sephadex, pH 6.8	21.2	1.77	101	4.75	12.2

(a) Preparation of freeze–dried mitochondria. Freeze–dried mitochondria were prepared from the cortex of fresh sheep kidney. The cortex was separated from the medulla, connective and adipose tissue and 100 g of cortex were homogenized in 350 ml of 0.25 M sucrose containing $1 \cdot 10^{-4}$ M EDTA for 1 min. The homogenate was centrifuged at 600 × *g* for 20 min. The supernatant was then centrifuged at 23 000 × *g* for 15 min. The sedimented material was evenly suspended in $1 \cdot 10^{-4}$ M EDTA to a final volume of half that of the original homogenate and centrifuged at 23 000 × *g* for 20 min. The residue was suspended in a minimum volume of $1 \cdot 10^{-4}$ M EDTA, frozen quickly in a dry ice–ethanol mixture and lyophilized.

(b) Extraction and $(NH_4)_2SO_4$ fractionation. Mitochondrial powder (50.0 g) was

extracted with 1 l of $5 \cdot 10^{-2}$ M potassium phosphate, pH 6.0, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, with stirring for 15 min, and the insoluble material wasre moved by centrifuging at 23 000 × *g* for 15 min. Solid $(NH_4)_2SO_4$ (24.3 g per 100 ml of extract) was added to the extract, the pH being maintained at pH 6.5 with KOH. After standing for 10 min, the precipitated protein was removed by centrifuging at 23 000 × *g* for 15 min and the supernatant recovered. Further $(NH_4)_2SO_4$ (13.2 g per 100 ml) was added to the supernatant maintaining the pH at 6.5. The precipitated protein was recovered by centrifuging at 23 000 × *g* for 15 min. The sedimented protein was suspended in a minimum volume of $2 \cdot 10^{-2}$ M potassium phosphate, pH 6.8, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, and the protein was brought into solution by dialysing against the above buffer for 1 h. At this stage, the volume of the enzyme solution was 30–35 ml.

(c) Sephadex G-100 gel filtration. The enzyme solution was applied to a Sephadex G-100 column (6 cm × 85 cm) equilibrated with $2 \cdot 10^{-2}$ M potassium phosphate, pH 6.8, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, and eluted with the same buffer. The fractions containing the highest specific activity were pooled and the protein precipitated with solid ammonium sulphate (39.0 g per 100 ml of solution) at pH 6.5. After centrifuging at 23 000 × *g* for 15 min, the sedimented protein was dissolved in $6 \cdot 10^{-2}$ M potassium phosphate, pH 6.2, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH (total volume, 5.0 ml). The solution was desalted using a Sephadex G-25 column and concentrated to about 3 ml using a Sartorius vacuum-filtration membrane.

(d) SE-Sephadex chromatography. The enzyme soltuion was applied to an SE-Sephadex column (C50, 4 cm × 56 cm) previously equilibrated with $6 \cdot 10^{-2}$ M potassium phosphate, pH 6.2, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, and eluted with the same buffer. The fractions with the highest specific activity were pooled and the protein precipitated with solid $(NH_4)_2SO_4$ (39.0 g per 100 ml of solution) at pH 6.5. The protein was recovered, freed from $(NH_4)_2SO_4$ and concentrated to about 3 ml as described above except that the buffer was $5 \cdot 10^{-2}$ M potassium phosphate, pH 6.8, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH.

(e) DEAE-Sephadex chromatography. The enzyme solution was applied to a DEAE-Sephadex column (A50, 30 cm × 2.7 cm) previously equilibrated with $5 \cdot 10^{-4}$ M potassium phosphate, pH 6.8, containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, and eluted with the same buffer. The fractions with the highest specific activity were pooled and the enzyme was stored as a suspension in $(NH_4)_2SO_4$ (40 g per 100 ml of solution, pH 6.5). The enzyme was stable for several months in this form.

(f) General comments. The difficulty in purifying PEP carboxylase from this source was markedly increased by its instability when bound to any chromatographic support and because of this, neither of the previously published procedures[13,17] could be used. However, the enzyme was obtained in a form which was essentially homogeneous by ultracentrifugal analysis and electrophoretic analysis in the presence of sodium dodecyl sulphate. A minor contaminant of slightly lower molecular weight was detected.

