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Pig Liver Pyruvate Carboxylase

PURIFICATION, PROPERTIES AND CATION SPECIFICITY

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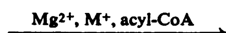
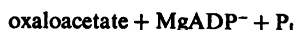
1. Pyruvate carboxylase was purified to apparent homogeneity from pig liver mitochondria and shown to be free of all kinetically contaminating enzymes. 2. The enzyme has a mol. wt. of 520000 and is composed of four subunits, each with a mol. wt. of 130000. 3. The enzyme can exist as the active tetramer, dimer and monomer, although the tetramer appears to be the form in which the enzyme is normally assayed. 4. For every 520000g of the enzyme there are 4 mol of biotin, 3 mol of zinc and 1 mol of magnesium. No significant concentrations of manganese were detected. 5. Analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis indicates three polypeptide chains per monomer unit, each with a mol. wt. of 47000. 6. The amino acid analysis, stoicheiometry of the reaction and the activity of the enzyme as a function of pH are also presented. 7. The enzyme is activated by a variety of univalent cations but not by $Tris^+$ or triethanolamine$^+$. 8. The activity of the enzyme is dependent on the presence of acetyl-CoA; the low rate in the absence of added acetyl-CoA is not due to an enzyme-bound acyl-CoA. The dissociation constant for enzyme-bound acetyl-CoA is a marked function of pH.

Pyruvate carboxylase (EC 6.4.1.1) has been purified to apparent homogeneity from chicken liver (Scrutton & Utter, 1965), yeast (Cazzulo & Stoppani, 1967; Young *et al.*, 1969), rat liver (Seufert *et al.*, 1971; McClure *et al.*, 1971*a*), turkey and calf liver (Scrutton *et al.*, 1972) and has been shown to catalyse the reaction;





$$\text{Pyruvate} + HCO_3^- + MgATP^{2-} \xrightarrow{Mg^{2+},\ M^+,\ \text{acyl-CoA}} \text{oxaloacetate} + MgADP^- + P_i$$

where M^+ represents a univalent cation (typically K^+) and the acyl-CoA is typically acetyl-CoA. Pyruvate carboxylase is thought to be involved in the regulation of such diverse metabolic pathways as gluconeogenesis (see Newsholme & Gevers, 1967; Scrutton & Utter, 1968; Exton, 1972) and lipogenesis (see Kornacker & Ball, 1965; Rognstad & Katz, 1966), and a detailed analysis of the reaction mechanism is an essential prerequisite to a deeper understanding of the role of potential regulatory factors of the enzyme *in vivo*. Reaction mechanisms for pyruvate carboxylase have been proposed for the enzyme purified from chicken liver (Barden *et al.*, 1972), rat liver (McClure *et al.*, 1971*b*) and *Aspergillus niger* (Feir & Suzuki, 1969); although the basic mechanism appears to be the same in each case, there are errors which probably invalidate the proposed reaction pathways. These errors are discussed in detail in the following paper (Warren & Tipton, 1974). In an attempt to resolve these difficulties the enzyme from pig liver has been purified to apparent homogeneity and its physical and chemical properties have been studied before a thorough analysis of the reaction mechanism.

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Materials

Yeast hexokinase (EC 2.7.1.1) was purified by the method of Schulze *et al.* (1966), with the omission of the bentonite-adsorption step. Yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was purified as described by Noltmann *et al.* (1961). Unfortunately, citrate synthase (EC 4.1.3.7) activity represented 0.7% of the activity of the enzyme purified in this way. Since the activity of glucose 6-phosphate dehydrogenase was 100–300 times the activity of pyruvate carboxylase that was normally present in the assay described below, the citrate synthase activity represented a major kinetic contamination. Glucose 6-phosphate dehydrogenase was applied to a DEAE-cellulose (Whatman DE52) column equilibrated and washed with 60mM-potassium phosphate buffer, pH 7.6. Citrate synthase did not bind to the ion exchanger under these conditions, and when the majority of this enzyme had been removed, glucose 6-phosphate dehydrogenase was eluted with 200mM-potassium phosphate buffer, pH 7.6. The activity of citrate synthase in the eluate now represented only 0.004% of the activity of

glucose 6-phosphate dehydrogenase. All other enzymes were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Avidin was purified by the method of Green & Toms (1970).

Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938). Sigma technical-grade pyruvic acid was twice distilled under N_2, at a reduced pressure, and the middle, colourless fractions were collected each time. After dilution with water to 1 M, it was stored at −20°C (Von Korff, 1969). Acetyl-CoA was prepared from CoA by treatment with acetic anhydride (Simon & Shemin, 1953) and purified on DEAE-cellulose (Whatman DE52) by the method of Moffatt & Khorana (1961). Other components of the assay systems for pyruvate carboxylase were obtained from Boehringer Corp. (London) Ltd. Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Dithiothreitol and (−)-carnitine hydrochloride were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. $Tris_2ATP$ from equine muscle was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. *Lactobacillus arabinosus* (culture 8014) was obtained from N.C.I.B., Torrey Research Station, Aberdeen, U.K. All other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity available. Distilled water was passed through a Permutit Mark II portable deionizer before use.

Methods

Spectrophotometer

The basic unit of the spectrophotometer used in these studies was a Beckman DU monochromator fitted with a sample chamber for 1 cm light-path cuvettes. Light was detected by an end-on photomultiplier (EMI 9526B) and the absorbance converter, constructed as described by Gordy *et al.* (1964), utilized the principle of Gilford & Wood (1961). The output was fed to a 1 mV Rikadenki recorder [Model B-024; 10 in (25 cm) chart width] and stable backing-off facilities were provided by an attentuated supply from mercury batteries. A highly stable light source was provided by a 150 W quartz–iodine lamp, cooled by a low-powered fan. The spectrophotometer was linear from 0 to 1.0 *E* unit and at 1.0 *E* unit there was no detectable noise or short-term (up to 15 min) drift on the 0 to 0.1 *E* unit scale of the recorder.

Enzyme assays

All enzymes, except yeast alcohol dehydrogenase, were assayed in 100 mM-triethanolamine hydrochloride–KOH buffer, pH 8.0, at 30°C. Lactate dehydrogenase, malate dehydrogenase, yeast alcohol dehydrogenase and pyruvate kinase were assayed in the manner described by the manufacturers. The following enzymes were assayed by the method described by the authors whose names are given in parentheses: hexokinase (Joshi & Jagannathan, 1966); glucose 6-phosphate dehydrogenase (Noltmann *et al.*, 1961); β-galactosidase (Craven *et al.*, 1965); citrate synthase, acetyl-CoA deacylase (Srere, 1969); acetyl-CoA carboxylase (Matsuhashi, 1969); ATPase (adenosine triphosphatase) (Warren *et al.*, 1974); phosphoglucose isomerase (Noltmann, 1966); phosphofructokinase (Ling *et al.*, 1966). Glucose 6-phosphatase was assayed in a novel fashion in a mixture containing 100 mM-triethanolamine hydrochloride–KOH, pH 8.0, 1 mM-glucose 6-phosphate, 1 mM-ATP, 10 mM-P_i, 5 mM-$MgSO_4$, 0.1 mM-NADH and 1 unit each of lactate dehydrogenase, pyruvate kinase and hexokinase in a total volume of 2.0 ml. Controls were carried out in the absence of glucose 6-phosphate. Glucose 6-phosphatase activity was monitored at 340 nm via the three coupled substrates (see McClure, 1969) glucose, ADP and pyruvate.

