Development of Gluconeogenesis in Neonatal Rat Liver

EFFECT OF TRIAMCINOLONE

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1. The normal development of the key enzymes of gluconeogenesis in rat liver, glucose 6-phosphatase, hexose diphosphatase, phosphopyruvate carboxylase and pyruvate carboxylase, was measured during the neonatal period. 2. Glucose 6-phosphatase, hexose diphosphatase and pyruvate carboxylase are all present in the late foetal liver, but all the enzymes show an increase in activity after birth. 3. Phosphopyruvate carboxylase is not present in liver extracts from foetal rats, but activity appears immediately after birth and increases rapidly over the first day and then more slowly to reach its maximum at the fourth postnatal day. 4. The fluorinated synthetic glucocorticoid, triamcinolone, was administered to foetal rats at various gestation times by intraperitoneal injection *in utero* and the animals were killed at intervals between 4 and 48 hr. later. 5. The administration of triamcinolone results in slight depression of glucose 6-phosphatase, and a more significant depression of hexose diphosphatase to about one-half its normal activity in foetal rat liver. 6. Triamcinolone injection is without effect on pyruvate carboxylase activity and does not result in premature appearance of phosphopyruvate carboxylase in foetal rat liver. 7. Pyruvate kinase and aspartate aminotransferase activities in foetal rat liver are both depressed by triamcinolone treatment, whereas phosphofructokinase activity is elevated. 8. Tyrosine aminotransferase activity in foetal rat liver is markedly elevated in animals exposed to triamcinolone for 10 hr. or more, but the effect is only observed in animals close to term. 9. The results are discussed in relation to mechanisms involved in the initial synthesis of tissue-specific enzymes in developing tissues, and it is concluded that glucocorticoids do not initiate the synthesis of the gluconeogenic enzymes.

Previous work has shown that gluconeogenesis from pyruvate and amino acids in rat liver is initiated only in early postnatal life and that the process is almost totally absent from foetal liver (Ballard & Oliver, 1963; Yeung & Oliver, 1967a).

Information on the development of the key enzymes of gluconeogenesis in rat liver has been fragmentary, but low activities of both hexose diphosphatase (EC 3.1.3.11) (Ballard & Oliver, 1962) and glucose 6-phosphatase (EC 3.1.3.9) (Weber & Cantero, 1955; Kretchmer, 1959; Coquoin-Carnot & Roux, 1960; Dawkins, 1961, 1963; Burch *et al.* 1963) were reported to occur in late foetal life and both enzymes reach maximum activities, which are much higher than adult values, soon after birth. In the present paper it is shown that pyruvate carboxylase (EC 6.4.1.1) is readily detectable in foetal liver from 18 days of gestation and develops rapidly to reach adult levels of activity at term. On the other hand, phospho-

pyruvate carboxylase (EC 4.1.1.32) is virtually absent from foetal liver and appears very rapidly after birth.

The mechanisms that control the production of these enzymes in the neonatal animal are unknown, but in the adult rat increases in the activity of glucose 6-phosphatase and hexose diphosphatase (Weber, Allard, De Lamirande & Cantero, 1955; Weber, Banerjee & Bronstein, 1961, 1962; Weber, Srivastava & Singhal, 1965b; Ashmore, Hastings, Nesbitt & Renold, 1956; Kvam & Parks, 1960), phosphopyruvate carboxylase (Shrago, Lardy, Nordlie & Foster, 1963; Ashmore, Wagle & Uete, 1964; Foster, Ray & Lardy, 1966) and pyruvate carboxylase (Henning, Seiffert & Seubert, 1963; Henning, Stumpf, Ohly & Seubert, 1966) of liver have been reported after glucocorticoid administration. Although these increases are rather small compared with those that occur in neonatal rat liver, it is possible that similar hormonal mechanisms operate in both foetal and adult animals to promote the appearance of enzyme activity or

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to accelerate biosynthesis. The present paper reports experiments designed to test the hypothesis.

