Phosphoenolpyruvate Carboxykinase of Kidney

SUBCELLULAR DISTRIBUTION AND RESPONSE TO ACID-BASE CHANGES

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1. A method for the assay of phosphoenolpyruvate carboxykinase is presented, based on the enzymic determination of the phosphoenolpyruvate produced by the enzyme reaction. 2. The subcellular distribution of phosphoenolpyruvate carboxykinase in the kidney of several animal species resembled the distribution in the liver. 3. The rise in enzyme activity in the kidney cortex of rats made acidotic by feeding with ammonium chloride was not prevented by administration of ethionine or actinomycin. 4. The possibility is suggested that in the kidney acidosis causes activation of an inactive form of the enzyme already present.

It has now been well established that in the rat metabolic acidosis produces an increase in the gluconeogenic capacity of kidney cortex (Goodman, Fuisz & Cahill, 1966; Goorno, Rector & Seldin, 1967; Kamm, Fuisz, Goodman & Cahill, 1967; Alleyne, 1970). Studies from this laboratory have described the changes in metabolic intermediates accompanying this increased gluconeogenic capacity and have also shown that there is a rapid increase in the activity of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase [GTP-oxaloacetate carboxy-lyase (transphosphorylating); EC 4.1.1.32] (Alleyne & Scullard, 1969; Alleyne, 1970). The gluconeogenesis induced by acidosis differed from that observed in starvation or during steroid treatment in that it was found to be confined to the kidney and no increase of other gluconeogenic enzymes, glucose 6-phosphatase or fructose 1,6-diphosphatase, could be demonstrated (Alleyne & Scullard, 1969; G. A. O. Alleyne & G. H. Scullard, unpublished work). Acidosis also enhanced the steroid-induced rise in the activity of phosphoenolpyruvate carboxykinase, but this effect was not observed with glucose 6-phosphatase (Alleyne & Scullard, 1969). During steroid treatment increases in RNA and protein synthesis have been shown to accompany the increases in activity of the key gluconeogenic enzymes in liver (Weber, 1963), but in the kidney cortex from the acutely acidotic rat RNA synthesis has been found to be actually depressed (Alleyne, 1970). These pieces of evidence suggest that the mechanisms by which acidosis and steroids increase renal phosphoenolpyruvate carboxykinase activity are different.

Results are presented below to support this

hypothesis. A method of assay of phosphoenolpyruvate carboxykinase suitable for crude preparations of the enzyme and information on species variation in the subcellular distribution of this enzyme in the kidney are also presented.

MATERIALS AND METHODS

White albino rats of 150–250 g body wt. fed on a commercial diet (Purina Lab Chow; Ralston Purina Co., St Louis, Mo., U.S.A.) were used. The rabbits, guinea pigs and dogs were from local sources. The specimen of human kidney was obtained when a kidney was removed from a 38-year-old man because it contained a solitary renal-cell carcinoma at the lower pole. There was no evidence of spread of the tumour to the upper pole, from which our samples were taken.

Production of acidosis. Metabolic acidosis was produced by infusion of NH_4Cl as previously described (Goodman *et al.* 1966; Alleyne & Scullard, 1969). The duration of acidosis was 24 h in the studies with actinomycin and 12 h in studies with ethionine.

Drugs and doses. Ethionine and actinomycin D (Sigma Chemical Co., St Louis, Mo., U.S.A.) were dissolved in 0.9% NaCl and injected intraperitoneally in doses of 50 mg and 6.25 $\mu\text{g}/100\text{ g}$ body wt. respectively each 12 h. Triamcinolone acetonide (E. R. Squibb and Sons Ltd., Montreal, Canada) was injected intramuscularly in doses of 2.5 mg/rat daily for 2 days. The injections of actinomycin D and ethionine were given $\frac{1}{2}$ h before any other treatment. Control animals received injections of 0.9% NaCl in equal volumes.

Handling of samples. Rabbits were killed by a blow on the head. All other animals were anaesthetized with 40 mg of sodium pentobarbital/kg body wt. Blood was withdrawn from the abdominal aorta into heparinized syringes; the kidneys were rapidly removed and slices of cortex taken and homogenized in 10 vol. of 0.25 M-sucrose.

Homogenates were centrifuged for 1 h at 100 000 *g* at 0°C in an MSE SS65 preparative ultracentrifuge. When required, mitochondria were isolated from the homogenates as described by Gevers (1967). This preparation was sonicated for 30 s at 20 000 Hz in an ultrasonic disintegrator before the enzyme assay.