Pyruvate carboxylase was assayed at 30°C and pH 8.0 unless otherwise stated. The K_m values of the coupling enzymes for the coupled substrates were determined, in each case, under the conditions of the assay for pyruvate carboxylase. The K_m values were used to derive the time taken (coupling time) before the rate of production of the measured product reached 99% of the steady-state rate of the pyruvate carboxylase reaction (McClure, 1969). In addition, the components of the coupled assays were found to have no significant effect on the activity of pyruvate carboxylase itself. Pyruvate carboxylase was assayed in the forward reaction by using a coupled assay for oxaloacetate (the oxaloacetate-coupled assay). The assay system contained, in a total volume of 2.0 ml, 100 mM-triethanolamine hydrochloride–KOH buffer, pH 8.0, 5 mM-potassium pyruvate, 1 mM-ATP, 5 mM-$MgSO_4$, 15 mM-$KHCO_3$, 0.1 mM-acetyl-CoA, 0.1 mM-NADH and 10 units of malate dehydrogenase. The reaction was started by the addition of 10–50 μg of pyruvate carboxylase and the rate of reaction was measured by the decrease in absorbance at 340 nm; the coupling time was less than 3 s. Unless otherwise stated, the ionic strength (*I*) of the assay system was maintained at 150 ± 15 mM by the addition of 1 M-KCl; variation within these limits had no detectable effect on the reaction rate. Maintenance of *I* at a fixed value was necessitated by the sensitivity of the $MgATP^{2-}$ stability constant to this parameter (O'Sullivan & Perrin, 1964).

Pyruvate carboxylase was assayed in the back reaction by using a coupled assay for ATP (the ATP-coupled assay). The assay system contained, in a total volume of 1.0 ml, 100 mM-triethanolamine

hydrochloride–KOH buffer, pH 8.0, 0.5mM-ADP, 10mM-P_i, 3.36mM-$MgSO_4$, 1mM-oxaloacetic acid, 0.1mM-acetyl-CoA, 2.5mM-glucose, 0.2mM-$NADP^+$, 25 units of hexokinase and 3 units of glucose 6-phosphate dehydrogenase. The reaction was started by the addition of 50–120μg of pyruvate carboxylase and the rate of reaction was measured by the increase in absorbance at 340nm; the coupling time was approx. 35s. Unless otherwise stated I was maintained at 200±20mM by the addition of 1M-KCl.

For both assays, a plot of reaction rate against enzyme concentration was linear up to the highest concentrations of pyruvate carboxylase used.

Substrate and effector assays

Pyruvate and oxaloacetate were assayed as described by Bergmeyer (1963*a*,*b*), acetyl-CoA as described by Chase (1967) and NH_4^+ as described by Lang (1958). All other components were assayed by weight.

Protein assay

This was carried out by the micro-biuret method of Goa (1953). For the experiments involving the measurement of the metal and biotin content of the enzyme, the specific extinction coefficient of pyruvate carboxylase in neutral buffers was derived. The $E_{1\,cm}^{0.1\%}$ of 1.06 at 280nm was lower than that obtained by using the micro-biuret method, and the latter results would have to be multiplied by 0.78 to give the correct answer.

Purification of pyruvate carboxylase

This was done by a modification of the method of Scrutton *et al.* (1969).

Preparation of freeze-dried mitochondria. All operations were performed at 0–4°C. Fresh pig liver (2.5kg) was freed from connective tissue and large blood vessels and was immersed in 2 litres of 0.3M-sucrose. Portions (250g) were added to 750ml of 0.3M-sucrose in a Kenwood blender attached to a Kenwood Mix-master. Homogenization was carried out at speed 3 until the homogenate had a smooth reddish-brown appearance (approx. 25s). The homogenate was centrifuged at 1000g for 10min. The supernatant was carefully poured through a large-mesh sieve to remove floating material, which included glycogen, until the loose reddish-brown layer of the sediment started to spill over. The supernatant was centrifuged at 1450g for 60–70min, which was sufficient time to sediment more than 70% of the mitochondria. The supernatant was carefully decanted and the mitochondrial sediment was suspended in 1.5 litres of 0.5mM-EDTA adjusted to pH 7.0–7.1 with 1M-KOH. The mixture was slowly stirred for 5–10min and centrifuged at 16000g for 30min. The supernatant was slowly decanted until the main part of the mitochondrial sediment started to emerge. The sediment was re-suspended in 50ml of 0.5mM-EDTA, pH 7.0–7.1, and the suspension was equally divided between two 5-litre round-bottomed Quickfit flasks. The mitochondria were then freeze-dried and stored at −20°C in a sealed flask. The 2.5kg of pig liver yielded 60–80g of freeze-dried mitochondria, which could be stored for at least 6 months with no loss of pyruvate carboxylase activity.

Preparation of pyruvate carboxylase from freeze-dried mitochondria. All operations were performed at 20–25°C unless otherwise stated. Freeze-dried mitochondria (100g) were slowly added to 1250ml of rapidly stirred 50mM-Tris–acetate buffer, pH 7.0, containing 5mM-$MgSO_4$, 0.2mM-ATP and 0.5mM-EDTA. The pH fell to 6.5–6.7 and was maintained between these limits by the addition of 1M-Tris base; at no time was the pH allowed to fall below 6.4, since pyruvate carboxylase became rapidly inactivated. The stirring speed was lowered and after 10–15min, 1M-Tris base was added to raise the pH to 7.0–7.2. The mixture was centrifuged at 15000g for 60min. The red supernatant was removed from the fairly firm precipitate and the pH was re-adjusted to 7.0–7.1 with 1M-Tris base.

The solution was still very turbid and was clarified by generating calcium phosphate gel in the solution itself (cf. Illingworth, 1972). To do this 2M-$CaCl_2$ (in 0.25M-Tris base) was added to a final concentration of 50mM (this included the volume of 1M-K_2HPO_4 that was subsequently added); a whitish precipitate appeared and the pH rose to 7.3. The solution was stirred rapidly and 1M-K_2HPO_4 was rapidly thrown into the central vortex to a final concentration of 100mM. A white gelatinous precipitate formed and the pH slowly fell to 7.1–7.2 after 5–10min of stirring. The mixture was centrifuged at 23000g for 15min. A clear red supernatant was decanted from a very hard precipitate. The pH of the supernatant was adjusted to 7.0–7.2 with 1M-Tris base and 180g of finely ground $(NH_4)_2SO_4$ was added to a litre. The pH was maintained by the addition of 1M-Tris base and the mixture was stirred for 15min. After centrifugation at 15000g for 30min the clear red supernatant was discarded. The firm precipitate was taken up in 250ml of 0.5M-sucrose containing 2mM-ATP, 5mM-$MgSO_4$ and 15mM-$KHCO_3$. The pH was adjusted to 7.0–7.1 with 1M-Tris base, before and after the suspension of the protein. The clear yellow solution was placed in a 1-litre round-bottomed Quickfit flask and rapidly heated to 45°C in an 80°C water bath. The flask was transferred to a 45°C water bath fitted with a Rank underwater stirrer and stirred at this temperature for 10min. The flask was rapidly cooled in a solid CO_2–ethanol bath to 25°C. The pH rose to 7.4–7.5 during this procedure. The pH was adjusted to 6.4–6.5 by using 1M-acetic acid, and calcium phosphate gel was added until