Weber *et al.* (1965b) reported that the synthetic fluorinated glucocorticoid, triamcinolone, was more effective than cortisone in promoting some of the enzyme effects described above and this substance was used in the present study. The direct administration of triamcinolone to foetal rats *in utero* was tested for its effects on the activity of the four obligatory enzymes of gluconeogenesis, pyruvate carboxylase, phosphopyruvate carboxylase, glucose 6-phosphatase and hexose diphosphatase. In addition, the activity of aspartate aminotransferase (EC 2.6.1.1), recently shown to increase after birth (Yeung & Oliver, 1967a) and to be implicated in gluconeogenesis in rat liver (Lardy, Paetkau & Walter, 1965), was also followed. Pyruvate kinase (EC 2.7.1.40) and phosphofructokinase (EC 2.7.1.11) were measured to assess the specificity of effects of hormone administration, and tyrosine aminotransferase (EC 2.6.1.5) activity was followed as a check on the efficacy of the hormone treatment, since the normal postnatal synthesis of this enzyme in rat liver is dependent on the presence of adrenal-cortical hormones (Sereni, Kenney & Kretchmer, 1959).

MATERIALS AND METHODS

Chemicals. ATP, ADP, AMP, ITP, CoA and phosphoenolpyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glucose 6-phosphate, fructose 1,6-diphosphate, potassium pyruvate, NADP, oxaloacetic acid, fructose 6-phosphate, pyridoxal phosphate and NADH₂ were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. L-Glutamic acid, L-tyrosine, sodium diethyldithiocarbamate and α -oxoglutaric acid were obtained from British Drug Houses Ltd., Poole, Dorset. Na₂¹⁴CO₃ (36 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Triamcinolone diacetate (Aristocort) (16 α ,21-diacetoxy-9 α -fluoro-11 β ,17 α -dihydroxypregnane-1,4-diene-3,2-dione) was a generous gift from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., U.S.A.

Acetyl-CoA. This was prepared from CoA by acetylation with acetic anhydride in neutral aqueous solution at 0°. The solution was tested for free SH groups with the nitroprusside reaction, and when acetylation was complete the solution was stored at -25°.

p-Hydroxyphenylpyruvic acid. This was prepared by condensation of p-hydroxybenzaldehyde and hippuric acid in the presence of anhydrous sodium acetate and acetic anhydride. The condensation product was hydrolysed in 4N-NaOH, and the p-hydroxyphenylpyruvic acid was isolated from the acidified solution after initial precipitation of benzoic acid (modified from Johnson, 1942). The twice-recrystallized product had corrected m.p. 219–220° (decomp.) (literature value 220°; see *Dictionary of Organic Compounds*, 1965). It readily reduced ammoniacal AgNO₃

and its ethanolic solution gave a transient green colour with FeCl₃.

Purified enzymes. Pyruvate kinase, aspartate aminotransferase, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glucose phosphate isomerase (EC 5.3.1.9), triose phosphate isomerase (EC 5.3.1.1) and glycerol phosphate dehydrogenase (EC 1.1.1.8) were obtained from C. F. Boehringer und Soehne G.m.b.H. Lactate dehydrogenase (EC 1.1.1.27), type III, and aldolase (EC 4.1.2.7) were obtained from Sigma Chemical Co. Malate dehydrogenase (EC 1.1.1.37) A grade was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Animals. Wistar albino rats were used. Foetal ages were established from a foetal age-weight curve established on the animal colony. Female rats (4–6 months old) were mated overnight for a period of 12 hr. only, and the males then removed. Pregnancy was determined after 15 days by manual examination. Animals that were pregnant were assumed to have conceived at the end of the mating period. In this colony birth occurs after 22 days of gestation and the average weight of newborn animals is 5.7 ± 0.2 g.

