Phosphoenolpyruvate Carboxykinase and Pyruvate Carboxylase in Developing Rat Liver

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(Received 6 January 1967)

1. Phosphoenolpyruvate carboxykinase and pyruvate carboxylase were measured in foetal, newborn and adult rat liver extracts by a radiochemical assay involving the fixation of [^{14}C]bicarbonate. 2. Pyruvate-carboxylase activity in both foetal and adult liver occurs mainly in mitochondrial and nuclear fractions, with about 10% of the activity in the cytoplasm. 3. Similar studies of the intracellular distribution of phosphoenolpyruvate carboxykinase show that more than 90% of the activity is in the cytoplasm. However, in the 17-day foetal liver about 90% of the activity is in mitochondria and nuclei. 4. Pyruvate-carboxylase activity in both particulate and soluble fractions is very low in the 17-day foetal liver and increases to near adult levels before birth. 5. Phosphoenolpyruvate-carboxykinase activity in the soluble cell fraction increases 25-fold in the first 2 days after birth. This same enzyme in the mitochondria has considerable activity in the foetal and adult liver and is lower in the newborn. 6. Kinetic and other studies on the properties of phosphoenolpyruvate carboxykinase have shown no differences between the soluble and mitochondrial enzymes. 7. It is suggested that the appearance of the soluble phosphoenolpyruvate carboxykinase at birth initiates the rapid increase in overall gluconeogenesis at this stage.

The foetal rat liver does not synthesize glucose or glycogen until birth, at which time the gluconeogenic pathway begins to function (Ballard & Oliver, 1963, 1965). In adult liver specific enzymes of gluconeogenesis are present to by-pass thermodynamically unfavourable glycolytic reactions. Although glucose 6-phosphatase (EC 3.1.3.9) (Weber & Cantero, 1957; Dawkins, 1966) and fructose 1,6-diphosphatase (EC 3.1.3.11) (Ballard & Oliver, 1962) increase in activity before and after birth, both enzymes are present in liver from rat fetuses late in gestation and consequently the lack of the overall pathway cannot be caused by the absence of these enzymes. In the present investigation the activity during development as well as other properties of pyruvate carboxylase (EC 6.4.1.1) and PEP* carboxykinase (EC 4.1.1.32) are presented.

MATERIALS AND METHODS

Animals. Albino rats were obtained from Carworth Farms Co., New City, N.Y., U.S.A. All adult rats used were males.

Chemicals. Malate dehydrogenase (EC 1.1.1.37), PEP and

sodium pyruvate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., ATP, ADP, IDP, UDP, GDP, CDP, acetyl-CoA and NADH were from P-L Biochemicals, Milwaukee, Wis., U.S.A., and $\text{NaH}^{14}\text{CO}_3$ was from New England Nuclear Corp., Boston, Mass., U.S.A. Propionyl-CoA was prepared from propionic anhydride and CoA (Stadtman, 1957) and used immediately after synthesis.

Subcellular fractionation of liver. Livers were homogenized in a buffered iso-osmotic medium containing sucrose (0.2M), triethanolamine (20mM), glutathione (1mM) and EDTA (1mM) at pH 7.5 (Henning, Stumpf, Ohly & Seubert, 1966) by using a coaxial homogenizer with Teflon pestle. The buffered solution was used for the determination of pyruvate-carboxylase activity, as previous experiments (Ballard & Hanson, 1967b) showed that the stability of the enzyme from adipose tissue was variable if 0.25M-sucrose was used for homogenization. Contrary to the findings of Wagle (1964), homogenization and centrifugation at room temperature did not increase either the pyruvate-carboxylase or the PEP-carboxykinase activity in the supernatant fractions. The homogenate was centrifuged at 100000g for 30min., and the pellet was suspended in buffered sucrose, freeze-dried and dissolved in water. The activities of both enzymes were low in untreated particulate fractions and were increased by freezing and thawing or ultrasonic treatment. However, the greatest activities were detected in freeze-dried preparations and this treatment was used in all subsequent experiments.

For the isolation of nuclei, mitochondria, microsomes and

* Abbreviation: PEP, phosphoenolpyruvate.

supernatant, successive fractions were obtained by centrifuging at 600g for 10 min. (International PR-1 centrifuge, rotor 840), 10000g for 15 min. (International PR-1 centrifuge, rotor 295) and 100000g for 30 min. (Beckman Spinco model L ultracentrifuge, rotor 50), all steps being carried out at 2°. All fractions were suspended in buffered sucrose and resedimented once. Particulate fractions were freeze-dried for enzyme assay.

