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Development of Gluconeogenesis in Neonatal Rat Liver

EFFECT OF PREMATURE DELIVERY

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1. An assay method for the determination of phosphopyruvate carboxylase activity is described in which improved sensitivity is obtained by separation of the enzyme from interfering pyruvate kinase by zone sedimentation. 2. The molecular weight of rat liver phosphopyruvate carboxylase determined by zone sedimentation is about 68000. 3. Premature delivery of rat foetuses by uterine section results in the rapid appearance of phosphopyruvate carboxylase, but hexose diphosphatase and pyruvate carboxylase, already present in the foetal rat liver, are not significantly affected, and glucose 6-phosphatase activity is only slightly affected. 4. The rate of incorporation of [^{14}C]pyruvate into glucose by liver slices is also greatly increased by premature delivery and there is a highly significant linear correlation between this process and the phosphopyruvate carboxylase activity.

Although the mechanism involved in the neonatal development of liver enzymes is unknown, the premature delivery of foetal animals by uterine section is known to result in the precocious appearance of at least two enzymes. These enzymes normally develop maximal activity or make their initial appearance in early postnatal life. Thus the precocious development of glucose 6-phosphatase (EC 3.1.3.9) in the liver of prematurely delivered rabbits has been reported (Dawkins, 1961) and tryptophan oxygenase (EC 1.13.1.12) activity can be similarly induced in rat liver (Nemeth, 1959, 1963).

Hepatic gluconeogenesis occurs only in the postnatal rat and is virtually absent from the foetal liver (Ballard & Oliver, 1963, 1965), but three of the four enzymes required in the system are already present in late foetal liver although their maximal activities occur in early postnatal life (Yeung, Stanley & Oliver, 1967). The last enzyme to make its appearance is phosphopyruvate carboxylase (EC 4.1.1.32), which is absent from foetal liver. However, the activity of this enzyme can be detected within a few hours of birth.

In recent experiments (Yeung *et al.* 1967) it has been shown that treatment of foetal animals with the powerful glucocorticoid, triamcinolone, does not result in the appearance of phosphopyruvate carboxylase activity. In the present paper it is shown that premature delivery of rat foetuses results in the rapid appearance of high activities of the enzyme. Parallel experiments on the incorporation of [^{14}C]pyruvate into glucose by liver slices from such animals indicate that the appearance of this enzyme initiates the overall process of gluconeogenesis.

MATERIALS AND METHODS

Chemicals. Phosphoenolpyruvate (sodium salt), ITP and ADP were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Oxaloacetic acid and NADP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Sodium [2-^{14}C]pyruvate (10 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks.

Purified enzymes. Pyruvate kinase (EC 2.7.1.40) was obtained from C. F. Boehringer und Soehne G.m.b.H., and lactate dehydrogenase (EC 1.1.1.27), type III, from the Sigma Chemical Co.

Animals. Wistar albino rats were used.

Pregnant animals were killed by a blow on the head and the whole uterus was removed from each and chilled in crushed ice. Foetuses were removed from the uterus, chilled in ice, washed free from amniotic fluid, blotted dry and weighed. The chilled foetuses were killed by cervical fracture, and the livers were removed, washed in cold 0·9% NaCl, blotted on filter paper and weighed for preparation of homogenates. Their age was established from the age–weight curve previously established for the colony (Yeung *et al.* 1967). Postnatal animals were killed by decapitation and the livers similarly treated.

Premature animals were obtained by uterine section. The pregnant animal was lightly anaesthetized with ether and the uterus exposed by laparotomy. The uterus was opened to expose one animal at a time, the head was swabbed with 0·9% NaCl and the umbilical cord tied.

When all foetuses were exposed, the cords were severed and the animals placed in an O_2-enriched atmosphere in an incubator at 37° until killed. Zero-time control animals were chilled in ice immediately after delivery and livers removed as soon as possible. All prematurely delivered animals were killed by cervical fracture.

Assay of glucose 6-phosphatase. Activity was assayed in homogenates of foetal livers prepared in 0·25M-sucrose according to the procedure described by Yeung *et al.* (1967).

Assay of pyruvate carboxylase (*EC* 6.4.1.1). Activity was assayed in extracts of freeze-dried liver homogenates by a modification of the method of Utter & Keech (1963) described by Yeung *et al.* (1967).

Assay of hexose diphosphatase (*EC* 3.1.3.11). Activity was assayed by the spectrophotometric procedure of Underwood & Newsholme (1965) in liver extracts prepared as described by Yeung *et al.* (1967).

