

## Gluconeogenesis from Amino Acids in Neonatal Rat Liver

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1. The utilization of amino acids for gluconeogenesis by rat liver develops in postnatal life, reaching maximum activity at the fifth day. 2. The activity of aspartate transaminase shows a similar trend in postnatal development and the increased activity appears to be due to the soluble enzyme. 3. The activity of alanine transaminase is low in foetal and postnatal rat liver and increases in activity at about the twentieth day. 4. Aspartate, glutamate and alanine make a major contribution to gluconeogenesis in the postnatal rat liver.

In earlier work (Ballard & Oliver, 1962, 1963, 1965; Ballard, 1965) it was demonstrated that the capacity for hepatic gluconeogenesis is acquired in the rat during the first few days of extrauterine life and that the late foetal liver is inactive in this function. In the earlier studies the development of gluconeogenesis was followed by determination of several of the obligatory enzymes of gluconeogenesis and by measurement of the incorporation of labelled pyruvate into glycogen and glucose by liver slices.

Since the milk diet of young mammals is rich in protein, these earlier results have now been extended by enzymic and isotope studies of the utilization of amino acids for carbohydrate synthesis in the neonatal rat liver.

### MATERIALS AND METHODS

#### *Animals*

Foetal, postnatal and adult rats of the Wistar albino strain of the species *Rattus norvegicus* were bred in the preclinical animal house of this University. Adult male rats were 3 months old.

#### *Experiments with liver slices*

Livers were removed from animals immediately after decapitation and chilled in an oxygenated medium of composition 70 mM-KCl-20 mM-MgCl<sub>2</sub>-40 mM-KHCO<sub>3</sub>-10 mM-CaCl<sub>2</sub> (adjusted to pH 7.45 with 0.01 N-HCl). Slices of 0.3 mm. uniform thickness were prepared as described by Ballard & Oliver (1963). Liver slices were blotted on filter paper and 150 mg. of tissue was transferred to a flat-bottomed flask containing 2 ml. of the oxygenated medium and 0.7  $\mu$ C of <sup>14</sup>C-labelled algal-protein hydrolysate (The Radiochemical Centre, Amersham, Bucks.). Then 0.1 ml. of a solution of carrier amino acids was added and the flasks were gassed with O<sub>2</sub>+CO<sub>2</sub> (95:5). The flasks were stoppered and shaken at 80 oscillations/min. at 37° for 2 hr. The solution of carrier amino acids consisted of a mixture of 15 L-amino acids of equivalent composition to the <sup>14</sup>C-labelled algal-protein hydrolysate. The concentrations in

mg./100 ml. were as follows: alanine, 5; arginine, 5; aspartic acid, 6; glutamic acid, 7; glycine, 3; histidine, 2; leucine, 9; isoleucine, 4; lysine, 6; phenylalanine, 4; proline, 5; serine, 2; threonine, 2; tyrosine, 4; valine, 4 (details supplied by The Radiochemical Centre).

The reaction was stopped by addition of 2 ml. of 60% (w/v) KOH. The tissue was digested by heating at 100° for 7 min. with shaking. Glycogen was purified by the procedure of Cowgill & Pardee (1957). The glycogen precipitates were washed free from soluble radioactive compounds by solution in hot water and reprecipitation with ethanol several times. They were dissolved in water for measurement of radioactivity and glycogen was determined with the anthrone reagent (Scott & Melvin, 1953). Oyster glycogen was used as standard.

Radioactivity was determined by liquid-scintillation counting. The purified glycogen was diluted in water to contain less than 2.5 mg./ml. and duplicate 0.2 ml. samples were pipetted into clear-glass counting vials. Then 5 ml. of Ditol (Herberg, 1960) was added and radioactivity determined in the Ekco liquid-scintillation counter. The efficiency of detection of <sup>14</sup>C was 50%. Addition of internal standards of <sup>14</sup>C-labelled glucose showed that no quenching corrections were necessary.