10–20% of the pyruvate carboxylase activity was removed (approx. 2mg of calcium phosphate gel/mg of protein was needed). The pH was maintained by the addition of 1M-Tris base and the mixture was stirred for 5min. The mixture was centrifuged at 23000g for 15min. The clear yellow supernatant was adjusted to pH7.0–7.2 with 1M-Tris base and 200g of finely ground $(NH_4)_2SO_4$ was added to 1 litre. The pH was maintained by the addition of 1M-Tris base and the mixture was stirred for 15min. The mixture was centrifuged at 15000g for 30min. Solutions for the serial $(NH_4)_2SO_4$ extractions were prepared by mixing 50%-saturated (at room temp.) $(NH_4)_2SO_4$ (adjusted to pH7.0 with 1M-Tris base) and 250mM-potassium phosphate buffer, pH7.0, containing 2mM-EDTA; the pH values of the solutions fell to 6.5–6.7 and were not readjusted. The percentages of $(NH_4)_2SO_4$ in the solutions were as follows: 32, 30, 28, 24, 22, 20, and 18%.

The initial precipitate was suspended in sufficient 32% $(NH_4)_2SO_4$ to give 15–20 units of pyruvate carboxylase activity/ml and the volume needed defined the volume of all subsequent solutions. The suspension was stirred in a 100ml polypropylene tube for 10min; the stirrer was removed and the mixture was centrifuged at 40000g for 10min. The supernatant was removed, the next $(NH_4)_2SO_4$ solution was added and the procedure was repeated. The supernatants from the 24%-, 22%- and 20%-$(NH_4)_2SO_4$ extractions were pooled and 260g of $(NH_4)_2SO_4$/litre was added. The pH was maintained at 7.0–7.2 with 1M-Tris base and the mixture was stirred for 15min. Centrifugation was carried out at 15000g for 45min. The precipitate was resuspended in 100mM-potassium phosphate buffer containing 1M-sucrose and 250g of $(NH_4)_2SO_4$/litre (final volume). The pH was re-adjusted to 7.0 with 1M-KOH and the suspension (containing 3–5mg of protein/ml) was stored at 4°C. Under these conditions the activity of pyruvate carboxylase was stable for at least 5 months. The whole purification procedure from fresh liver was completed in 2 days and the purification of the enzyme from freeze-dried mitochondria was completed in 10–12h. Stored pyruvate carboxylase was prepared for use by gel-filtering the enzyme on Sephadex G-25 into 100mM-triethanolamine hydrochloride–KOH buffer, pH8.0, containing 25% (v/v) glycerol. Acetyl-CoA was then added to a final concentration of 150μM. Under these conditions the enzyme was totally stable for at least 10h at room temperature. Coupling enzymes, which were stored as the $(NH_4)_2SO_4$ suspension, were treated in a similar fashion and stored at 0°C.

Intracellular location

Samples were taken at each stage of the preparation of mitochondria and freeze-dried. All the samples were extracted with 50mM-Tris–acetate buffer, pH7.0, and the activity of citrate synthase was determined. $(NH_4)_2SO_4$ (200g/litre) was added and the precipitate (which was now free of most of the lactate dehydrogenase activity, but retained all of the pyruvate carboxylase activity) was assayed for pyruvate carboxylase by using the oxaloacetate-coupled assay in the presence and absence of acetyl-CoA. Since the activity of citrate synthase in the whole homogenate was approximately the same as that derived by the above procedure, the latter appears to be a valid means of extracting intramitochondrial proteins.

Polyacrylamide-gel electrophoresis

This was performed by a modification of the method of Davis (1964). Gels were prepared by mixing the following reagents: acrylamide, 4.5g; *NN'*-methylenebisacrylamide, 0.23g; *NNN'N'*-tetramethylethylenediamine, 0.083ml; 1M-Tris–HCl buffer, pH8.1, 10.0ml; 100mM-potassium persulphate, 0.8ml; and water to 100ml. The electrode compartments contained 100mM-Tris–100mM-glycine buffer, pH8.9. Samples of pyruvate carboxylase (10–100μg) in 100mM-triethanolamine hydrochloride–KOH buffer, pH8.0, containing 25% (v/v) glycerol were mixed with 0.01% Bromothymol Blue (which was shown to have no effect on the mobility of pyruvate carboxylase) and applied carefully to the gel surface. The gels were subjected to electrophoresis for 30min at room temperature and 4mA/gel and stained by using Amido Schwarz in 7% (v/v) acetic acid.

Molecular-weight determination

Glycerol gradient centrifugation. Linear gradients from 10 to 25% (v/v) glycerol were prepared in 100mM-triethanolamine hydrochloride–KOH buffer, pH8.0, to a final volume of 5.0ml. In some experiments these gradients contained 0.2mM-acetyl-CoA with or without 5mM-$MgSO_4$ present at a constant concentration throughout the gradient. Samples of pyruvate carboxylase were prepared by gel filtration on Sephadex G-25 into 100mM-triethanolamine hydrochloride–KOH buffer, pH8.0, containing 0.2mM-acetyl-CoA. The gradients were centrifuged on a SW-50L rotor at 45000rev./min and 20°C for 6h on a Beckman-Spinco model L2-50 ultracentrifuge. The four molecular-weight markers described below were run in parallel to the samples containing pyruvate carboxylase, and a standard curve, derived from their activity peaks, was used to determine the molecular weights of the protein and activity peaks of pyruvate carboxylase.

Gel filtration on Sephadex G-200. Molecular-weight estimation by gel filtration on Sephadex G-200 was carried out by the method of Andrews (1970). The

column was equilibrated in 100mM-Tris–HCl buffer, pH 8.0, containing 25% (v/v) glycerol, and in some experiments the buffer also contained 0.1 mM-acetyl-CoA or 10mM-$MgSO_4$. Pyruvate carboxylase was dialysed against the column buffer for several days before use and the column was calibrated by using four molecular-weight markers described by Andrews (1970). The markers (with the molecular weight given in parentheses) were malate dehydrogenase (65000–70000), yeast alcohol dehydrogenase (150000), pyruvate kinase (235000) and β-galactosidase (520000).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

This was performed as described by Weber & Osborn (1969) with one modification. As soon as sodium dodecyl sulphate was added to the protein samples, they were heated at 100°C for 5min to destroy any proteinases (cf. Pringle, 1970).

Assay of biotin

Biotin was assayed by using *Lactobacillus arabinosus* as described by Wright & Skeggs (1944). Pyruvate carboxylase was prepared for the biotin assay by desalting the enzyme into 100mM-potassium phosphate buffer, pH 7.0, followed by hydrolysis in 3M-H_2SO_4 at a pressure of 15lb/in^2 (1.03×10^5 Pa) for 1 h. Results are expressed as the mean ± S.E.M. (9).