Surgical procedures. Pregnant animals were lightly anaesthetized with ether and the whole uteri exposed by laparotomy. Triamcinolone suspensions and sterile 0.9% NaCl were administered by injection directly through the uterine wall into the foetal peritoneum, from an Agla micrometer syringe (Burroughs Wellcome and Co.) equipped with polythene cannula tubing and 30-gauge needle. A 20 μ l. sample of triamcinolone suspension (25 mg./ml.) was administered to alternate foetuses, the other animals each receiving 20 μ l. of sterile 0.9% NaCl. Then 1 ml. of sterile 0.9% NaCl containing 50 000 units of sodium penicillin G [Glaxo-Allenbury's (Australia) Pty. Ltd., Sydney] was added to the maternal peritoneal fluid and the abdominal wall was closed by sutures. Chloramphenicol sodium succinate (Parke, Davis and Co., Hounslow, Middx.) was applied locally to the wound and the skin was closed with clips. Operative technique was clean but not sterile. The average duration of the operation was 7 min. In some experiments foetuses were injected with 0.9% NaCl only.

Pregnant operated animals were killed by cervical fracture 5–48 hr. after foetal injection and the whole uteri removed and chilled in ice. Foetuses were removed from the uteri and chilled in ice, and the average weights of control and hormone-injected animals were recorded. 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. Postnatal animals were killed by decapitation and the livers similarly treated.

Assay of glucose 6-phosphatase. In view of the finding of Imsande & Ephrussi (1964) that citrate buffers inhibit colour development in standard procedures for the determination of inorganic phosphate, an assay method was developed for glucose 6-phosphatase that overcomes this.

Pooled livers were homogenized in ice-cold 0.25 M-sucrose (4 ml./g. of liver) and assayed within 20 min. The assay medium contained 0.8 ml. of 0.1 M-tris-2 mM-EDTA brought to pH 6.8 with M-maleic acid, and 0.1 ml. of 0.1 M-glucose 6-phosphate dissolved in the same buffer. After preincubation at 30°, the reaction was started by addition of 0.1 ml. of homogenate. Incubation was for 0, 5 and 10 min. and the reaction was stopped by addition of 1.0 ml. of 6% (w/v) trichloroacetic acid. After centrifugation in the cold,

1.0-ml. samples of the supernatant solution were analysed for inorganic phosphate by the method of Taussky & Shorr (1953).

The addition of EDTA prevented a decrease in the rate of the reaction that otherwise occurred after the first 5 min. The use of tris-maleate buffer instead of citrate buffer results in a sixfold increase in the sensitivity of the assay for inorganic phosphate.

A sample of the homogenate was used for the assay of pyruvate kinase and aspartate aminotransferase described below.

Assay of pyruvate kinase. Activity was assayed in the supernatant solution after centrifugation of sucrose homogenates, prepared as described above, at $200000g_{\max}$, for 30 min. at 5° in a Spinco L-2 preparative ultracentrifuge (rotor 50) (Beckman Instrument Co., Spinco Division, Palo Alto, Calif., U.S.A.). The assay procedure was that of Bücher & Pfeleiderer (1955) as modified by Krebs & Eggleston (1965).

Assay of aspartate aminotransferase. Activity was assayed at 37° in liver extracts prepared as described for the pyruvate kinase assay above. The procedure was essentially that of Karmen (1955) with minor modifications (Yeung & Oliver, 1967a).

Assay of tyrosine aminotransferase. Pooled livers were homogenized in ice-cold 0.25 M-sucrose-5 mM- β -mercaptoethanol (4 ml./g. of liver). A sample of the homogenate was removed for assay of phosphofructokinase, phosphopyruvate carboxylase and pyruvate carboxylase described below. Tyrosine aminotransferase was assayed in 0.1-ml. samples of the whole homogenate according to the procedure of Sereni *et al.* (1959) except that incubations were carried out for 0 and 15 min. at 37° . Linearity of the assay with adult liver homogenates, which contained more activity than any of the foetal livers, indicated this procedure to be satisfactory for quantitative assay.