Assays of pyruvate carboxylase and phosphoenolpyruvate carboxykinase. These assays are in principle similar to those used by Utter & Keech (1960) and Chang & Lane (1966) respectively, and measured the incorporation of [^{14}C]-bicarbonate into oxaloacetate. One unit of activity is the amount of enzyme that catalyses the fixation of 1 μmole of [^{14}C]bicarbonate/min. at 37°.

The complete assay system for pyruvate carboxylase contained up to 0.01 unit of enzyme, 25 μmoles of tris-HCl buffer, pH 7.4, 10 μmoles of sodium pyruvate, 2.5 μmoles of sodium ATP, 0.75 μmole of acetyl-CoA, 50 μmoles (2 μC) of KHCO_3 and 5 μmoles of MgCl_2 in a total volume of 1 ml. The final pH was 7.5.

The reaction mixture for PEP carboxykinase contained up to 0.008 unit of enzyme, 100 μmoles of imidazole-HCl buffer, pH 6.6, 2 μmoles of MnCl_2 , 1 μmole of GSH, 1.25 μmoles of sodium IDP, 50 μmoles (2 μC) of KHCO_3 , 2.5 μmoles of NADH, 1.5 μmoles of PEP and 2 units of malate dehydrogenase in a volume of 1 ml. The final pH was 6.9–7.1.

After incubation for 10, 20 and 30 min. at 37°, the reactions were stopped by the addition of 0.5 ml. of 10% (w/v) trichloroacetic acid. After centrifugation, any remaining CO_2 was removed by bubbling unlabelled CO_2 through the solution, and 1 ml. portions of these solutions were counted in a Packard model 314E liquid-scintillation spectrometer with Ditol (Herberg, 1953) as solvent. In the absence of substrates, or enzyme, or if zero-time controls were carried out, a blank incorporation of 400–1000 disintegrations/min. was found. The variation in this value was due to different amounts of labelled impurities in each batch of the $\text{NaH}^{14}\text{CO}_3$. This value was subtracted from all assay measurements. For routine determinations, blanks with

acetyl-CoA omitted (for pyruvate carboxylase) or IDP omitted (for PEP carboxykinase) were used. At all incubation times and with tissue from all sources, these blanks were less than 100 disintegrations/min. above the impurity level. Up to the amounts of enzyme noted above, incorporation rates were proportional to both time and concentration of enzyme. In most assays, 1000–100000 disintegrations/min. above blank values were incorporated into products.

Identification of reaction products. Previous assays of pyruvate carboxylase in adipose tissue (Ballard & Hanson, 1967b) indicated that the radioactive product was not oxaloacetate but citrate, and it was concluded that in this tissue there was sufficient citrate synthetase in both soluble and particulate preparations to convert the oxaloacetate product into citrate in the presence of the acetyl-CoA used as activator. This was confirmed by the replacement of acetyl-CoA with propionyl-CoA, when no ^{14}C -labelled citrate was formed. Under these conditions all the fixed radioactivity was found in oxaloacetate. Similar experiments were carried out with liver supernatant and particulate fractions in which portions of the radioactive products of either enzyme assay were chromatographed on Dowex 1 (formate form) by using the method of Busch, Hurlbert & Potter (1952) with unlabelled citrate, malate or pyruvate. In addition, the radioactivity in the β -carboxyl carbon atom of oxaloacetate was determined by treating another portion of the reaction product with aniline citrate (Krebs & Eggleston, 1945), collecting the CO_2 evolved on filter paper saturated with NaOH and determining the radioactivity in these papers by the method of Buhler (1962). Sample experiments with supernatant enzymes from adult liver and particulate enzymes from foetal liver are shown in Table 1.

These experiments show that under normal assay conditions oxaloacetate was not a major product of pyruvate carboxylase and 70–75% of the radioactivity incorporated was in citrate. By replacing acetyl-CoA with propionyl-CoA in the assay this incorporation into citrate was prevented and oxaloacetate was found to be the major reaction product. Further, in the assay with foetal liver mitochondria, randomization of label in oxaloacetate via malate

Table 1. *Products of liver pyruvate carboxylase and phosphoenolpyruvate carboxykinase*

Supernatant fractions of adult liver and total particulate fractions from livers of 17-day fetuses were assayed for pyruvate-carboxylase and PEP-carboxykinase activities and the radioactivity in oxaloacetate, malate and citrate as well as the total incorporated radioactivity was measured. The results are expressed as percentages of the total radioactivity incorporated in each enzyme assay and are the means of duplicate experiments. Cofactor concentrations were: acetyl-CoA, 0.75 mM; propionyl-CoA, 0.5 mM. Additional experimental details are given in the Materials and Methods section.