Metal content

All glassware was soaked successively in 5% (v/v) Decon 90, water, 5mM-EDTA and deionized water. Pyruvate carboxylase was gel-filtered on Sephadex G-25 into 100mM-potassium phosphate buffer, pH 7.0, containing 2mM-EDTA. The efficiency of the desalting procedure was demonstrated by the removal of more than 98% of NH_4^+ from pyruvate carboxylase on the column. The specific activity of the enzyme was unchanged by passage through the column. Magnesium, zinc and manganese were assayed by using a Unicam SP.90 atomic absorption spectrophotometer. Linear standard curves for all three metals in the concentration range 0–10μM were unaffected by the presence of 10μM of either of the other two metals or by the presence of 2mg of bovine serum albumin/ml. The results are expressed as the mean ± S.E.M. (5).

Amino acid analysis

Purified pyruvate carboxylase (20mg) was dialysed exhaustively against water at 4°C and then freeze-dried. The protein was then incubated in 100mM-Tris–HCl buffer, pH 8.0 containing 8M-urea and 5mM-dithiothreitol in a total volume of 2ml; incubation was carried out at room temperature and under N_2 for 20min. Neutralized iodoacetic acid (recrystallized from *n*-heptane before use) was added to a final concentration of 40mM and incubation was continued as above and in the dark for 1 h. A drop of 2-mercaptoethanol was added to react with excess of alkylating reagent and the protein was dialysed exhaustively against water at 4°C and then freeze-dried. Samples (1mg) of the carboxymethylated protein were hydrolysed in 6M-HCl *in vacuo* at 105°C for 1, 2 and 4 days and the freeze-dried hydrolysates were analysed by using a Technicon amino acid autoanalyser. Tryptophan was determined separately by the method of Edelhoch (1967).

Stoicheiometry

Pyruvate carboxylase (200μg) was added to 100mM-triethanolamine hydrochloride–KOH buffer, pH 8.0, containing 5mM-$MgSO_4$, 1mM-ATP, 15mM-$KHCO_3$ and 0.1mM-acetyl-CoA in a total volume of 2ml. After 45min of incubation at 30°C, 0.1 ml of 1M-HCl was added, followed after 5min by 0.1ml of 1M-KOH. Controls involved the addition of 1M-HCl before the addition of pyruvate carboxylase. The samples and controls were each incubated in triplicate and each was assayed in duplicate. This system was only used to indicate the role of acetyl-CoA in the reaction mechanism, since HCl destroyed the oxaloacetic acid generated by the carboxylation of pyruvate. The synthesis of oxaloacetate was monitored by the disappearance of acetyl-CoA when the above experiment was repeated in the presence of 1 unit of citrate synthase and 0.5mM-acetyl-CoA.

Cation specificity

Additional precautions were taken for these studies to lower the concentration of all univalent cations except $Tris^+$ and triethanolamine$^+$ to a very low value. To do this 100mM-triethanolamine hydrochloride–KOH buffer, pH 8.0, was replaced by 100mM-Tris adjusted to pH 8.0 with CO_2 (oxaloacetate-coupled assay) or by 100mM-triethanolamine adjusted to pH 8.0 with conc. H_3PO_4 (ATP-coupled assay). Samples of pyruvate carboxylase were dialysed exhaustively against these buffers [containing 25% (v/v) glycerol] at room temperature, and all coupling enzymes, stored as the precipitate in $(NH_4)_2SO_4$ solutions, were dialysed exhaustively against these buffers at 4°C. $Tris^+$ was used to replace all other K^+ in the system.

Results

A typical purification is shown in Table 1. From the results in Table 2 and the observation that

Table 1. *Typical purification of pig liver pyruvate carboxylase*

Details of the purification procedure are given in the text and the table represents the purification of the enzyme from 80g of freeze-dried mitochondria.

Stage		Volume (ml)	Total units	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification
Tris–acetate supernatant		720	1980	13400	0.15	100	1.0
Calcium phosphate supernatant		780	1990	11200	0.18	101	1.2
$(NH_4)_2SO_4$ precipitate in sucrose solution		205	1090	840	1.3	55	8.7
Heat-treated mixture		205	840	900	0.93	42	6.2
Calcium phosphate supernatant		288	760	417	1.8	38	12.0
$(NH_4)_2SO_4$ precipitate in 32% solution		45	604	238	2.5	31	16.7
Supernatant from $(NH_4)_2SO_4$ extractions	32%	40	—	54			
	30%	40	4	16			
	28%	40	27	22			
	24%	40	134	48	2.8		
	22%	40	134	35	3.8		
	20%	40	79	18	4.4		
	18%	40	32	12			
Combined supernatants from 24%, 22%, 20% solutions		116	312	97	3.2	16	21.4

approx. 60% of the dry weight of liver is protein, the enzyme has been purified over 250-fold with a 6% yield from fresh liver. On polyacrylamide-gel electrophoresis only one protein band was obtained, despite deliberate overloading, and the mobility of this band was 0.25, compared with the mobility of Bromothymol Blue. It is not, however, sufficient to demonstrate the purity of an enzyme solely on the basis of one detectable protein species. The presence of small quantities of enzymes (particularly those with high specific activities) capable of interfering with a kinetic analysis of the enzyme may not be detected by the methods normally used to demonstrate the homogeneity of the protein. The effective purification of pyruvate carboxylase was primarily achieved by two procedures. First, the isolation of mitochondria removed most of the cytoplasmic protein and most of the lactate dehydrogenase activity. Secondly, the precipitation of pyruvate carboxylase before 31% saturation with $(NH_4)_2SO_4$ removed a large amount of protein and almost all of the kinetically contaminating enzymes; 99% of the lactate dehydrogenase and citrate synthase activities and 99.9% of the malate dehydrogenase activity were removed at this step. Of even greater importance are the activities of kinetically contaminating enzymes in the coupling enzymes themselves, since the activities of the latter are normally two to three orders of magnitude greater than the activity of the enzyme being assayed.

Pig liver pyruvate carboxylase and all of the coupling enzymes were assayed, where appropriate, for the following kinetically contaminating enzymes: acetyl-CoA carboxylase (EC 6.4.1.2), acetyl-CoA deacylase (EC 3.1.2.1), ATPase (EC 3.6.1.3), citrate synthase (EC 4.1.3.7), glucose 6-phosphatase (EC 3.1.3.9), lactate dehydrogenase (EC 1.1.1.27; NADH and NADPH activity), malate dehydrogenase (EC 1.1.1.37; NADH and NADPH activity), NADH oxidase (EC 1.6.99.3), NADPH oxidase (EC 1.6.99.1), oxaloacetate decarboxylase (EC 4.1.1.3), phosphofructokinase (EC 2.7.1.11), phosphoglucose isomerase (EC 5.3.1.9) and pyruvate carboxylase (EC 6.4.1.1). The activity of these enzymes was expressed as a percentage of the lowest activity of pyruvate carboxylase present in the assay system (10μg in the forward reaction and 50μg in the back reaction). The activity of phosphoglucose isomerase in the ATP-coupled assay was very high (20000%), but in the absence of any phosphofructokinase (<0.01%) it would merely serve to buffer the coupled substrate, glucose 6-phosphate. The next highest degree of contamination was that with citrate synthase (present in purified glucose 6-phosphate dehydrogenase), which represented 1.2% of the activity of pyruvate carboxylase in the ATP-coupled assay. A degree of contamination of less than 1% is quite satisfactory for the majority of kinetic studies.