Assay of hexose diphosphatase. Homogenates prepared as described for the tyrosine aminotransferase assay above were centrifuged at $200000g_{\max}$, for 30 min. at 5° in rotor 50 of the Spinco L-2 ultracentrifuge. Enzyme activity was assayed at 37° in the supernatant solution by the continuous spectrophotometric procedure of Underwood & Newsholme (1965).

Assay of phosphofructokinase. Activity was assayed in the soluble liver supernatant prepared as described above, by the spectrophotometric procedure of Mansour (1963).

Assay of phosphopyruvate carboxylase. Activity was assayed as described in the next paper (Yeung & Oliver, 1967b).

Assay of pyruvate carboxylase. Activity was assayed by a modification of the method of Utter & Keech (1963) suggested by Dr D. B. Keech (personal communication).

Livers were homogenized at room temperature in a buffer consisting of 40 mM-tris-0.1 mM-EDTA-0.5 mM-ATP, adjusted to pH 7.8 with 2 N-HCl (3 ml. of buffer/g. of liver). ATP was added to stabilize the enzyme (M. F. Utter, personal communication). A measured volume of the homogenate was freeze-dried and the solid resuspended in the same volume of distilled water to solubilize the enzyme. The preparation was then centrifuged for 2 hr. at $25000g_{\max}$ in rotor 295 of the International refrigerated centrifuge (model PR2) at 10° with the high-speed attachment. The supernatant was diluted tenfold with homogenization medium for assay of enzymic activity.

Reaction mixtures contained 100 μ moles of tris-HCl buffer, pH 7.8, 5 μ moles of potassium pyruvate, 5 μ moles of glutamic acid, 3.3 μ moles of $MgCl_2$, 1.2 μ moles of ATP, 0.65 μ mole of acetyl-CoA, 20 μ g. of aspartate aminotransferase, 5 μ g. of pyridoxal phosphate and 20 μ moles of $NaHCO_3$ containing 1 μ C of $Na_2^{14}CO_3$. The final volume was 0.450 ml. Samples (50 μ l.) of diluted enzyme were added to start the reaction. Incubations at 37° were carried out for 0, 5 and 10 min. and the reaction was stopped by addition of 50 μ l. of 36% (w/v) trichloroacetic acid. Controls, which were incubated for 10 min., consisted of the above system minus the acetyl-CoA and ATP.

After incubation, the remaining $^{14}CO_2$ was 'chased' out with solid CO_2 (five additions with shaking). The mixture was centrifuged and 100 μ l. samples of the supernatant were taken for assay of ^{14}C .

In all enzyme assays involving spectrophotometry, a Beckman model DU Spectrophotometer fitted with a Gilford Instruments (Oberlin, Ohio, U.S.A.) model 2000 Multiple Sample Absorbance Recorder was used.

Determination of protein. All protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Homogenate proteins were determined on samples that had been diluted in 2% (w/v) Na_2CO_3 in 0.1 N-NaOH and incubated overnight at 37° . The protein in liver extracts was determined on samples diluted 50-fold in the same alkaline reagent.

Assay of ^{14}C . For this, 10 ml. of Diotol (Herberg, 1960), 100 μ l. of Hyamine hydroxide (1 M in methanol solution) and 100 μ l. of the ^{14}C -labelled sample were pipetted into vials and counted in a Nuclear-Chicago Corp. model 6860 (Mk. I) Automatic Liquid Scintillation Spectrometer. Channels-ratio data were obtained for quenching corrections, but no significant variation in the ratio was observed in the experiments described. The efficiency of the counter for ^{14}C was 80%. All samples were counted to at least 1000 counts above the background.