Assay of pyruvate kinase (*EC* 2.7.1.40). Activity was assayed according to the procedure of Bücher & Pfleiderer (1955) as modified by Krebs & Eggleston (1965).

Separation of phosphopyruvate carboxylase and pyruvate kinase. The presence of pyruvate kinase in crude liver extracts should interfere with procedures for the assay of phosphopyruvate carboxylase in which the formation of phosphoenolpyruvate from oxaloacetate and ITP is measured (e.g. Nordlie & Lardy, 1963; Seubert & Huth, 1965). Preliminary experiments showed this to be the case.

Attempts to separate the two enzymes by chromatography on CM-cellulose and DEAE-cellulose were unsuccessful, but zonal centrifugation of crude liver extracts resulted in their clear separation. The following procedure was therefore used in the routine assay of phosphopyruvate carboxylase.

Pooled livers were homogenized in 4ml. of ice-cold 0·25M-sucrose/g. of tissue. The homogenate was centrifuged at $162000g_{max.}$ for 30min. at 4° in the type 50 rotor of the Spinco L–2 ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.) with $\frac{5}{16}$in. $\times 1\frac{5}{16}$in. cellulose tubes and the appropriate adaptors. Samples (0·2ml.) of the supernatant were centrifuged by the zonal technique on 5ml. of a sucrose concentration gradient in $\frac{1}{2}$in. $\times$ 2in. cellulose tubes (SW39 rotor). The linear sucrose concentration gradients were prepared by the method of Britten & Roberts (1960) with the concentration increasing from 5% (w/v) at the top to 20% (w/v) at the tube bottom. The sucrose solutions were buffered with sodium phosphate buffer, pH7·4 (5mM), and EDTA (5mM). The enzyme solution was layered on the top of the gradient by a device similar to a commercial design marketed by Beckman Instruments for the SW39 rotor. The layering takes place in our devices at about 4000 rev./min.

After gentle acceleration of the SW39 rotor the tubes were centrifuged for 12hr. at $176000g_{max.}$ at 2° and the rotor was slowed without braking. In preliminary experiments with extracts of adult liver the tube contents were fractionated from the bottom of the tube by a pinhole puncture, and 8 drops were collected in each fraction. Each fraction was assayed for pyruvate kinase and phosphopyruvate carboxylase. The distribution of the enzymes was reproducible over several separate runs. In routine assays, the fractions known to contain pyruvate kinase were first collected by drop-counting and discarded; the carboxylase fractions were then collected similarly as a pooled sample and assayed for enzyme.

Determination of molecular weight. A 100μl. sample of liver extract (adult rat liver), with 25μl. of a 5% (w/v) solution of crystalline bovine ferrohaemoglobin added, was zone-centrifuged as above. Centrifugation and fractionation were carried out exactly as described. Haem protein was determined by measuring E_{415} of diluted fractions.

Assay of phosphopyruvate carboxylase. The enzyme was assayed according to the method of Seubert & Huth (1965), with β-mercaptoethanol replacing GSH and with KCl omitted from the reaction medium; the enzymic reaction was terminated by addition of KBH_4, which reduces α-oxo acids to the hydroxy acids. The phosphoenolpyruvate formed from oxaloacetate (freshly prepared) and ITP was determined enzymically by the method of Czok & Eckert (1963). No pyruvate was ever found on addition of lactate dehydrogenase in the presence of $NADH_2$; the assay was then completed by addition of ADP and pyruvate kinase.

Assay of overall gluconeogenesis. Liver slices (150–200mg.) were incubated in the presence of 60mM-pyruvate containing 1μC of [2-^{14}C]pyruvate for 2hr. at 37° as described by Ballard & Oliver (1964, 1965). The reaction was stopped with $HClO_4$ and the tissue was homogenized and treated with Bio-Deminrolit (Permutit Co. Ltd., London) as described by Ballard & Oliver (1965) for the separation of radioactive glucose from [^{14}C]pyruvate.

Samples of the final glucose-containing solutions were dried *in vacuo* in scintillation pots, dissolved in 0·2ml. of water and counted after the addition of 10ml. of the scintillation solvent Diotol (Herberg, 1960). Radioactivity was determined (at 80% efficiency) in the Nuclear–Chicago Corp. model 6860 (Mk. I) Automatic Liquid Scintillation Spectrometer. At least 1000 counts above background were accumulated for each sample and appropriate quenching corrections were obtained by the channels-ratio method. Variations in this were negligible from sample to sample. Results were calculated as μmoles of pyruvate incorporated into glucose/g. wet wt. of liver slices.