Enzyme	Source	Cofactor	% of incorporated radioactivity		
			In oxaloacetate	In malate	In citrate
Pyruvate carboxylase	Adult supernatant	Acetyl-CoA	22	< 1	75
		Propionyl-CoA	69	—	—
	Foetal particles	Acetyl-CoA	23	< 1	70
		Propionyl-CoA	96	—	—
PEP carboxykinase	Adult supernatant	Complete	< 1	96	< 1
		— NADH	86	—	—
	Foetal particles	Complete	< 1	98	< 1
		— NADH	97	—	—

Table 2. *Intracellular distribution of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in foetal and adult rat liver*

Subcellular fractions of adult and 17-day foetal liver were assayed for pyruvate-carboxylase and PEP-carboxykinase activities as described in the Materials and Methods section. The activities are expressed as μmoles of $[^{14}\text{C}]$ bicarbonate incorporated/min./g. of tissue, with the average percentage distribution in parentheses assuming quantitative recovery of subcellular components.

Enzyme	Source	Expt.	Cellular fraction			
			Supernatant	Microsomes	Mitochondria	Nuclei
Pyruvate carboxylase	Adult	1	1580	52	7430	3310
		2	915	52	4630	2420
	Foetus		(12.1)	(0.5)	(58.9)	(28.5)
		1	16.9	0	202	70.1
		2	24.6	3.4	123	54.3
			(8.9)	(0.9)	(64.8)	(25.4)
PEP carboxykinase	Adult	1	2440	7.8	110.8	100.0
		2	3432	6.2	144.0	112.0
	Foetus		(92.3)	(0.3)	(4.0)	(3.4)
		1	55.8	7.8	190.8	130.1
		2	40.0	14.4	200.6	93.4
			(13.0)	(3.1)	(53.5)	(30.5)

and fumarate had not occurred, as 96% of the total radioactivity fixed was in the β -carboxyl carbon atom of oxaloacetate. In agreement with Utter & Keech (1963), the catalytic rate with propionyl-CoA was 70–80% of the rate with acetyl-CoA. No further increase in incorporation was found by increasing the propionyl-CoA concentration from 0.5 mM to 1 mM. Chromatographic separation of the products of the PEP-carboxykinase assay on Dowex 1 (formate form) (Busch *et al.* 1952) showed that only malate was radioactive. Omission of NADH prevented malate formation and under these conditions the $\text{NaH}^{14}\text{CO}_3$ was incorporated into oxaloacetate. No significant differences were found between the incorporation pattern of enzymes from foetal liver mitochondria and adult liver supernatant.

Nitrogen. This was determined by micro-Kjeldahl digestion followed by nesslerization (Johnson, 1941).

Molecular weights. These were determined by chromatography on Sephadex G-100 by the method of Andrews (1964).

RESULTS

The intracellular distributions of pyruvate carboxylase and PEP carboxykinase from 17-day foetal and adult liver are shown in Table 2. Approx. 60% of the pyruvate-carboxylase activity was in the mitochondrial fraction with some activity in the nuclei and supernatant. No apparent differences were noted between the two ages. In adult liver 92% of the PEP-carboxykinase activity was in the soluble fraction with 4% of the activity in the mitochondria.

PEP-carboxykinase activity was assayed in the supernatant and particles after centrifugation at 100 000g for 30 min. (Fig. 1). The soluble activity is extremely low in foetal liver and increases sharply at

Fig. 1. PEP-carboxykinase activity in (a) supernatant fraction or (b) particulate fractions of rat liver. The activities are expressed as $\mu\text{moles/min./g. of liver}$ (●) or $\mu\text{moles/min./mg. of tissue nitrogen}$ (○). Each point represents the mean $\pm$ S.E.M. of six to ten determinations. T, Term; A, adult.

birth to an activity of 2.61 units/g. compared with an activity of 2.38 units/g. in the adult. The particulate enzyme activity is highest in the 17-day foetus

Fig. 2. Pyruvate-carboxylase activity in (a) supernatant fraction or (b) particulate fractions of developing rat liver. The activities are expressed as μ moles/min./g. of liver ($\bullet$) or μ moles/min./mg. of tissue nitrogen ($\circ$). Each point represents the mean $\pm$ s.e.m. of six to ten determinations. T, Term; A, adult.

with an activity of 0.36unit/g. and falls to lower levels during the suckling period, with an increase in adult liver. When these results are expressed as units/mg. of nitrogen, the enzyme activities in foetal liver are relatively higher when compared with those of postnatal animals, as the nitrogen content of liver increases during development.