Assays. Protein was measured in supernatants by the method of Lowry, Rosebrough, Farr & Randall (1951). Liver triglycerides were measured as described by Carlson (1963). Blood pH was measured at 38°C in a micro-Astrup assembly (Radiometer, Copenhagen, Denmark). Phosphoenolpyruvate carboxykinase was assayed in the same medium as described by Nordlie & Lardy (1963) but the phosphoenolpyruvate formed as a result of the reaction was measured enzymically. The assay medium contained, in a final volume of 1 ml: 100 μ mol of tris-HCl buffer, pH 7.5; 13 μ mol of NaF; 15 μ mol of $MgSO_4$; 6 μ mol of ITP; 2 μ mol of GSH and 5 μ mol of the tris salt of oxaloacetic acid. The last three solutions were prepared fresh for each set of assays. This mixture was preincubated for 3 min at 37°C, after which 0.02–0.2 unit of phosphoenolpyruvate carboxykinase was added in 0.1 ml (one unit is the amount of enzyme which catalyses the decarboxylation of 1 μ mol of oxaloacetate/min). The reaction was stopped after 3 min with 1 ml of ice-cold 6% $HClO_4$. Then 5 M- K_2CO_3 was added to neutralize the $HClO_4$ with Methyl Orange as an internal indicator; the precipitate was discarded by centrifugation and the phosphoenolpyruvate in the supernatant was assayed in a medium containing, in 1 ml: 40 μ mol of triethanolamine-HCl buffer, pH 7.6; 60 μ mol of KCl; 6 μ mol of $MgSO_4$; 0.2 μ mol of ADP; 1.33 μ mol of NADH; 2.5 units of malate dehydrogenase; 2.5 units of lactate dehydrogenase and 0.5 ml of supernatant. After a constant E_{340} value had been obtained, 2 units of pyruvate kinase was added in a negligible volume. The phosphoenolpyruvate concentration in the incubation medium was calculated from the decrease in E_{340} produced by the addition of pyruvate kinase.

All the assays reported here were done in a Unicam SP.8000 recording spectrophotometer (Pye Unicam, Cambridge, U.K.).

Chemicals. All chemicals were AnalaR grade. Ethionine, actinomycin D, oxaloacetate, glutathione and all nucleotides and enzymes except malate dehydrogenase were obtained from Sigma Chemical Co. Malate dehydrogenase was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. It is essential that malate dehydrogenase and lactate dehydrogenase are free of pyruvate kinase activity.

RESULTS

Assay of phosphoenolpyruvate carboxykinase. The major advantage in the enzyme assay proposed here is the specificity of the determination of the enzymically produced phosphoenolpyruvate. In the method described by Nordlie & Lardy (1963) this was measured by determining the P_i produced by exposure of the phosphoenolpyruvate to Hg^{2+} ions. This method gives very high blank values owing to the considerable amount of P_i formed from the excess of ITP present. In the present method it is necessary

Fig. 1. Time-course of the phosphoenolpyruvate carboxykinase-catalysed decarboxylation of oxaloacetate. The enzyme was assayed as described in the Materials and Methods section with 0.46 mg of soluble protein from rat kidney. The reaction was stopped at the times indicated.

Fig. 2. Dependence of production of phosphoenolpyruvate on supernatant protein concentration. Phosphoenolpyruvate carboxykinase was assayed as described in the Materials and Methods section in different dilutions of a supernatant from rat kidney homogenate.

to remove the excess of oxaloacetate, which interferes with the phosphoenolpyruvate assay, owing to its spontaneous decarboxylation to pyruvate. The enzymic removal of oxaloacetate with malate dehydrogenase and NADH is the main difference between this assay and that proposed by Seubert & Huth (1965), in which the excess of oxaloacetate is reduced with potassium borohydride.

Figs. 1 and 2 show the linearity of the assay with

Fig. 3. Dependence of the phosphoenolpyruvate carboxykinase-catalysed production of phosphoenolpyruvate on oxaloacetate concentration. The different concentrations of substrate were obtained by dilution of a 10 mM solution of the tris salt of oxaloacetate (pH 7.5) in 0.2 M-tris-HCl buffer, pH 7.5. The assay was carried out as described in the Materials and Methods section but the concentration of NADH used for the determination of the phosphoenolpyruvate produced was adjusted according to the oxaloacetate concentration. The samples of protein-free supernatants used to determine phosphoenolpyruvate were also adjusted to obtain a ΔE_{340} between 0.090 and 0.460.

time and enzyme concentration respectively. The reproducibility of duplicate assays was $5.3 \pm 0.7\%$ throughout all the experiments reported here.