Determination of protein. Protein was determined in liver homogenates and soluble extracts by the method of Lowry, Rosebrough, Farr & Randall (1951). Homogenates were diluted 50-fold in 2% (w/v) Na_2CO_3 in 0·1N-NaOH and incubated overnight at 37° before protein assay.

Spectrophotometric procedures. These were carried out in a Beckman model DU Spectrophotometer at 37° equipped with a Gilford Instruments (Oberlin, Ohio, U.S.A.) model 2000 Multiple Sample Absorbance Recorder.

RESULTS

Fig. 1 shows the zone-sedimentation behaviour of pyruvate kinase, phosphopyruvate carboxylase and bovine ferrohaemoglobin. The separation of the two enzymes is clearly seen. The peak of the carboxylase activity is only one fraction behind that of haemoglobin. Thus the molecular weight of the enzyme is close to 68000.

In the run shown in Fig. 1, assay of the phosphopyruvate carboxylase activity in the unfractionated soluble extract indicated an enzyme activity of 0·41μmole of product/g. of liver/min. After the zone centrifugation, the area under the enzyme curve indicates an activity of 2·3μmoles of product/g. of liver/min. Thus the interfering effect of

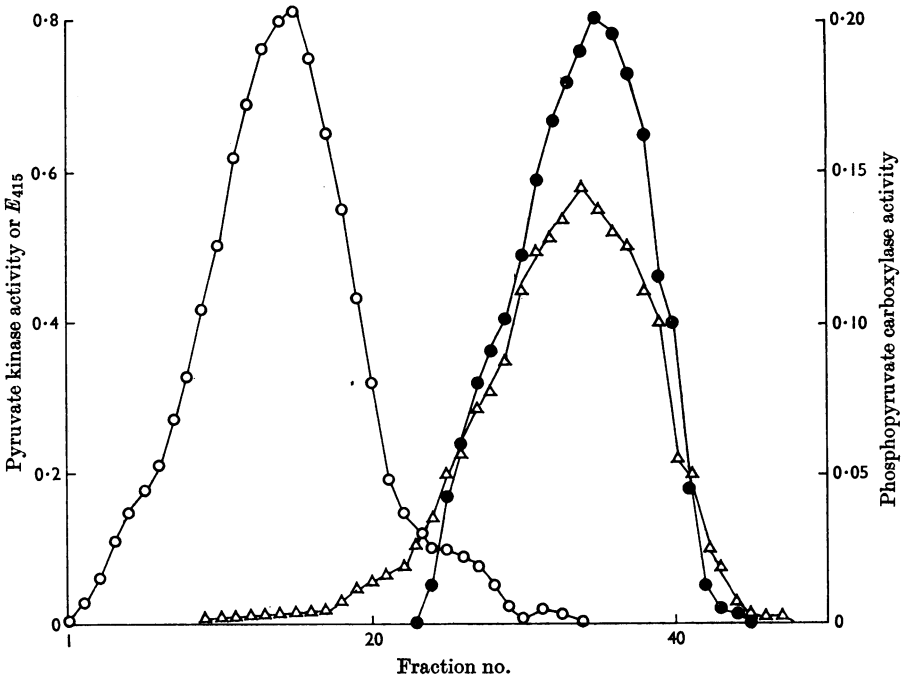

Fig. 1. Separation of pyruvate kinase and phosphopyruvate kinase by zone sedimentation. In this experiment, 0·1 ml. of adult liver extract containing added bovine ferrohaemoglobin was layered over 5 ml. of a linear concentration gradient of sucrose (5% at top to 20% at tube bottom) as described in the text. Centrifugation was for 12 hr. at 39460 rev./min. in rotor SW39 of the Spinco L–2 ultracentrifuge at 4°. After centrifugation, fractions (8 drops) were collected from each of three identical tubes by drop-counting from a pinhole in the tube bottom and assayed. Phosphopyruvate carboxylase activities are expressed as μmoles of phosphopyruvate formed/tube/hr. Pyruvate kinase activities are expressed as the change in E_{340}/2 min. For assay conditions see the text. Fraction 1 represents the tube bottom. ○, Pyruvate kinase activity; ●, phosphopyruvate carboxylase activity; △, E_{415} (haemoglobin).

pyruvate kinase on the assay procedure is clearly demonstrated.

Fig. 2 shows the normal development of hepatic phosphopyruvate carboxylase activity over the first 2 postnatal days and the effect of premature delivery on the enzyme activity. Considerable activity appears even 1½ hr. after delivery and there appears to be no obvious lag phase in the development of the enzyme activity.