Pyruvate-carboxylase activity (Fig. 2) is low in both particulate and supernatant fractions in the 17-day foetal liver. The supernatant and particulate activities are 0.37unit/g. and 5.91units/g. respectively at term and increase during later development.

Several properties of PEP carboxykinase from adult liver supernatant and liver mitochondria from 17-day foetal rats are summarized in Table 3. The Michaelis constant for PEP measured over the PEP concentration range 0.1–2.0mm, and the relative catalytic rates of IDP, GDP, UDP, CDP and ADP, are similar. The pH-activity curves showed a broad peak from pH 6.6 to 7.2 with an optimum of pH 7.0 and considerably lower activities above pH 7.5 for the enzyme from both sources. The molecular weights were approx. 80 000.

Table 3. Properties of phosphoenolpyruvate carboxykinase from mitochondrial and supernatant fractions of liver

PEP carboxykinase from the mitochondria of 17-day foetal livers or the supernatant of adult livers was assayed as described in the Materials and Methods section. The K_m for PEP and the pH optimum were obtained with IDP as nucleotide substrate. The molecular weights were determined by chromatography on Sephadex G-100 (Andrews, 1964).

Properties	Source of enzyme	
	Mitochondria	Supernatant
K_m for PEP (mm)	0.4	0.4
Relative velocity with:		
1 mm-IDP	(100)	(100)
1 mm-GDP	112	104
1 mm-UDP	1.5	1.2
1 mm-ADP	0	0.9
1 mm-CDP	0	0.3
pH optimum	7.0	7.0
Molecular weight	78000	82000

DISCUSSION

Gluconeogenesis, the synthesis of glucose from lactate, pyruvate and certain amino acids, differs from a direct reversal of glycolysis in that essentially irreversible glycolytic enzyme reactions are circumvented by glucose 6-phosphatase, fructose 1,6-diphosphatase and the dicarboxylic acid shuttle (Krebs, 1963). This last pathway involves the carboxylation of pyruvate to oxaloacetate and the phosphorylative decarboxylation of oxaloacetate to PEP.

The activities of the enzymes involved are known to change in adult liver in a pattern similar to the overall pathway of gluconeogenesis under conditions of starvation, re-feeding and under varied changes in hormonal balance (Ashmore, Wagle & Uete, 1964; Exton & Park, 1966; Henning *et al.* 1966; Shrago, Lardy, Nordlie & Foster, 1963). Gluconeogenesis and glyconeogenesis occur at minimal levels in foetal rat liver (Ballard & Oliver, 1963, 1965) and appear at birth. Jaquot, Kretschmer, Tsuboi, Taylor & McNamara (1961) reported that gluconeogenesis was active in foetal rat liver but their method of injecting 14 C-labelled pyruvate into adults and measuring the subsequent appearance of label in foetal liver glycogen has an alternative and logical explanation; the pyruvate is taken to the maternal liver and kidney, converted into glucose and transferred back to the foetus, where glycogen is deposited.

As has been noted with hormonal studies in the adult rat (Shrago *et al.* 1963; Henning *et al.* 1966) the activity changes in hepatic PEP carboxykinase

during development are greater than reported for the other key gluconeogenic enzymes. Further, soluble PEP carboxykinase has a very low activity in the liver of the term foetus, and, as with gluconeogenesis and glyconeogenesis, the activity increases 25-fold in the next 48 hr. The activity increases reported for pyruvate carboxylase in the present paper, and those previously published for glucose 6-phosphatase (see Dawkins, 1966) and fructose 1,6-diphosphatase (Ballard & Oliver, 1962), vary in the range 2–3.5-fold over the same 48 hr. period. From these findings it seems logical to propose that the appearance of one enzyme, PEP carboxykinase, initiates the effective activity of the complete gluconeogenic sequence.