Fig. 3 shows that the enzyme from rat kidney cortex follows standard Michaelis-Menten kinetics with respect to oxaloacetate concentration. For this study it was ensured that under the conditions of the assay oxaloacetate in the medium was stable for at least 13 min at 37°C.

The effect of pH on phosphoenolpyruvate carboxykinase activity was studied between pH 5.7 and 7.9. In this range there was no significant change in enzyme activity.

It was important to ensure that the phosphoenolpyruvate formed enzymically was not degraded during the assay by other enzymes present in the supernatant. Recovery experiments were done in which 0.03–0.18 μmol of phosphoenolpyruvate was added to the incubation mixture 10 s before the addition of the supernatants. The recovery of the added phosphoenolpyruvate was $97.5 \pm 4.3\%$ (mean $\pm$ S.D.)

Extraction of kidney cortex in 0.25 M-sucrose and 0.14 M-potassium chloride gave values for phosphoenolpyruvate carboxykinase activity that agreed within 0.7%.

Subcellular distribution of phosphoenolpyruvate carboxykinase in kidney. Table 1 shows the pattern

Table 1. *Subcellular distribution of kidney phosphoenolpyruvate carboxykinase*

The values shown are the mean of two to four determinations.

	Enzyme activity (units/g of kidney cortex)	
	Mitochondria	Soluble fraction
Rat	0.14	11.2
Man	0.25	8.0
Dog	0.48	2.6
Guinea pig	0.99	3.8
Rabbit	1.55	4.0
Human kidney tumour	0.05	0.3

of subcellular distribution in the kidneys from five animal species. In man and rat the enzyme is almost entirely cytoplasmic, whereas in guinea pig and rabbit there is considerable activity in the mitochondrial fraction. The dog is in an intermediate position and in the human kidney tumour there was virtually no enzyme activity in either fraction.

Effect of actinomycin D and ethionine on the increase in phosphoenolpyruvate carboxykinase activity produced by acidosis. Table 2 shows that the administration of actinomycin D had no effect on the enzyme activity in the control rats and did not suppress the increase in activity produced by acidosis. The values for blood pH indicate that the drug did not contribute to acid-base changes. The same results were obtained with doses of actinomycin of up to 50 $\mu\text{g}/100\text{g}$ body wt. With this high dose the animals appeared very ill and mortality was high. The injection of actinomycin completely inhibited the rise in phosphoenolpyruvate carboxykinase activity produced by steroids, as shown in Table 3. Thus the increase in enzyme activity produced by triamcinolone in kidney and liver may be due to a similar mechanism, dependent on protein synthesis (Shrago, Lardy, Nordlie & Foster, 1963; Lardy, Foster, Shrago & Ray, 1964).

The effects of ethionine administration are shown in Table 4. This inhibitor also failed to prevent the increase in enzyme activity produced by acidosis, but during the period of study the rats developed a fatty liver, as shown by the increase in the concentration of liver triglycerides. It is not clear why in this group of rats there was such a high enzyme activity in the control animals. The duration of acidosis here was only 12 h, hence there is a smaller increase in enzyme activity than that shown in Table 3 where the rats were acidotic for 24 h (Alleyne & Scullard, 1969).

Stability of the supernatant phosphoenolpyruvate carboxykinase activity. No differences in the stability of the enzyme activity in supernatants from control

Table 2. *Effect of actinomycin D on the acid-induced rise in rat kidney-cortex phosphoenolpyruvate carboxykinase*

The rats were made acidotic for 24 h by intragastric feeding with NH_4Cl . Actinomycin D ($6.25 \mu\text{g}/100 \text{g}$ body wt.) was injected intraperitoneally every 12 h, 30 min before the NH_4Cl . Control rats received 0.9% NaCl. Values shown are means $\pm$ s.d. for the numbers of animals given in parentheses. *P* refers to the difference from control values, calculated by using Student's '*t*' test corrected for unequal variances where necessary. NS, not significant.