Fig. 3 shows the relation of overall gluconeogenesis assessed in liver slices and the carboxylase activity in the livers of litter mates delivered by uterine section and maintained for various times before being killed. The linear relationship between the two parameters is highly significant (correlation coefficient = 0·973).

Determinations of the effect of premature delivery on the activity of the other obligatory enzymes of gluconeogenesis showed either no effects or only small effects. At 10 hr. after delivery, the activities of hexose diphosphatase and pyruvate carboxylase showed increases over those of the zero-time litter-mate controls of less than 20%, and this increase is similar in magnitude to that shown in the normal development curve of animals *in utero*. With glucose 6-phosphatase, 12 hr. after premature delivery, the increase in activity was 2·6-fold in 20-day foetuses and about 1·7-fold in 21-day foetuses. This exceeds the normal increase seen *in utero*, but is small compared with the effect on phosphopyruvate carboxylase.

DISCUSSION

In preliminary studies on the neonatal development of phosphopyruvate carboxylase, measurement of the enzyme activity in crude liver extracts by the method of Seubert & Huth (1965) showed activity to be present only after birth of the animal. However, the presence of pyruvate kinase in foetal liver could result in the masking of carboxylase activity until the enzyme activity reached

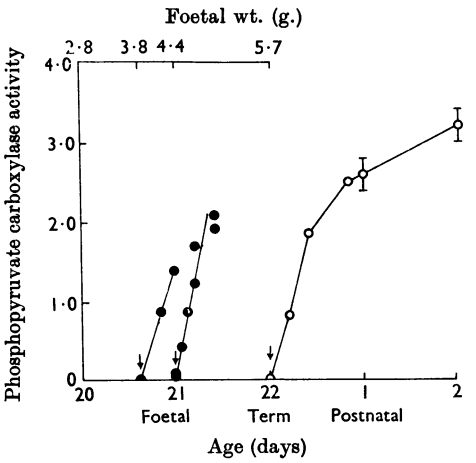

Fig. 2. Induction of phosphopyruvate carboxylase in foetal rat liver by premature delivery. The development of phosphopyruvate carboxylase activity in rat liver over the first 2 days after normal delivery is shown on the right of the Figure. The data at 1 and 2 days after birth were each obtained from four litters and the mean values are plotted; the vertical bars represent ±1 S.D. of the mean. The data up to 20hr. after normal delivery were obtained from a single litter; each point shows data obtained with two pooled livers. Prematurely delivered animals were killed in pairs after various times in an incubator and enzyme activity was determined in extracts of the pooled livers. The base-line points represent the age (shown at bottom) and weight ±0·1g. (shown at top). Prematurely delivered animals were obtained from several litters and the data show the time-course of development after delivery. Arrows show time of delivery. Phosphopyruvate carboxylase activities are expressed as μmoles of phosphoenolpyruvate produced/mg. of protein/hr. at 37°. ○, Postnatal animals; ●, prematurely delivered animals.

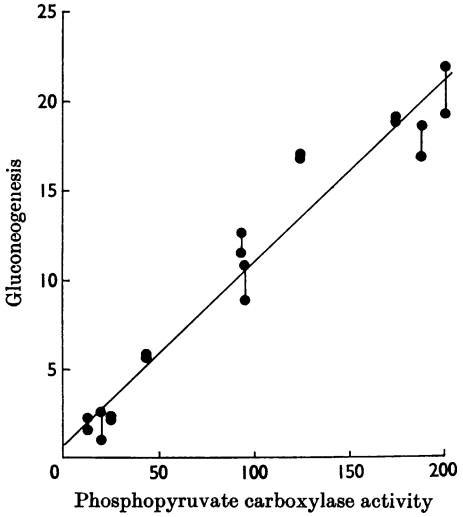

Fig. 3. Gluconeogenesis and phosphopyruvate carboxylase activity in liver of prematurely delivered foetal rats. Prematurely delivered animals of the same litter were killed in groups of four at various times after delivery. One pair of animals was used for phosphopyruvate carboxylase assay (pooled livers) and slices were prepared from the livers of the other pair for duplicate determinations of gluconeogenesis by incorporation of [^{14}C]pyruvate into glucose. Enzyme duplicates always agreed within 4% and the mean activities are plotted against the overall rate of gluconeogenesis. Since agreement was less reproducible in the latter determination, the individual values are plotted. The line of best fit was calculated by the method of least squares and the correlation coefficient calculated to be 0·973. Animals used for this experiment ranged in average weight from 3·8 to 4·4g. at delivery. Gluconeogenesis is expressed as μmoles of pyruvate incorporated into glucose/g. of liver slices/2hr. Phosphopyruvate carboxylase activities are expressed as μmoles of phosphoenolpyruvate produced/g. of liver slices/hr.

a level where the products of the reaction would accumulate in detectable amounts. Thus the first appearance of the enzyme in the liver could readily be obscured in this assay. Accordingly, the assay was carried out after separating the two enzymes. As the results of Fig. 1 show, this procedure results in a vastly improved sensitivity for phosphopyruvate carboxylase activity. The zone-centrifugation procedure, although it is time-consuming, does not result in degradation of the enzyme, since crude extracts of adult rat liver stored at 4° for the same time do not lose apparent activity.