Intracellular location

As shown in Table 2, the distribution of pyruvate carboxylase over the various subcellular fractions closely followed the distribution of citrate synthase;

Table 2. *Intracellular distribution of pig liver pyruvate carboxylase*

Details of the method are given in the text and results are presented as a percentage of the activity in the homogenate: pyruvate carboxylase, 2500 units/kg of fresh liver; citrate synthase, 5000 units/kg of fresh liver.

Source	Pyruvate carboxylase activity	Citrate synthase activity
Homogenate	100	100
Nuclei, erythrocytes, unbroken cells	40	40
Mitochondria	40	45
Supernatant	20	15

this suggests a mitochondrial location for pig liver pyruvate carboxylase. Our results agree with the more definitive studies that have been carried out with rat liver (Böttger *et al.*, 1969; Ballard *et al.*, 1970; Brech *et al.*, 1970), but disagree with the results of Seubert and his co-workers, who were the first to postulate an extra-mitochondrial location for pyruvate carboxylase (Henning & Seubert, 1964). In particular, our results disagree with the existence of an extramitochondrial form of pyruvate carboxylase in pig liver, which has been postulated by Dugal (1972).

U.v. spectrum

Pig liver pyruvate carboxylase in 100mm-potassium phosphate buffer, pH 7.0, exhibited a typical protein spectrum with no significant absorbance above 310nm. The E_{280}/E_{260} ratio of 1.8 was indicative of the absence of nucleic acid and any bound nucleotides.

Molecular-weight determination

A typical protein profile obtained by glycerol gradient centrifugation is shown in Fig. 1(*a*). The profile was not significantly altered by changes in the protein applied to the gradient from 0.3 to 0.8mg of protein or by a change of pH throughout the system from 8.0 to 7.0. The presence of 0.2mm-acetyl-CoA, with or without 5mm-Mg^{2+}, throughout the gradient, also had no significant effect on this

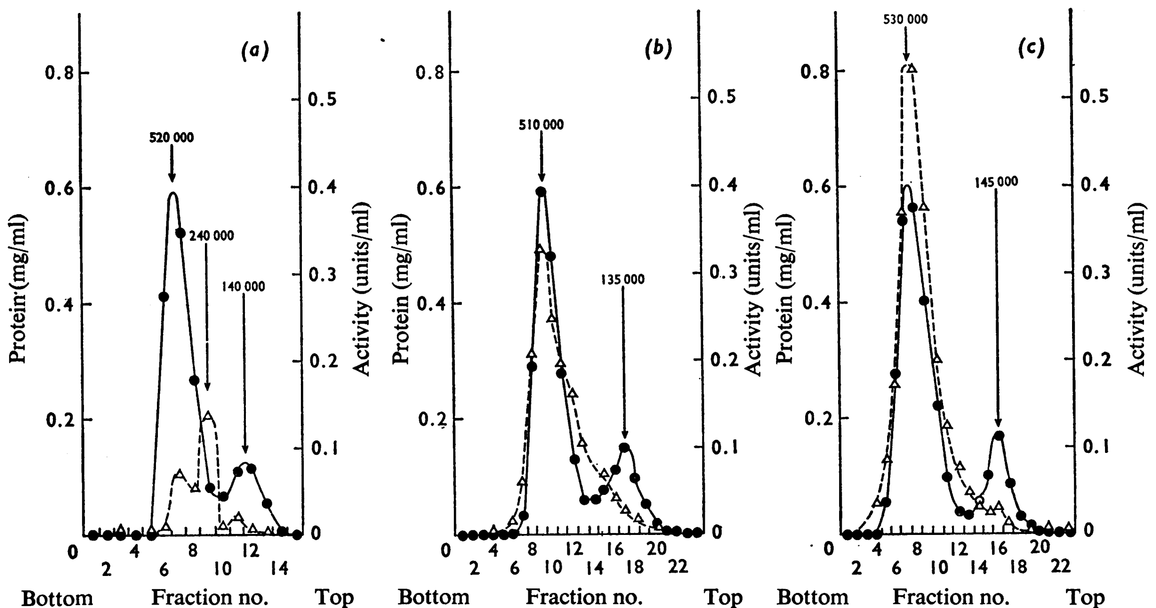

Fig. 1. *Glycerol gradient centrifugation of pig liver pyruvate carboxylase*

Details of the method are given in the text and 0.64mg of protein (specific activity = 2.8 units/mg) was loaded on the gradient in each case. (*a*) No additions to the gradient; (*b*) 0.2mm-acetyl-CoA throughout the gradient; (*c*) 0.2mm-acetyl-CoA and 5mm-Mg^{2+} throughout the gradient. ●, Protein concn. (mg/ml); △, pyruvate carboxylase activity (units/ml). All assays for pyruvate carboxylase were conducted in triplicate and the mean value was taken. The results did not differ by more than 10% from the mean value. The molecular weights of the protein and activity peaks indicated by arrows were derived by using standard molecular-weight markers as described in the text.

profile (Figs. 1*b* and 1*c*). The biotin and metal stoicheiometry (see below) suggested that the major protein peak (Fig. 1*a*) with a molecular weight of 520000 was a tetramer, and the minor protein peak (Fig. 1*a*) with a molecular weight of 140000 was therefore probably the monomer.

In the absence of acetyl-CoA or Mg^{2+} (Fig. 1*a*), three peaks of activity were separated. Two of the peaks of activity corresponded to the protein peaks obtained above, and the third peak of activity occurred at a position which was equivalent to a molecular weight of 240000; this was close to the molecular weight of 260000 that would be predicted for the dimer of pyruvate carboxylase. The overall recovery of activity was only 5–10% and this probably reflected the instability of the tetramer in the absence of acetyl-CoA and Mg^{2+}. The activity of the monomer was not due to the low, kinetically contaminating activity of lactate dehydrogenase, which had approximately the same molecular weight of 130000 (Andrews, 1970); the monomer had no activity in the absence of acetyl-CoA and it was found to contain biotin.

In the presence of acetyl-CoA, with or without Mg^{2+} (Figs. 1*b* and 1*c*) the major peak of activity resided in the tetramer. The dimer was still present, as shown by the 'shoulder' on the protein and activity peak corresponding to the tetramer. However, it was difficult to assess whether any increase in the activity of this fraction had occurred, as a result of the presence of acetyl-CoA, with or without Mg^{2+}. The activity of the monomer appeared to be unchanged and represented less than 1% of the activity of the applied sample. The overall recovery of activity over that of the starting material increased to 25–30% in the presence of acetyl-CoA (Fig. 1*b*) and to 40–50% in the presence of acetyl-CoA and Mg^{2+} (Fig. 1*c*).

Gel-filtration experiments on Sephadex G-200 gave essentially similar results. The protein profile was unchanged by protein concentration (in the range 2–20 mg of applied protein/ml; the protein concentration of the peak tube was some 10% of the applied protein concentration) or by the presence of 0.1 mM-acetyl-CoA or 10 mM-Mg^{2+}. For reasons which are still unclear, the peak of activity always corresponded to the tetramer, even under conditions where very low recoveries of activity were obtained. The dimer was always present as a 'shoulder' on the activity and protein peak of the tetramer, but no activity was ever found in the protein peak corresponding in elution volume to the monomer; this probably reflected the longer duration of the gel-filtration experiments (24 h) compared with the centrifugation experiments (8 h) before the assay of pyruvate carboxylase. In the presence of 0.1 mM-acetyl-CoA or 10 mM-Mg^{2+}, the recovery of activity over the starting material was as a routine 85–95% at all protein concentrations.