RESULTS

The experiments described involved over 900 foetuses. The foetal mortality rate over the whole series was 2.5%. All deaths occurred in the 40-48 hr. group and all had received triamcinolone. In a few experiments hormone-injected animals were allowed to come to normal term (more than 48 hr. after injection). The newborn animals appeared to be normal in appearance and activity. Foetal animals that had received triamcinolone were always slightly smaller than saline-injected litter mates. This difference was more pronounced the longer the time of exposure to the hormone.

Fig. 1 shows the normal developmental curves for glucose 6-phosphatase and hexose diphosphatase determined by the assay procedures described in this paper. Significant activities of both enzymes are seen in foetal liver, but there are large increases after birth. The hexose diphosphatase activity in foetal liver is inhibited 60% by 0.1 mM-AMP and 40% by 1 mM-fructose 1,6-diphosphate under the standard assay conditions, and thus shares the properties of the enzyme from

adult liver. Histochemical techniques have shown glucose 6-phosphatase activity to be localized in the endoplasmic reticulum membranes of paren-

chymal cells of foetal rat liver (W. F. C. Blumer, personal communication), as in adult liver.

Fig. 2 shows the development of pyruvate carboxylase and phosphopyruvate carboxylase in neonatal rat liver. There is no detectable activity of phosphopyruvate carboxylase in liver extracts before birth and the appearance of the enzyme appears to be initiated at birth (see Yeung & Oliver, 1967b). On the other hand, pyruvate carboxylase develops rapidly over the last few days before term and appears to show no birth-related increase in activity.

Fig. 3 shows the effect of the administration of triamcinolone to foetal rats *in utero* on the activity of tyrosine aminotransferase in the liver. It is apparent that the treatment results in increased activities of the enzyme provided that the time of exposure to the hormone is 10 hr. or more. The effect is also most marked in foetuses approaching term, and both the hormone-injected and saline-injected litter mates are affected.

Fig. 4 compares the specific activities of glucose 6-phosphatase in the liver of normal foetal rats and of those treated with triamcinolone. There is significant depression of activity by the hormone in foetuses younger than 21 days. The specific activities in hormone-treated animals and their saline-injected litter mates did not differ significantly.

Fig. 1. Neonatal development of glucose 6-phosphatase and hexose diphosphatase in rat liver. Each point represents the mean of determinations on four to six animals drawn from different litters. The vertical bars represent ± 1 s.d. of the mean. Glucose 6-phosphatase specific activity was determined in liver homogenates, hexose diphosphatase in soluble liver extracts (100000g-hr. supernatant). A, Adult male rats, 3 months old. O, Glucose 6-phosphatase activity (μ moles of inorganic phosphate produced/mg. of protein/hr. at 30°C); ●, hexose diphosphatase activity (μ moles of fructose 6-phosphate produced/mg. of protein/hr. at 37°C).

Fig. 2. Neonatal development of pyruvate carboxylase and phosphopyruvate carboxylase in rat liver. Each point represents the mean of determinations on four to six animals drawn from different litters. The vertical bars represent ± 1 s.d. of the mean. Phosphopyruvate carboxylase specific activity was determined on 100000g-hr. liver supernatant after zone centrifugation as described in the text. Pyruvate carboxylase activity was determined on 50000g-hr. extracts obtained from freeze-dried liver homogenates. A, Adult male rats, 3 months old. O, Pyruvate carboxylase activity (μ moles of oxaloacetate produced/mg. of protein/hr. at 37°C); ●, phosphopyruvate carboxylase activity (μ moles of phosphoenolpyruvate produced/mg. of protein/hr. at 37°C).