Physical properties of PEP carboxylase

(a) Molecular weight. Sedimentation velocity experiments were carried out at 9–12 °C in $5 \cdot 10^{-2}$ M potassium phosphate buffer, pH 6.8, containing $1 \cdot 10^{-4}$ M EDTA

and $1 \cdot 10^{-4}$ M GSH, with the enzyme concentration varying between 1.0 and 7.5 mg/ml. Sedimentation analysis using Schlieren optics revealed a single sedimenting boundary with no evidence of any association and/or dissociation of the enzyme. Fig. 1 presents a plot of the sedimentation data where $s_{20,w}$ is plotted as a function of enzyme concentration according to the equation,

$$s_{20,w} = s^{0}_{20,w} - k \cdot c$$

where c is the enzyme concentration in mg/ml and k is the slope of the line. The value of k (0.03135 $mg^{-1} \cdot ml$) indicates only a slight dependence of the sedimentation velocity on protein concentration, and a value of 4.58 S for $s_{20,w}$ compares with 5.21 S for the pig liver mitochondrial enzyme[13]. Diffusion experiments were performed at 9.5 °C in $5 \cdot 10^{-2}$ M potassium phosphate buffer, pH 6.8, containing $1 \cdot 10^{-4}$ M EDTA and $1 \cdot 10^{-4}$ M GSH. The diffusion coefficient ($5.858 \cdot 10^{-7}$) was determined by the method of Kawahara[24]. The partial specific volume of the enzyme, calculated from its amino acid composition was 0.73 (ref. 25). Fitting the above data to the Svedberg[26] equation yields a molecular weight for sheep kidney mitochondrial PEP carboxylase of 71 100 which compares with that of the pig liver mitochondrial enzyme (73 300; ref. 13) and rat cytosol enzyme (74 500; ref. 17).

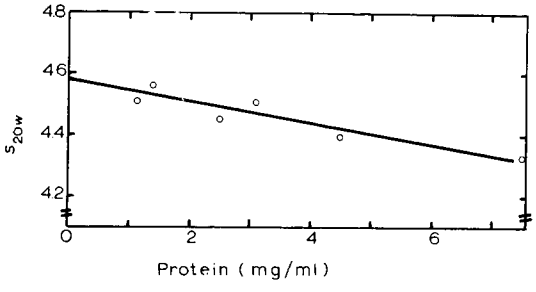


Fig. 1. Variation of the sedimentation coefficient as a function of enzyme concentration obtained from a series of measruements in $5 \cdot 10^{-2}$ M phosphate, $1 \cdot 10^{-4}$ M EDTA, $1 \cdot 10^{-4}$ M GSH, pH 6.8, over the concentration range of 1.0–7.5 mg of protein per ml. Rotor velocity was 59 780 rev./min.

A second molecular weight estimate was obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate[27,28]. The calibration curve for this method is shown in Fig. 2 and gave a molecular weight estimate of 72 000 which is in good agreement with the value obtained using the ultracentrifuge.

Despite the good agreement between the above two estimates, a value of only 47 800 was obtained for the molecular weight of this enzyme using gel filtration (Fig. 3). Since the enzyme also appears to behave as a polymer of molecular weight less than 71 000 on Bio-Gel P100, it would appear that the enzyme assumes a more compact conformation than that normally shown by globular proteins[29].

(b) Amino acid composition. Prior to acid hydrolysis, the protein was carboxymethylated by the method of Milne and Wells[30]. The protein was hydrolysed under vacuum in glass distilled constant boiling point HCl at 105–110 °C for 22 and 48 h. The hydrolysates were analysed using a Beckman 120C Amino Acid Analyser, using a single column with gradient elution according to the method of Piez and Morris[31]. Tryptophan was determined spectrophotometrically by the method of Goodwin and