Subunit analysis

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of pyruvate carboxylase and the carboxymethylated enzyme resolved a minor (high-molecular-weight) band and a major (low-molecular-weight) band. For pyruvate carboxylase the molecular weight of the minor band was 125000, which presumably reflected the undissociated 'monomer'. The molecular weight of the major band was 47000, which suggested that the 'monomer' of pyruvate carboxylase was composed of three polypeptide chains each with the same molecular weight. The molecular weight of the major band of the carboxymethylated enzyme was 59000 and the molecular weight of the minor band (extrapolated beyond the standard curve) was approx. 180000. This suggests that the increase in molecular weight of the polypeptide chain after carboxymethylation was reflected in the 'monomer' of pyruvate carboxylase. An increase in molecular weight after carboxymethylation has also been noted by Gibbons (1970) for aldolase.

The presence of the undissociated 'monomer' of pyruvate carboxylase suggests that it is fairly resistant to sodium dodecyl sulphate; similar observations have been noted for the enzyme purified from rat liver (McClure *et al.*, 1971*a*) and sheep kidney (R. Bais & D. B. Keech, unpublished work, quoted by Scrutton & Young, 1972).

Biotin content

Pig liver pyruvate carboxylase was inhibited by avidin, as shown in Fig. 2 indicating that biotin was essential for the activity of the enzyme. For every

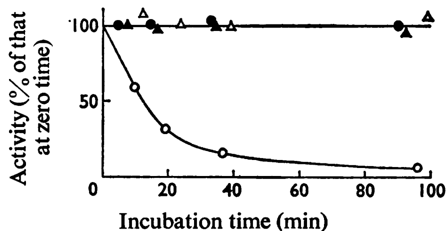

Fig. 2. *Effect of avidin on the activity of pig liver pyruvate carboxylase*

Pyruvate carboxylase (specific activity 2.6 units/mg) was incubated at 22°C in 100 mM-triethanolamine hydrochloride–KOH buffer, pH 8.0, containing 25% (v/v) glycerol at a final concentration of 2.0 mg of protein/ml. The incubation had the following additions: ●, none; ▲, 1.0 mg of biotin/ml; ○, 1.0 mg of avidin/ml; △, 1.0 mg of avidin–(biotin)$_4$ complex/ml, prepared by preincubating 1 mg of avidin and 1 mg of biotin for 5 min in a small volume of 100 mM-triethanolamine hydrochloride–KOH buffer, pH 8.0.

520000g of pyruvate carboxylase there were 3.8±0.4mol of biotin, and this suggested that the enzyme was a tetramer.

Metal content

For every 520000g of pyruvate carboxylase there were 2.7±0.07mol of zinc, 1.08±0.05mol of magnesium and 0.30±0.10mol of manganese. Thus pig liver pyruvate carboxylase appears to be a magnesium–zinc metalloenzyme and is the first mammalian enzyme that has been shown to contain no manganese. The method of analysis does not, however, distinguish between a homogeneous population of tetramers each of which contains 3 molecules of zinc and 1 molecule of magnesium and a heterogeneous population of tetramers in which one-quarter of the tetramers contain magnesium and three-quarters contain zinc.

Amino acid analysis

The amino acid analysis is given in Table 3 and shows a slight excess of polar over non-polar amino acids. The results are quite similar to those obtained for chicken liver pyruvate carboxylase (Scrutton & Utter, 1965).

Table 3. *Amino acid composition of pig liver pyruvate carboxylase*

The results are presented as mol of amino acid/130000g of pyruvate carboxylase, and are corrected for the time-dependent destruction of serine and threonine. No attempt was made to calculate the amide content of the protein.

Amino acid	Content (mol/130000g of enzyme)
Cystine	13
Aspartic acid	94
Threonine	55
Serine	65
Glutamic acid	107
Proline	58
Glycine	88
Alanine	82
Valine	66
Methionine	16
Isoleucine	57
Leucine	70
Tyrosine	29
Phenylalanine	42
Lysine	63
Tryptophan	14
Histidine	29
Arginine	54

Stability

Purified pig liver pyruvate carboxylase is a very unstable enzyme. In 100mM-triethanolamine hydrochloride–KOH buffer, pH8.0, the enzyme was inactivated at 22°C in an apparently first-order reaction with a half-life of 10min. Acetyl-CoA at a final concentration of 0.1mM was completely effective at stabilizing the enzyme in this buffer, whereas 25% (v/v) glycerol or 5mM-Mg^{2+} were only partially

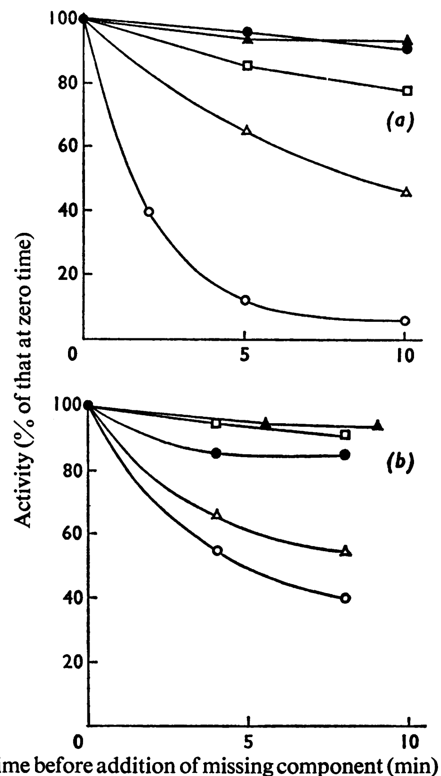

Fig. 3. *Stability of pyruvate carboxylase in the assay medium*

Pyruvate carboxylase was incubated at 30°C for various times in the assay medium in the absence of one assay component and the residual activity was determined by the addition of the missing component. (*a*) Forward reaction. The final concentration of pyruvate carboxylase (specific activity = 2.7 units/mg) in the oxaloacetate-coupled assay was 20μg/ml and the missing components were: ○, 0.1mM-acetyl-CoA; △, 5mM-Mg^{2+}; □, 5mM-pyruvate; ●, 1mM-ATP; ▲, 50mM-K^+. (*b*) Back reaction. The final concentration of pyruvate carboxylase (specific activity = 2.6 units/mg) in the ATP-coupled assay was 100μg/ml and the missing components were: ○, 0.1mM-acetyl-CoA; △, 3.35mM-Mg^{2+}; □, 1mM-oxaloacetate; ●, 0.5mM-ADP; ▲, 50mM-K^+.

Table 4. *Stoicheiometry of the pyruvate carboxylase reaction*

Details of the method are given in the text and the results are expressed as the mean ± S.E.M. (6).