	Enzyme activity (units/g of protein)	<i>P</i>	Blood pH
Control (6)	106.4 ± 13.4	—	7.38 ± 0.04
Actinomycin (5)	99.2 ± 9.7	NS	7.39 ± 0.13
Acidotic (6)	239.5 ± 27.2	<0.001	7.17 ± 0.19
Acidotic + actinomycin (9)	225.3 ± 31.1	<0.001	7.20 ± 0.16

Table 3. *Effect of actinomycin D on the steroid-induced rise in rat kidney-cortex phosphoenolpyruvate carboxykinase*

Triamcinolone was given intramuscularly in doses of 2.5 mg/rat daily, and actinomycin D ($6.25 \mu\text{g}/100 \text{g}$ body wt.) was injected every 12 h. The rats were killed at 48 h. Expression of results is as in Table 2.

	Enzyme activity (units/g of protein)	<i>P</i>
Controls (6)	138.0 ± 24.9	—
Triamcinolone (4)	227.2 ± 11.2	<0.05
Triamcinolone + actinomycin (4)	156.0 ± 24.7	NS

Table 4. *Effect of ethionine on the acid-induced rise in rat kidney-cortex phosphoenolpyruvate carboxykinase*

The rats were made acidotic for 12 h by intragastric feeding with NH_4Cl . Ethionine ($50 \text{mg}/100 \text{g}$ body wt.) was injected intraperitoneally. The results are expressed as in Table 2.

	Enzyme activity (units/g of protein)	<i>P</i>	Liver triglycerides (mg/g of liver)	Blood pH
Control (5)	181.0 ± 34.2	—	7.8 ± 0.2	7.39 ± 0.04
Ethionine (3)	199.7 ± 19.3	NS	39.5 ± 0.9	7.37 ± 0.05
Acidotic (7)	259.0 ± 23.8	<0.02	9.8 ± 0.2	7.21 ± 0.07
Acidotic + ethionine (8)	316.9 ± 73.7	<0.005	41.4 ± 3.7	7.19 ± 0.09

and acidotic rats were found. In both cases the activity was stable for 13 min at 37°C and for at least 12 h at 0°C .

Mixing of supernatants. Since it was considered possible that acidosis could produce changes in the concentration of an activator or inhibitor of phosphoenolpyruvate carboxykinase in the kidney cortex that would be apparent even at the dilution used for the assay, the enzyme was assayed separately and in mixtures of supernatants from acidotic and control rats. The activity in the mixtures was that predicted from the original supernatants.

DISCUSSION

The present study shows that phosphoenolpyruvate carboxykinase in kidney of several species varies in its subcellular distribution in a manner

similar to that described for the liver (Nordlie & Lardy, 1963). The significance of the mitochondrial activity in the guinea pig and rabbit is not clear and it will be of interest to determine the effects of stimuli such as acidosis on both fractions. Ballard & Hanson (1969) have shown immunological differences between the soluble and mitochondrial enzymes in rat liver, and Nordlie & Lardy (1963) have shown that only the cytoplasmic form is inducible by steroids or starvation.

Alleyne & Scullard (1969) suggested that there would be significant phosphoenolpyruvate carboxykinase activity in rat kidney mitochondria, an error that could probably be attributed to the enzyme assay method then used.

From previous studies it was concluded that an increase in gluconeogenesis was an early and rapid response to acidosis and that the key enzyme

involved was phosphoenolpyruvate carboxykinase (Alleyne, 1968). An increase in enzyme activity was indeed demonstrated as early as 4h after the intra-gastric administration of ammonium chloride (Alleyne & Scullard, 1969). At this time overall synthesis of RNA appeared depressed (Alleyne, 1970). This provided indirect evidence that protein synthesis was not involved in the acid-induced rise in enzyme activity. The present experiments in which two inhibitors of protein synthesis failed to block the increase of this enzyme activity support this view and make it highly probable that the effect of acidosis is on the activation of a form of the enzyme already present. The involvement of a soluble factor that could cause reversible changes in the activity of the enzyme is made unlikely by the experiments in which the enzyme activity was measured in mixed supernatants from acidotic and control rats.

In view of the close association between renal gluconeogenesis and ammonia production in the acidotic rat (Goodman *et al.* 1966; Alleyne, 1970) it is notable that Goldstein (1965) was not able to suppress the increase in ammonia production of acidotic rats by administration of actinomycin D. Similarly, renal-cortex slices from acidotic rats treated with actinomycin D still show an increased gluconeogenic capacity (G. A. O. Alleyne, unpublished work).

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