Fig. 3. Effect of triamcinolone on the activity of liver tyrosine aminotransferase in foetal rats *in vivo*. Each point represents the specific activity found in homogenates prepared from two pooled livers of foetal rats each injected *in utero* with 0.5 mg. of triamcinolone. The animals were killed (a) 5 hr., (b) 10 hr., (c) 20 hr. and (d) 44 hr. after triamcinolone administration as shown. The enclosed area represents the mean enzyme activity ± 2 S.D. of the mean over the foetal weight and age range shown of control unoperated animals. Sham laparotomy and foetal injection with saline did not alter these values. ▲, Triamcinolone-injected animals; △, saline-injected litter-mate controls plotted on the same vertical axis as the test animals. The weights represented are the average weights of saline-injected animals. Activities are expressed as μ moles of *p*-hydroxyphenylpyruvate produced/mg. of protein/hr. at 37°.

Fig. 4. Effect of triamcinolone on liver glucose 6-phosphatase activity of foetal rats *in vivo*. Each point represents the specific activity found in homogenates prepared from two pooled livers of foetal rats each injected *in utero* with 0.5 mg. of triamcinolone. The animals were killed 5, 10, 20 and 44 hr. after injection, but, since no effect of exposure time was discernible, the results are plotted as a scatter diagram. The enclosed area represents the mean specific activity ± 2 S.D. of the mean of liver glucose 6-phosphatase in normal unoperated foetal rats (data from Fig. 1). Activities were determined at daily intervals from 17 days of gestation to term. Activities are expressed as μ moles of inorganic phosphate produced/mg. of protein/hr. at 37°.

Fig. 5 compares the specific activities of hexose diphosphatase in the liver of normal foetal rats and those treated with triamcinolone. There is significant depression of activity to about half the mean specific activity of the enzyme in the untreated control animals. The specific activities in hormone-treated animals and their saline-injected litter mates did not differ significantly.

Fig. 6 compares the specific activities of pyruvate carboxylase in the liver of normal foetal rats and those treated with triamcinolone. No significant effect can be seen. The enzymic activities in hormone-treated animals were sometimes depressed 10% below those in the saline-injected litter mates.

For phosphopyruvate carboxylase, Fig. 2 shows there to be no activity in foetal liver and no activity

was ever detected in the triamcinolone-treated or saline-injected litter-mate animals.

The effect of triamcinolone on the activities of the two glycolytic enzymes in foetal rat liver was anomalous. With pyruvate kinase, which shows little change in specific activity during late foetal development, the activity was depressed to a mean level about 75% of the normal mean values found in foetal liver between 17 days of gestation and term. For phosphofructokinase, treatment with triamcinolone resulted in an increase of activity to a mean level about 175% of the normal values. After the treatment with triamcinolone, aspartate aminotransferase activity was depressed to 65% of the values found in the normal foetal livers.

Fig. 5. Effect of triamcinolone on liver hexose diphosphatase activity of foetal rats *in vivo*. Details are as given in Fig. 4. Activities are expressed as μ moles of fructose 6-phosphate produced/mg. of protein/hr. at 37°.

With these three enzymes there was, again, no significant difference between activities in saline-injected animals and the hormone-injected litter mates. With the exception of tyrosine aminotransferase, there was no correlation of the time of exposure to hormone and the observed effects on enzyme activity.

When saline alone was injected into all foetuses, none of the enzymic activities described above differed significantly from those in normal unoperated animals.

DISCUSSION

The normal development of hexose diphosphatase activity in neonatal rat liver was previously reported by Ballard & Oliver (1962), who found that the major part of the activity developed after birth. This enzyme was reinvestigated because the original method of assay is now known to underestimate the activity considerably, owing to inhibition of the enzyme at the high substrate concentration used (Krebs, 1963; Weber, 1964; Underwood & Newsholme, 1965). The data reported here confirm the previous results with respect to the developmental process, but the absolute activities are much higher than the original values if expressed on the same basis. The spectrophotometric assay of Underwood &

Fig. 6. Effect of triamcinolone on liver pyruvate carboxylase activity of foetal rats *in vivo*. Each point represents the specific activity in soluble extracts of freeze-dried homogenates prepared from two pooled livers of foetal rats each injected *in utero* with 0.5 mg. of triamcinolone. The enclosed area represents the mean specific activity ± 2 S.D. of the mean of liver pyruvate carboxylase activity in normal unoperated foetal rats (data from Fig. 2). Other details are as given in Fig. 4. Activities were determined at daily intervals from 18 days of gestation to term. Activities are expressed as μ moles of oxaloacetate produced/mg. of protein/hr. at 37°.