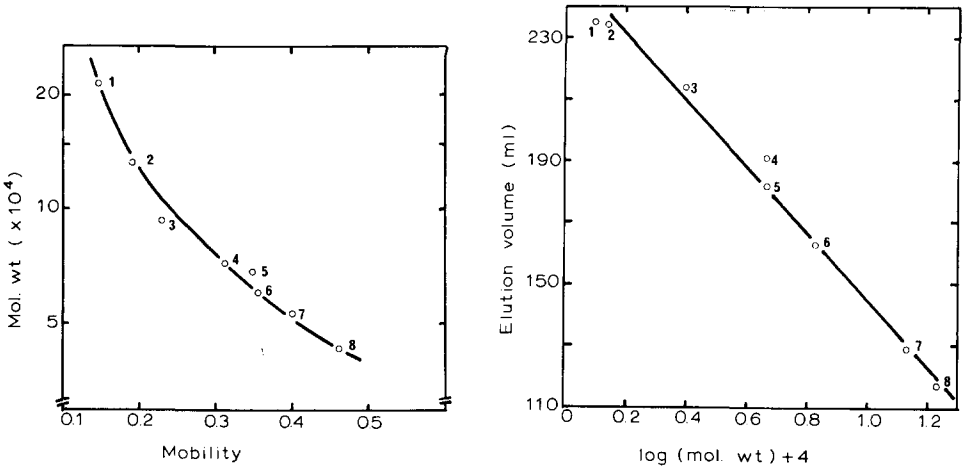


Fig. 2. Molecular weight determination of PEP carboxylase from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. A semilog plot of polypeptide molecular weight against distance of migration relative to the migration of the dye. The proteins used were: 1, myosin; 2, β-galactosidase; 3, phosphorylase b; 4, PEP carboxylase; 5, bovine serum albumin; 6. catalase; 7, glutamate dehydrogenase; 8, ovalbumin.

Fig. 3. Determination of the molecular weight of PEP carboxylase by gel filtration. A Sephadex G-150 column (185 cm × 1.5 cm), previously equilibrated with $5 \cdot 10^{-2}$ M potassium phosphate, pH 6.8 containing $5 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-4}$ M GSH, was calibrated with the following proteins: 1, cytochrome *c*; 2, pancreatic ribonuclease; 3, chymotrypsinogen; 4, ovalbumin; 6, bovine serum albumin monomer; 7, bovine serum albumin dimer; 8, cytochrome b_2. Elution volume was plotted against log molecular weight[29]. PEP carboxylase (5) was detected using the CO_2-fixation assay.

TABLE II

AMINO ACID COMPOSITION OF PEP CARBOXYLASE

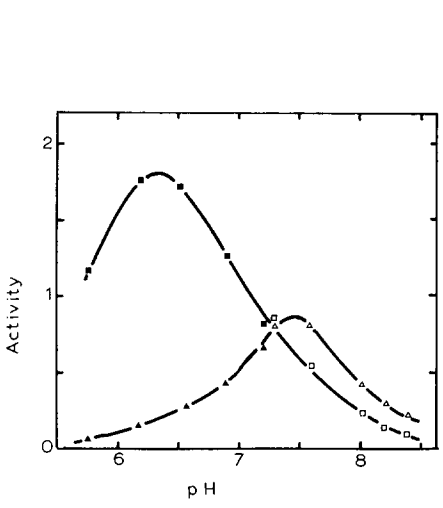
Amino acid	*Residues per mole (sheep kidney)*[a,b]	*Residues per mole (pig liver)*[c]
Cysteine[d]	13	15
Aspartic acid	57	53
Threonine[e]	38	31
Serine[e]	39	31
Glutamic acid	79	70
Proline[e]	54	56
Glycine	63	64
Alanine	55	55
Valine	42	44
Methionine	14	20
Isoleucine	29	28
Leucine	52	58
Tyrosine	12	12
Phenylalanine	29	27
Lysine	34	26
Histidine	13	12
Tryptophan[f]	13	13
Arginine	38	48

[a] Molecular weight, 71 100.
[b] Average of four determinations.
[c] Molecular weight, 73 000[13].
[d] Determined as carboxymethylcysteine.
[e] Corrected for destruction during hydrolysis.
[f] Determined spectrophotometrically by the method of Goodwin and Morton[32].

Morton[32]. The amino acid composition of sheep kidney and pig liver mitochondrial PEP carboxylases are compared in Table II.