Intracellular distribution has been of particular interest in studies of pyruvate carboxylase and PEP carboxykinase. The early work of Keech & Utter (1963) and Freedman & Kohn (1964) implied that hepatic pyruvate-carboxylase activity is localized in the mitochondria. However, Henning & Seubert (1964), Henning *et al.* (1966), Wagle (1964) and Barritt, Keech & Ling (1966) have reported soluble pyruvate-carboxylase activity. Henning *et al.* (1966) have shown that the pyruvate carboxylase in both soluble and particulate fractions of liver and kidney changes in activity under conditions of starvation, adrenalectomy and cortisol treatment in directions similar to the change in the overall pathway of gluconeogenesis. Krebs (1966) could not show a variation in pyruvate-carboxylase activity in alloxan-diabetes, in contrast with the findings of Prinz & Seubert (1964). In the present work we have also noted pyruvate-carboxylase activity in both the soluble and mitochondrial fractions of cells from both foetal and adult liver. However, there was little change in pyruvate-carboxylase activity at birth, although there was a 30-fold increase in gluconeogenesis at this time.

Nordlie & Lardy (1963), Holton & Nordlie (1965) and Lardy, Foster, Shrago & Ray (1965) investigated the intracellular distribution of PEP carboxykinase in livers from several mammalian species and found large variations, ranging from total activity in the supernatant (mouse, hamster) to total activity in particulate fractions (rabbit). Although soluble PEP carboxykinase from the rat and guinea pig responds to dietary stimuli, a careful study of possible differences between the two enzymes in guinea-pig liver (Holton & Nordlie, 1965) revealed no kinetic differences. However, the mitochondrial enzyme, which contains 70% of the total activity in this species, does not change under hormonal treatments that may alter the soluble activity up to fourfold (Lardy *et al.* 1965).

The highest particulate PEP-carboxykinase activity noted in the present work was in livers from 17-day foetuses. Livers from animals of this age

have extremely high rates of lipogenesis, and the mitochondrial PEP carboxykinase may in some unexplained manner be involved in this process. Unlike gluconeogenesis, hepatic lipogenesis decreases tenfold at birth (Ballard & Hanson, 1967a). A study of certain enzymes involved in lipogenesis in foetal liver has shown that ATP citrate lyase and the enzymes required to support the citrate-cleavage pathway, with the exception of NADP malate dehydrogenase, were active (Ballard & Hanson, 1967a). The changes in particulate PEP-carboxykinase activity with age are similar to the pattern for ATP citrate lyase. The cleavage of citrate in the cytoplasm provides not only a source of extramitochondrial acetyl-CoA (Srere, 1959; Spencer & Lowenstein, 1962) but also oxaloacetate. In the adult liver and adipose tissue (Young, Shrago & Lardy, 1964; Ballard & Hanson, 1967b) oxaloacetate is converted into pyruvate with a simultaneous transhydrogenation of NADH to NADPH via the NAD and NADP malate dehydrogenases. In the absence of NADP malate dehydrogenase, the extramitochondrial oxaloacetate formed from ATP citrate lyase probably re-enters the mitochondria to support citrate synthesis. Our findings of only 5% pyruvate-carboxylase activity in the 17-day foetal liver as compared with that of the adult liver (Fig. 2a) further emphasize the importance of a continual source of oxaloacetate to maintain citrate concentrations for ATP citrate lyase and soluble acetyl-CoA formation. The striking increase of extramitochondrial PEP carboxykinase would therefore contribute to the decrease in lipogenesis associated with birth by drawing from the oxaloacetate pool needed to support lipogenesis and at the same time stimulate gluconeogenesis. It is noteworthy that a similar mechanism has been suggested by Krebs (1966) to explain the inverse relationship between ketogenesis and gluconeogenesis in adult liver.

The rat foetus is dependent on the maternal supply of glucose, and the lactate formed by glycolysis in extrahepatic tissues cannot be converted into glucose in the liver but is passed to the mother. The situation in other species is not clear. Recent studies (Ballard & Oliver, 1965) have demonstrated an active pathway of gluconeogenesis in foetal sheep liver, and indirect measurements suggest that the pathway is also present in the human foetus (Villee, 1953; Shelley, 1961). At birth the dependence on maternal glucose ends and hepatic gluconeogenesis begins. The results of the present paper suggest that this process is initiated in the newborn rat by a sharp increase in the activity of PEP carboxykinase.

The authors acknowledge the assistance of Miss Joan Krassenstein and Mr Alan J. Garber. We are also indebted to Dr Sidney Weinhouse for critically reading the manuscript. This work was supported by National Institutes of

Health Grants AM-05487 and CA-07174 and American Cancer Society Grant P-119B.

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