Newsholme (1965) also allowed characterization of the enzyme present in the foetal liver. It appears to be the specific hexose diphosphatase since the activity is inhibited by AMP and fructose 1,6-diphosphatase, properties found for the partially purified enzyme from adult rat liver (Underwood & Newsholme, 1965; J. Zalitis & I. T. Oliver, unpublished work).

The modified assay procedures for glucose 6-phosphatase, which overcome the problems outlined by Imsande & Ephrussi (1964), confirm the presence of activity in the late foetal liver, but it has not been possible to characterize the enzyme further, except with respect to its intracellular location. The assay procedure utilized for pyruvate carboxylase is highly specific since the activity is demonstrable only in the presence of acetyl-CoA. The enzyme is very active in the foetal liver, reaching adult values just before term and showing the familiar 'overshoot' phenomenon in early postnatal life.

The only key enzyme of gluconeogenesis not detectable in foetal liver is phosphopyruvate carboxylase. The next paper (Yeung & Oliver, 1967b) describes an assay procedure that, despite its sensitivity, fails to detect activity in the late foetal liver.

In the rat, Nordlie & Lardy (1963) found that more than 90% of the phosphopyruvate carboxylase activity is in the soluble fraction of liver homogenates, and Dr F. J. Ballard (personal communication) has confirmed this finding by using the assay method of Chang & Lane (1966). Dr Ballard also showed that there was essentially no activity in the soluble fraction of foetal rat liver and only a low activity in the particulate fraction from the same source. Yeung & Oliver (1967b) were unable to detect activity in the washed particulate fraction from foetal rat liver. The postnatal development of the enzyme that Dr Ballard found is similar to the results reported here.

Yeung & Oliver (1967b) have presented evidence that the development of phosphopyruvate carboxylase completes the chain of enzymes involved in gluconeogenesis and thus leads to the functional activity of the overall pathway, a process that normally occurs only in the postnatal rat liver. Premature delivery of rat foetuses leads to precocious development of the enzyme and of gluconeogenesis assessed by incorporation of [^{14}C]pyruvate into glucose by liver slices, and the two processes are related by a linear function.

Glucocorticoids have been reported on numerous occasions to promote elevated activities of the four key hepatic enzymes of gluconeogenesis in adult animals *in vivo*. However, none of the studies were carried out simultaneously on all four enzymes. Weber *et al.* (1955, 1961, 1962, 1965b), Ashmore *et al.* (1956) and Kvam & Parks (1960) reported elevation of the activities of hepatic glucose 6-phosphatase or hexose diphosphatase in adult rats treated with cortisone derivatives and triamcinolone. Similarly, the elevation of the activities of pyruvate carboxylase (Henning *et al.* 1963, 1966) and phosphopyruvate carboxylase (Shrago *et al.* 1963; Ashmore *et al.* 1964; Henning *et al.* 1966) was shown to follow treatment with cortisone derivatives. However, Foster *et al.* (1966) have shown that the effect on phosphopyruvate carboxylase is only transient and an indirect effect on enzyme synthesis has been postulated. Weber, Singhal & Srivastava (1965a) have suggested that the synthesis of the four key gluconeogenic enzymes is controlled by steroid hormones at the genetic level and, further, that the structural genes specifying the enzymes are organized in an operon, similar to the bacterial models proposed by Jacob & Monod (1961).