Kinetic properties

(a) Relationship between pH and activity. The effect of pH on the activity of PEP carboxylase was examined over the range, pH 5.7 to 8.8, for both the CO_2-fixation reaction (Fig. 4) and the $^{14}CO_2$–oxaloacetate exchange reaction (Fig. 5) in the presence of Mn^{2+} and Mg^{2+} as the activating metal ion. With the carboxylation reaction, the pH optimum is 6.5 with Mn^{2+} but with Mg^{2+} the pH optimum shifts to 7.5 with a lower maximum velocity. This shift in pH optimum could be important mechanistically and could reflect either a different CO_2 species being used by the two activating metal ions or the different pK_a values of metal-bound water molecules which have been implicated in the PEP carboxylase reaction (unpublished observation). On the other hand, these metal-bound hydroxyl ions would not be involved in the $^{14}CO_2$–oxaloacetate exchange reaction and it would be expected that the pH optimum would be independent of the activating metal ion. This was in fact observed (Fig. 5) although the pH optimum varied with the added nucleotide, *viz.* pH 6.5 with the triphosphate but pH 7.0 with the diphosphate.



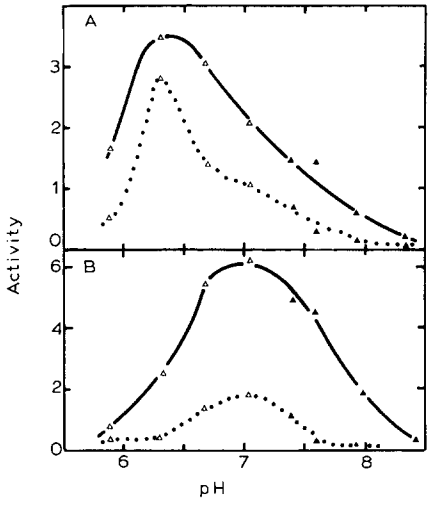


Fig. 4. Effect of pH on the PEP carboxylation activity with Mn^{2+} or Mg^{2+} as the activating divalent cation. Assay conditions were as described in Materials and Methods except that the buffers used were potassium 3,3′-dimethyl glutarate (open symbols) and Tris–HCl (closed symbols, both at 0.1 M). ■—■ and □—□, Mn^{2+}; ▲—▲ and △—△, Mg^{2+}.

Fig. 5. Effect of pH on the $^{14}CO_2$–oxaloacetate exchange activity with Mn^{2+} or Mg^{2+} as the activating divalent cation and in the presence of ITP (A) or IDP (B). Assay conditions were as described in Materials and Methods except that the buffers used were potassium 3,3′-dimethyl glutarate (open symbols) and Tris–HCl (closed symbols, both at 0.1 M). ———, Mn^{2+}; · · · · · ·, Mg^{2+}.

(b) Effect of Mn^{2+} and Mg^{2+} with inosine or guanosine nucleotides on the reaction rates. The relative magnitude of the carboxylation reaction, the $^{14}CO_2$–oxaloacetate exchange reaction and the decarboxylation reaction was assessed using either inosine or guanosine nucleotides in the presence of either Mn^{2+} or Mg^{2+} as activating me-

tal ion (Table III). The exchange reaction was faster than the decarboxylation reaction which, in turn, was faster than the carboxylation reaction. Similar results have been reported for the pig[14] and chicken[33] liver mitcohondrial activities. However, there appear to be species differences with respect to the relative rates of the three reactions since Chang *et al.*[14] report a 32-fold difference in rates between the exchange reaction and the carboxylation reaction.

TABLE III

COMPARISON OF THE RATES OF THE ENZYME-CATALYSED REACTIONS WITH INOSINE OR GUANOSINE NUCLEOTIDES AND Mn^{2+} AND Mg^{2+}

Standard assay conditions were as described in Materials and Methods except that nucleotide and metal ion were added as indicated above. When both cations were present, each was added to a concentration half that normally present.