Pyruvate	Oxaloacetate	ATP	ADP	Acetyl-CoA
−0.41 ± 0.0061	+0.43 ± 0.0025	−0.34 ± 0.014	+0.39 ± 0.0095	−0.00022 ± 0.0037

effective. Pyruvate, ATP^{4-}, $MgATP^{2-}$ and HCO_3^-, the concentrations normally present in the oxaloacetate-coupled assay, were completely ineffective in stabilizing the enzyme activity. When the enzyme was added to the assay medium in the absence of one of the reaction components the stability depended on the presence of acetyl-CoA and Mg^{2+} (Figs. 3*a* and 3*b*). Kinetic analyses at sub-saturating concentrations of either or both of these effectors yielded very curved rate-plots, and the only successful means of ameliorating this curvature was to increase the concentration of pyruvate carboxylase in the assay medium. This probably explains the greater stability of the enzyme in the back reaction in the absence of acetyl-CoA or Mg^{2+} (Fig. 3*b*). The instability of pig liver pyruvate carboxylase under the conditions of assay validates the analysis of the coupled assay systems; such an analysis not only conserves expensive coupling enzymes and lowers the concentration of kinetically contaminating enzymes, introduced by the coupling enzymes, but also allows one to control the coupling time so that meaningful initial rates are measured. Moreover, the relatively long coupling time for the ATP-coupled assay was compatible with the higher concentrations of pyruvate carboxylase that were used in this system as a routine.

Stoicheiometry

Before any valid kinetic analysis can be carried out it is essential to verify the stoicheiometry of the reaction in question. The results shown in Table 4 suggest that 1 mol of pyruvate is converted into 1 mol of oxaloacetate with the concomitant conversion of 1 mol of ATP into 1 mol of ADP. The lack of any significant change in the concentration of acetyl-CoA suggests that it is not used as a substrate in the reaction. The results agree with those obtained in studies of pyruvate carboxylase from chicken liver (Utter & Keech, 1963) and yeast (Cazzulo & Stoppani, 1967).

pH–activity curve

Pig liver pyruvate carboxylase was maximally active ($V_{max.}$) at pH 8.0 (Fig. 4). Since three of the reaction components existed predominantly in the form utilized by the enzyme at this pH ($MgATP^{2-}$, HCO_3^- and HPO_4^{2-}), pH 8.0 was chosen for most of the studies on this enzyme.

Cation specificity

In the absence of any univalent cation other than $Tris^+$ the rate of reaction in the forward direction was approx. 7% of that obtained in the presence of saturating concentrations of K^+. This blank rate was unaffected by variations in the concentration of $Tris^+$ from 5 to 100 mM and disappeared when the pH of the assay medium was lowered to 7.5. Thus pig liver pyruvate carboxylase, unlike the enzyme from chicken liver (Scrutton, 1971) but similar to that purified from rat liver (McClure *et al.*, 1971*a*), was not activated by $Tris^+$. In the back reaction the blank rate was less than 1% of the maximum velocity for activation by K^+, suggesting that triethanolamine$^+$ was similarly non-activating. For both sets of experiments the blank rates were substracted from all reaction rates and no attempt was made to compensate for changes in I caused by the addition of univalent cations. Table 5 clearly shows that K^+, NH_4^+, Rb^+ and Cs^+ all activated the enzyme in the forward reaction with low K_m values and high V values, whereas the converse was true for Li^+ and Na^+. The K_m values obtained (0.6–1.2 mM, excluding those for Na^+ and Li^+) were lower than those obtained for pyruvate carboxylase from rat liver (1.3–2.3 mM; McClure *et al.*, 1971*a*),

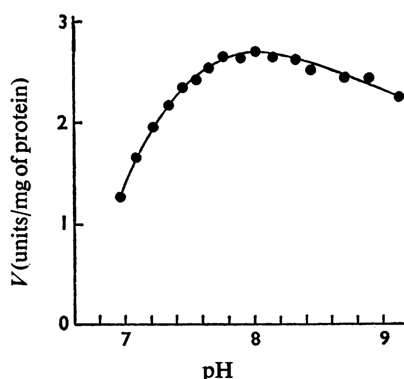

Fig. 4. *pH–activity curve of pig liver pyruvate carboxylase*

Pyruvate carboxylase (12.5 μg; specific activity 2.7 units/mg) was added to the oxaloacetate-coupled assay; the pH of the assay mixture was adjusted with 1 M-KOH and was unaffected by the presence of the enzyme. The use of triethanolamine hydrochloride buffer in this context was advantageous, since the addition of 1 M-KOH had no effect on the ionic strength of the buffer.

Table 5. *Univalent cation specificity of pyruvate carboxylase in the forward and back reactions*

Details of the method are given in the text. K_m^f and K_m^b are the K_m values for the foward and back reactions respectively. $V_{\%}^f$ and $V_{\%}^b$ represent the V for a particular cation as a percentage of the V for activation by K^+ in the forward and back reactions respectively. ND, not done because of precipitation.

Cation	Forward reaction		Back reaction		K_m^f/K_m^b
	K_m^f (mM)	$V_{\%}^f$	K_m^b (mM)	$V_{\%}^b$	
Li^+	34.5	18	—	—	
Na^+	23.5	40	—	—	
K^+	1.14	100	20.0	100	17.5
NH_4^+	0.92	114	13.3	88	14.4
Rb^+	0.66	117	9.3	103	14.1
Cs^+	1.10	101	ND	ND	

chicken liver and yeast (10–30mM; Scrutton & Young 1972). In the back reaction, K^+, NH_4^+ and Rb^+ activated pyruvate carboxylase with K_m values that were 14–18-fold higher than those for the forward reaction; this not only suggests that all the univalent cations activate the enzyme at the same site, but also suggests a reason for the lack of activation of pyruvate carboxylase in the back reaction by Na^+ and Li^+. The predicted K_m value for Li^+ and Na^+ in the back reaction would be in excess of 400mM; since inhibition of the reaction by high I sets in at approx. 100mM in the back reaction (see Fig. 6), Na^+ and Li^+ would start to activate the enzyme significantly just as inhibition set in. This could also explain the apparent inhibitory effect of Li^+ on pyruvate carboxylase from chicken liver (Scrutton & Young, 1972) and the inhibitory effect of Na^+ and Li^+ in the present of K^+ on the enzyme from yeast (Cazzulo & Stoppani, 1967) and *A. niger* (Feir & Suzuki, 1969).

The effectiveness of the cation is not solely dependent on the ionic volume, as shown in Fig. 5, since V increased linearly with ionic volume only for Na^+, Li^+ and K^+. Moreover, the non-linearity of the plot of the apparent first-order rate constant (V/K_m) against ionic volume suggests that the anionic field strength is also important (see Eisenman, 1961). High concentrations of K^+ inhibit pyruvate carboxylase in the forward and back reactions (Fig. 6). In contrast with the results of McClure *et al.* (1971*a*), inhibition does not become apparent at the same concentration of K^+ in the forward and back reactions, even though the I of the starting assay media

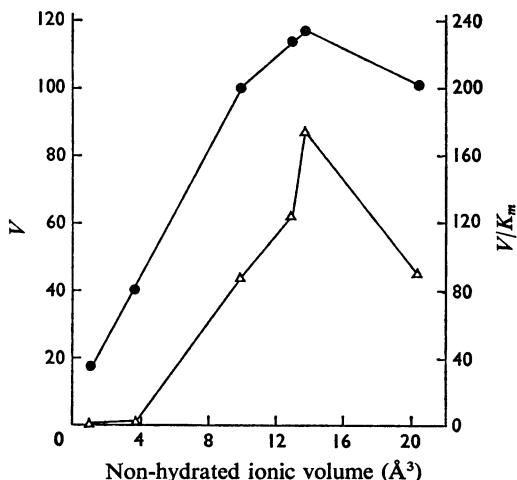

Fig. 5. *Ionic volume of activating univalent cations as a function of V and V/K_m*

The experimental points were derived from the results presented in Table 5 for the forward reaction and the V values were equivalent to $V_{\%}^f$. ●, V; △, V/K_m.