The four enzymes make their first appearance in

rat liver during perinatal development and, if the postulates of Weber *et al.* (1965a) are correct, treatment of the foetus with steroid hormones might promote the precocious appearance of activity, or would elevate activity after it had appeared. Experimental results reported here and elsewhere give evidence against the specific activation of a 'gluconeogenic operon' by glucocorticoid hormones.

The triamcinolone regime depressed the activity of hexose diphosphatase in foetal liver, but the effect was less marked on glucose 6-phosphatase. There was no significant effect on pyruvate carboxylase activity and the hormone treatment did not promote appearance of phosphopyruvate carboxylase in the foetal liver. There appeared to be no dependence of these effects on the time of exposure to hormone. Although the hormone dose was high (500 $\mu\text{g.}$ /foetus), unabsorbed hormone was always found in the abdominal cavity and the elevation seen in tyrosine aminotransferase and phosphofructokinase activities argues against severe toxicity, as does the low foetal mortality rate in the experiments.

Triamcinolone promoted the prenatal appearance of tyrosine aminotransferase activity after 10 hr. of exposure to the hormone. Sereni *et al.* (1959) were unable to detect any significant effect of hydrocortisone when foetuses were exposed to the hormone for 5 hr., a result that is consistent with the data in this paper. However, the increases in tyrosine aminotransferase activity shown after prolonged exposure indicate that the hormone was reaching a target system in the liver, whatever the precise nature of the system might be. It is also apparent that hormone was transferred between foetuses because the saline-injected litter mates showed elevated tyrosine aminotransferase activity (see Fig. 3). This effect was not due to the operative procedure, since sham-operated saline-injected foetuses show activities within the range found in normal foetuses.

The results indicate that glucocorticoids do not initiate the synthesis of gluconeogenic enzymes and other mechanisms must be sought. However, the effects of glucocorticoids on the activity of hepatic enzymes in adult animals *in vivo* suggest that such hormones play a part in control mechanisms for regulation of enzyme activities once synthesis is initiated.

Korner (1966) has recently summarized data from a wide variety of work on hormonal effects on enzyme and protein synthesis. Some of the data are inconsistent with the notion that the prime action of hormones in enzyme synthesis is at the genetic level (Karlson, 1961; Weber *et al.* 1965a). For mammalian systems both Korner (1966) and Schimke (1966) have emphasized that hormone treatment increases the activities of enzymes that

are already present and has not been shown to initiate enzyme synthesis. In this respect, it is difficult to explain the data on tyrosine aminotransferase in terms of direct hormonal activation or depression of the relevant genetic system, since the effects on enzyme activity cannot be evoked until the rat foetus has reached a certain stage of development. This fact suggests that a complex mechanism is involved in this system, in which, for example, gene activation or de-repression occurs without the immediate production of the final gene product and a more distal step in the chain of events leading to production of active enzyme is potentiated by the hormone.

Similar interpretations are possible for an unrelated liver enzyme that develops postnatally. It has been clearly shown that insulin is necessary for the resynthesis of hepatic glucokinase [high- K_m (glucose) type] in alloxan-diabetic rats (Sharma, Manjeshwar & Weinhouse, 1963; Salas, Viñuela & Sols, 1963). This enzyme is absent from the liver of foetal and newborn rats and first appears about 16 days after birth (Walker, 1963; Ballard & Oliver, 1964). The enzyme appears to be newly synthesized, since the development is blocked by actinomycin D and *p*-fluorophenylalanine (Walker & Holland, 1965). Insulin administration to postnatal rats does not advance the time at which glucokinase appears, although the presence of the hormone appears to be necessary for the production of the enzyme, since starvation and alloxan-diabetes both repress the normal development of activity. These findings again suggest the operation of a basic 'trigger' mechanism that sets up the potentiality for enzyme synthesis. In this case, since the insulin status of the postnatal rat appears to be like that of adults (Walker & Holland, 1965), active enzyme production rapidly follows the primary event. It is obviously of interest in developmental biology to know more of the properties of such initial 'trigger' mechanisms.

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