Components	*% Activity**
Carboxylation	
IDP + Mn^{2+}	100
GDP + Mn^{2+}	83
IDP + Mg^{2+}	3
GDP + Mg^{2+}	5
IDP + Mn^{2+} + Mg^{2+}	46
GDP + Mn^{2+} + Mg^{2+}	46
$^{14}CO_2$–oxaloacetate exchange	
ITP + Mn^{2+}	260
GTP + Mn^{2+}	213
IDP + Mn^{2+}	132
GDP + Mn^{2+}	140
ITP + Mg^{2+}	185
GTP + Mg^{2+}	161
IDP + Mg^{2+}	8
— + Mn^{2+}	8
— + Mg^{2+}	Trace
IMP + Mn^{2+}	19
Decarboxylation	
ITP + Mn^{2+}	130
GTP + Mn^{2+}	68
ITP + Mg^{2+}	Trace
GTP + Mg^{2+}	Trace
ITP + Mn^{2+} + Mg^{2+}	131
GTP + Mn^{2+} + Mg^{2+}	68

* Expressed as % activity relative to carboxylation with IDP and Mn^{2+}.

(c) $^{14}CO_2$–oxaloacetate exchange reaction. The only detectable exchange reaction catalysed by the sheep kidney mitochondrial PEP carboxylase was the $^{14}CO_2$–oxaloacetate exchange reaction which appeared not to be dependent on the presence of nucleoside triphosphate (Table III). This is contrary to the results obtained using pig liver mitochondrial enzyme where the $^{14}CO_2$–oxaloacetate exchange activity is dependent on the presence of Mn^{2+}, is not activated by Mg^{2+} but is stimulated 15-fold by IDP (or GDP) and 30-fold by ITP (or GTP). Further evidence to support the conclusion that the exchange reaction is not dependent upon the presence of nucleoside triphosphate is indicated by the fact that, (i) Mg^{2+} was ineffective in activating the exchange reaction at pH 6.5 although activity was observed with Mg^{2+}

and ITP (or GTP) (Table III); (ii) Attempts to detect nucleotides in the nucleotide-free exchange reaction assay solution were unsuccessful; (iii) The steady-state rate equation for the incorporation of $^{14}CO_2$ into oxaloacetate predicts intersecting initial velocity patterns when the reciprocal of the initial velocity is plotted as a function of the reciprocal of the substrate concentration (see Appendix). This was observed

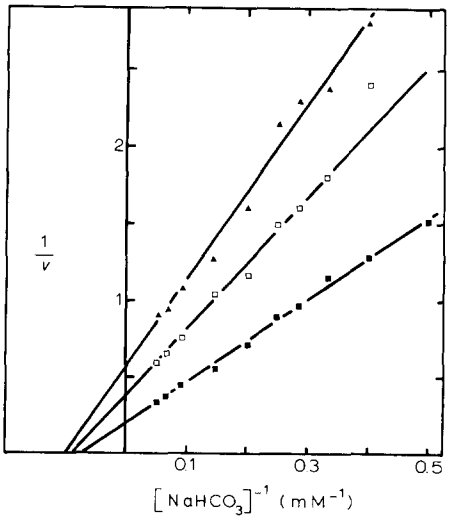


Fig. 6. Double-reciprocal plots of the initial velocity of the $^{14}CO_2$–oxaloacetate exchange reaction against the $NaHCO_3$ concentration. The concentrations of oxaloacetate were: ■—■, 2.0 mM; □—□, 0.6 mM; ▲—▲, 0.4 mM. The assay mixtures contained: imidazole–HCl, pH 6.5, 0.1 mM; $MnCl_2$, 4.0 mM; reduced glutathione, 1.6 mM; enzyme, 0.01 unit. Incubation was for 4 min.

(Fig. 6). The results of the initial velocity studies of the nucleotide-independent exchange reaction are presented in Figs 6 and 7.

The mechanism of the stimulation of the exchange reaction in the presence of nucleotide remains unexplained. Both IDP and ITP complete an oxaloacetate decarboxylation system giving either pyruvate or PEP, respectively[12,23] so that reversal of these sequences would effect an exchange of CO_2 into oxaloacetate. However, the

TABLE IV

CONTRIBUTION OF THE REVERSAL OF THE CARBOXYLASE REACTION TO THE $^{14}CO_2$ OXALOACETATE EXCHANGE REACTION

Components common to all assay mixtures (0.5 ml) were: 0.1 M imidazole–HCl, pH 6.5, (adjusted at 30 °C; 4 mM $MnCl_2$; 2 mM oxaloacetate; 2 mM ITP (or IDP); 20 mM $NaH^{14}CO_3$ ($6 \cdot 10^5$ cpm/μmole); 1.6 mM GSH. In addition, 0.5 mM ADP, 1 mM NADH, 50 mM KCl, 5 units pyruvate kinase and 4.5 units lactate dehydrogenase were added as indicated. The reaction was started by the addition of oxaloacetate and incubation was for 2 min at 30 °C except for the control where incubation was for 4 min.