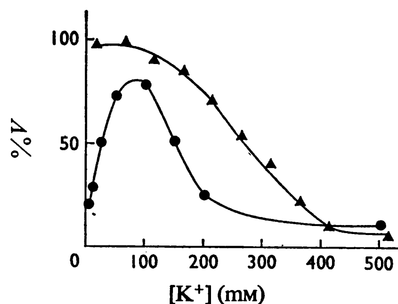

Fig. 6. *Inhibition of pig liver pyruvate carboxylase by univalent cations*

The I of the ATP- and oxaloacetate-coupled assays in the absence of added K^+ was approx. 60mM. The V for the forward and back reactions was determined from reciprocal plots (Lineweaver & Burk, 1934) at non-inhibitory concentrations of K^+. ▲, forward reaction; ●, back reaction.

were approximately the same. The reason for this difference is still unclear, and the situation is complicated by the fact that high I, brought about by raising the concentration of triethanolamine$^+$, inhibited the enzyme at non-inhibitory concentrations of K^+.

Activation by acetyl-CoA

As with all mammalian and avian enzymes examined so far, pig liver pyruvate carboxylase is dependent on the presence of acetyl-CoA for activity. In the absence of added acetyl-CoA, the initial rate was approx. 2% of the maximum forward rate, which was well above the activity (0.3%) of contaminating lactate dehydrogenase present in pure preparations of this enzyme. Similar observations have been noted for pyruvate carboxylase purified from rat liver (McClure *et al.*, 1971*a*; Scrutton & White, 1972) and sheep kidney (Ashman *et al.*, 1972). In these cases pyruvate carboxylase was preincubated with citrate synthase and oxaloacetate to remove any available enzyme-bound acetyl-CoA. However, pyruvate carboxylase is not activated solely by acetyl-CoA (see Scrutton & Young, 1972). Pig liver pyruvate carboxylase was therefore incubated with excess of carnitine acetyltransferase and (−)-carnitine to convert any available acyl-CoA into a non-activating/non-inhibitory acyl-carnitine; the acetyl-CoA-independent rate of reaction was not affected by this procedure. Acetyl-CoA appears to be a true allosteric effector of pyruvate carboxylase rather than a catalytic component in the reaction mechanism.

At pH8 and 30°C the dissociation constant for enzyme-bound acetyl-CoA in the forward and back reactions was 22μM, and the Hill coefficient (n) was 2.1 and 2.5 respectively. If purified acetyl-CoA was used the dissociation constant in the forward reaction was considerably lower (5.7μM) and n was 2.6. This suggests that the impurities normally present in acetyl-CoA are very inhibitory, and results obtained by using such preparations of acetyl-CoA may be very misleading when applied to physiological systems. As shown in Table 6, K_s, V and n are markedly affected by pH, with the dissociation constant increasing fourfold from pH 8.0 to 7.0.

Table 6. *Characteristics of activation by acetyl-CoA as a function of pH*

Pyruvate carboxylase (10μg; specific activity 2.7 units/mg) was added to the oxaloacetate-coupled assay, the pH of which had been adjusted with 1M-KOH. V is expressed as a percentage of the maximum velocity at pH 8.0; K_s^{AcCoA} is the dissociation constant for enzyme-bound acetyl-CoA; n is the Hill coefficient for the activation of pyruvate carboxylase by acetyl-CoA.

pH	V	K_s^{AcCoA} (μM)	n
7.0	23	23	1.7
7.5	78	10.5	2.5
8.0	100	5.7	2.6
8.5	98	4.5	2.8

Discussion

Unlike other biotin carboxylases (e.g. acetyl-CoA carboxylase; see Moss & Lane, 1971), pyruvate carboxylases from a number of higher organisms exhibit remarkably similar physical properties; 130000g of pig liver pyruvate carboxylase contains 1mol of biotin and 1mol of metal, which is in very good agreement with results obtained for pyruvate carboxylase from chicken liver (Scrutton *et al.*, 1966), turkey and calf liver (Scrutton *et al.*, 1972), rat liver (McClure *et al.*, 1971*a*) and yeast (Scrutton *et al.*, 1970). This, together with other evidence in the present paper, suggests that pig liver pyruvate carboxylase is homogeneous, and yet the specific activity is rarely greater than 3 units/mg, a value that is approximately an order of magnitude lower than that obtained for most other purified pyruvate carboxylases. However, Seufert *et al.* (1971) have purified rat liver pyruvate carboxylase to homogeneity by using a purification procedure that actually caused a substantial fall in the specific activity to a value of 2.8–4.0 units/mg. Since the glycerol gradient centrifugation and Sephadex G-200 experiments described above suggest that catalytic inactivation is not accompanied by gross changes in the aggregation state of the enzyme, the low final specific activity may reflect the presence of inactive tetramers in pure preparations of the pig liver enzyme.

The separated monomer, dimer and tetramer of pig liver pyruvate carboxylase have been shown to exhibit activity, although the form of the enzyme during the assay was unknown. Since acetyl-CoA and Mg^{2+} not only stabilized the activity of the tetramer separated on glycerol gradients (Figs. 1*b* and 1*c*), but also the activity of the enzyme in the cuvette (Figs. 3*a* and 3*b*), the tetramer of pyruvate carboxylase appears to be the form which is normally assayed. The allosteric interaction of acetyl-CoA with pyruvate carboxylase could be effected by changes in the oligomeric state of the protein. This does not appear to be true for the pig liver enzyme, because acetyl-CoA does not affect the aggregation state of the protein (Figs. 1*a*, 1*b* and 1*c*) and plots of reaction rate against enzyme concentration are linear at all concentrations of acetyl-CoA. This agrees with published results for the chicken liver and yeast enzymes (Scrutton & Utter, 1967; Taylor *et al.*, 1972). The physiological function of the monomer and dimer of pyruvate carboxylase is unclear.

The metal content of pyruvate carboxylase is markedly species-dependent (Scrutton *et al.*, 1972) and the pig liver enzyme is no exception. It is, however, the first mammalian enzyme that has been shown to contain no bound manganese.

Finally, the 'monomer' of pig liver pyruvate carboxylase appears to contain three polypeptide chains, each with a molecular weight of 47000. This situation is reminiscent of the three functional subunits of *Escherichia coli* acetyl-CoA carboxylase [see Alberts & Vagelos (1972) for a review], although the molecular weights of individual polypeptide chains are very different in this case. Nevertheless, the three polypeptide chains of the 'monomer' of pig liver pyruvate carboxylase must at least be dissimilar with respect to their biotin and (possibly) their metal content. Three functional subunits may indeed be a common feature of all biotin carboxylases.

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