The zone-centrifugation procedure also gives information about the size of the enzyme, and it appears to be similar in sedimentation characteristics to haemoglobin with a molecular weight of about 68000. Chang & Lane (1966) have reported a sedimentation-equilibrium molecular weight of 73300 for the enzyme purified from pig liver mitochondria.

In the present studies, phosphopyruvate carboxylase was assayed only in the soluble fraction of rat liver, since Nordlie & Lardy (1963) have shown that in this species more than 90% of the total activity occurs in this fraction. In foetal rat livers, the 'particulate' fraction, which consists of whole haematopoietic cells, parenchymal-cell nuclei, mitochondria, microsomes and free ribosomes, shows no activity in the unmodified assay method of Seubert & Huth (1965). Nordlie & Lardy (1963) were unable to detect activity in freshly prepared mitochondria from adult rat liver. The particulate fraction was therefore discarded in routine preparations.

Premature delivery of the rat foetus results in the rapid appearance in the liver of enzyme activity that is detectable as early as 1½hr. after delivery. After 10hr. the activity is quantitatively similar to that found 10–12hr. after normal birth (see Fig. 2),

and, although the prematurely delivered rats were not fed during maintenance in the incubator, starvation is probably not a factor influencing the development of enzyme activity, since the time-course of premature development shows no lag period. After normal birth young rats begin to feed after about 4 hr. The appearance of enzyme activity may be due to activation of an inactive form of the enzyme already present in the tissue or it may be due to very rapid synthesis, either from preformed messenger RNA or from rapidly synthesized messenger RNA. The effect of premature delivery on the carboxylase activity is similar to results reported for glucose 6-phosphatase (Dawkins, 1961) and tryptophan oxygenase (Nemeth, 1959) in the rabbit. Nemeth & de la Haba (1962) and Nemeth (1962) tested the effect of puromycin and 5-fluorouracil on the adaptive formation of tryptophan oxygenase in premature rabbits, and Nemeth (1963) reported the effects of low oxygen pressure, high temperature, progesterone, oestradiol, glucose and adrenalectomy on the birth-induced increase in activity. Fluorouracil and puromycin partially prevented the increase, showing that some new enzyme synthesis occurred; the other agents were without effect, and Nemeth (1963) concluded only that some factor in the uterine environment repressed the formation of the liver enzyme.

Phosphopyruvate carboxylase appears to be the last of the four obligatory enzymes of gluconeogenesis to make its appearance in developing rat liver, and normally appears during the first postnatal day (Yeung *et al.* 1967; F. J. Ballard, personal communication). Earlier results showed that overall gluconeogenesis assessed in liver slices (Ballard & Oliver, 1963, 1965) is absent from the foetal liver and makes a rapid appearance within the first postnatal day. Thus it is likely that the overall process is dependent on the appearance of this enzyme, since it has been implicated in gluconeogenesis by many authors (e.g. Shrago, Lardy, Nordlie & Foster, 1963; Utter, 1963; Young, Shrago & Lardy, 1964). In Fig. 3 evidence is presented for this view. Gluconeogenesis at various times after premature delivery was measured by the incorporation of [^{14}C]pyruvate into glucose by liver slices and compared with the carboxylase activity in the livers of litter-mate animals incubated for the same time. The data show a highly significant linear relation between gluconeogenesis and the carboxylase activity.

Under the conditions of the experiments the normal foetal activities of liver pyruvate carboxylase and hexose diphosphatase (Yeung *et al.* 1967) were unaffected by premature delivery, since their activities increased by values consistent with the increase *in utero* at the same stages of development. Glucose 6-phosphatase activity was increased slightly above the normal increment found *in utero*, but the effect was small compared with the increment in phosphopyruvate carboxylase activity. Thus the adaptive increase in phosphopyruvate carboxylase activity brought about by premature delivery is highly specific and the appearance of the enzyme leads to functional activity of the gluconeogenic pathway.

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