Components	*Activity (cpm incorporated)*
Control (no nucleotide)	97
(a) $MnCl_2$ + oxaloacetate + ITP + $NaH^{14}CO_3$ + KCl	1460
(a) + pyruvate kinase + ADP	1500
(b) $MnCl_2$ + oxaloacetate + IDP + $NaH^{14}CO_3$	880
(b) + lactate dehydrogenase + NADH	850

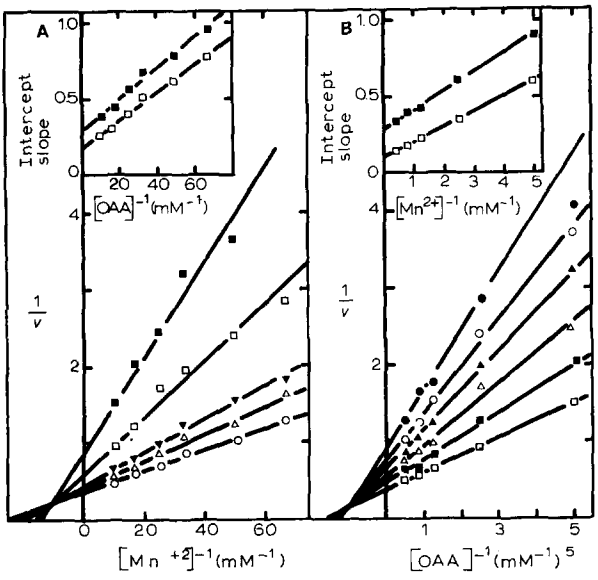


Fig. 7. Double reciprocal plots of the initial velocity of the $^{14}CO_2$–oxaloacetate exchange reaction with varying amounts of $MnCl_2$ (A) and oxaloacetate (B). Reaction mixtures contained: imidazole–HCl, pH 6.5, 0.1 M; $NaH^{14}CO_3$, 20.0 mM; reduced glutathione, 1.6 mM; enzyme, 0.02 unit. Incubation was for 4 min. A. $MnCl_2$ was varied at the constant oxaloacetate levels: ○—○, 2.0 mM; △—△, 1.2 mM; ▲—▲, 0.8 mM; □—□, 0.4 mM; ■—■, 0.2 mM. B. Replot of the data in A. Oxaloacetate was varied at the constant $MnCl_2$ levels: □—□, 0.1 mM; ■—■, 0.06 mM; △—△, 0.04 mM; ▲—▲, 0.03 mM; ○—○, 0.02 mM; ●—●, 0.015 mM. Inset: replots of the intercepts (■—■) and slopes (□—□), obtained from the related primary plots, *versus* the reciprocal concentration. OAA = oxaloacetate.

data presented in Table IV where the decarboxylation products were continuously removed by coupling systems negates this possibility. Also, it is possible that the exchange reaction in the absence of added nucleotide could be an abortive pathway in which case the exchange reaction in the presence of nucleotide would be energy dependent. However, the results presented in Table V demonstrate that the $^{14}CO_2$–oxaloacetate exchange reaction is independent of phosphoryl transfer in the presence and absence of nucleotide.

These results are consistent with the hypothesis that during carboxylation of PEP catalysed by sheep kidney mitochondrial PEP carboxylase, the central complex, consisting of enzyme, Mn^{2+}, IDP, PEP and CO_2 undergoes a two-step conversion *via* an enzyme-bound three-carbon intermediate to give a complex consisting of enzyme, Mn^{2+}, ITP and oxaloacetate. This proposed mechanism is in contrast to the concerted conversion of the central complexes proposed for the pig liver mitochondrial PEP carboxylase[35]. The fact that the exchange activity is independent of phosphoryl transfer (Table V) indicates that the nucleotide becomes an activator of this reaction (*cf.* Bridger *et al.*[36]), and that the phosphoryl transfer or the splitting of the high energy phosphate bond would not be essential for carboxylation or decarboxylation *per se*. Attempts to demonstrate a three-carbon enzyme-bound intermediate by a carboxylation of pyruvate or an exchange of pyruvate into oxaloacetate have been unsuccessful.

TABLE V

COMPARISON OF CO_2 AND NUCLEOTIDE TURNOVER IN THE $^{14}CO_2$–OXALOACETATE EXCHANGE REACTION

The reaction was started with oxaloacetate and incubation, at 30 °C, was stopped after 0, 2.25 and 4.5 min with 0.05 ml of 5 M formic acid. Denatured protein was removed by centrifuging and aliquots of the supernatant were chromatographed on PEI-paper[34] using 0.3 M NH_4HCO_3. The ultraviolet-absorbing regions were cut out and counted in a Packard-Tricarb scintillation spectrometer.

Activating nucleotide	*Turnover (μmole/min)*	
	*$^{14}CO_2$**	*[^{32}P]Nucleotide***
GTP	0.0236	0.0152
GDP	0.0159	0.0
No nucleotide	0.0095	—

* Experimental conditions were the same as described in Table IV and samples assayed for acid-stable radioactivity at 2.25 and 4.5 min.

** Conditions were as above except that non-radioactive $NaHCO_3$ was used as well as [α-^{32}P]GTP or [β-^{32}P]GDP.

This inability of the enzyme to bind or utilise pyruvate is reminiscent of the enzyme PEP carboxytransphosphorylase. PEP carboxytransphosphorylase carboxylates PEP in the presence of orthophosphate to yield oxaloacetate and pyrophosphate[37] but also fails to utilise pyruvate even though pyruvate is formed from PEP in the absence of CO_2. The enzyme also appears to catalyse a Mn^{2+}-depednent, pyrophosphate-stimulated exchange of CO_2 into oxaloacetate[38]. Therefore, it is quite feasible that these two related PEP carboxylating enzymes may have similar mechanisms.

APPENDIX

Consider Eqn 1 which is the sequence whereby $^{14}CO_2$ is incorporated into oxaloacetate.

$$\begin{array}{ccccccc} & A & & P^* & & Q & \\ & k_1 \downarrow\uparrow k_2 & & k_3 \uparrow\downarrow k_4 & & k_5 \uparrow\downarrow k_6 & \\ E & \text{———} & EA & \text{———} & EQ & \text{———} & E \end{array} \qquad (1)$$

(where A = oxaloacetate; P = $^{14}CO_2$; Q = pyruvate; E = enzyme; and * represents a ^{14}C-labelled component).

From steady-state kinetics,

$$\frac{\mathrm{d}\,E^*A}{\mathrm{d}t} = k_4\,P^*\,EQ - (k_2 + k_3)\,E^*A = 0 \qquad (2)$$

$$\therefore\ E^*A = \frac{k_4\,P^*\,EQ}{(k_2 + k_3)} \qquad (3)$$

$$v = \frac{\mathrm{d}\,A^*}{\mathrm{d}t} = k_2\,E^*A$$

$$= \frac{k_2 k_4 P^* EQ}{(k_2 + k_3)} \qquad (4)$$

where v is the initial velocity of the isotope exchange reaction. Using the distribution equation derived by the method of King and Altman[39] and putting $Q = 0$

$$\frac{EQ}{E_t} = \frac{k_1k_3A}{k_5(k_2 + k_3) + k_1(k_3 + k_5)A + k_1k_4AP + k_2k_4P} \quad (5)$$

Substituting for EQ in Eqn 4

$$v = \frac{k_1k_2k_3k_4A\ P^*E_t}{(k_2 + k_3)\,[k_5(k_2 + k_3) + k_1(k_3 + k_5)A + k_1k_4AP + k_2k_4P]} \quad (6)$$

$$= \frac{\frac{k_2k_3}{k_2 + k_3}\,AP^*E_t}{\frac{k_5(k_2 + k_3)}{k_1k_4} + \frac{(k_3 + k_5)}{k_4}\,A + \frac{k_2}{k_1}\,P + AP} \quad (7)$$

which is an equation for a straight line if $1/v$ is plotted as a function of $1/A$. If P is held at fixed non-saturating concentrations then a series of straight intersecting lines will be obtained.

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