Results were calculated as counts/min. incorporated into the total glycogen present in 1 g. of liver (counts/min./g. of liver). The specific radioactivity of the glycogen was not used as a measure of incorporation from amino acids since its value is dependent on the amount of glycogen initially present in the slices, and the concentration of glycogen in the liver varies considerably with the age of the neonatal animal (see Ballard & Oliver, 1964). Previous papers have discussed the requirements for quantitative recovery of glycogen from neonatal rat liver slices (Ballard & Oliver, 1964) and these requirements were strictly observed in the present work. The incorporation rate of <sup>14</sup>C from amino acids in these experiments was linear for at least 2 hr. of incubation, which was the period used.

#### *Enzyme assays*

*Alanine transaminase* (L-alanine-2-oxoglutarate amino-transferase, EC 2.6.1.2). This was assayed in liver homogenates by the method of Rosen, Roberts & Nichols (1950)

with only minor modifications in procedure. Liver homogenates were prepared in 20–40 vol. of ice-cold distilled water. Incubations were at 37°.

*Aspartate transaminase* (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1). This was assayed in liver homogenates by the method of Tonhazy, White & Umbreit (1950). Liver homogenates were prepared in 10 vol. of ice-cold 0.25 M-sucrose solution. Immediately before assay, the homogenate was diluted with 9 vol. of 0.05 M-potassium phosphate buffer (adjusted to pH 7.4 with N-KOH). Incubations were at 37°.

#### *Spectrophotometric assays of soluble transaminases*

*Alanine transaminase.* Livers from rats were homogenized in 5 vol. of ice-cold 0.25 M-sucrose solution in a Perspex co-axial homogenizer. The homogenates were centrifuged at 200000g<sub>av</sub> for 30 min. in rotor 50 of the Spinco L-2 preparative ultracentrifuge at 6°. The clear supernatants were removed and assayed for alanine transaminase by the 'optimal' method described by Bergmeyer & Bernt (1963). The reaction mixtures consisted of 75 mM-KH<sub>2</sub>PO<sub>4</sub>-0.8 M-L-alanine-0.2 mM-NADH<sub>2</sub> (adjusted to pH 7.6 with KOH) and contained 3 units of lactate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) in a volume of 1.05 ml. Microcuvettes were used in the Gilford Multiple-Sample Absorbance Recorder at 37°. Suitable diluted samples of the enzyme (50 μl.) were added and the reaction was started by addition of 0.1 ml. of 0.3 M-oxoglutarate neutralized with NaOH. Simultaneous determinations of glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] were also run in the presence of oxoglutarate and with alanine omitted. In no case did this rate exceed 4% of the rate due to transaminase. The rate of decrease of E<sub>340</sub> was recorded for 5–8 min. and the maximum rate of change used as the measure of enzymic activity.

*Aspartate transaminase.* Livers from rats were homogenized in 4 vol. of ice-cold 0.25 M-sucrose solution in a Perspex co-axial homogenizer. The homogenate was centrifuged at 25000g in the International refrigerated centrifuge (model PR2) for 2 hr. at 0°. The clear supernatant was removed, and the residue was resuspended by homogenization in 2 vol. of 0.25 M-sucrose and centrifuged for 30 min. at 25000g and 0°. The supernatant solutions were combined and assayed for aspartate transaminase by the method of Karmen (1955) with the following modifications. The assay medium in a final volume of 1.1 ml. consisted of 0.15 mM-NADH<sub>2</sub>-10 mM-oxoglutarate-50 mM-KH<sub>2</sub>PO<sub>4</sub> (adjusted to pH 7.4 with N-KOH). Excess (3 units) of malate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H.) and an appropriate sample of liver extract was added to the microcuvette, and when the extinction was steady the reaction was started by the addition of 10 μmoles of aspartate. The rate of oxidation of NADH<sub>2</sub> was measured at 340mμ in a Gilford Multiple-Sample Absorbance Recorder at 37°.

#### *Protein estimation*

In both whole homogenates and liver extracts, protein was measured by the method of Lowry, Rosebrough, Farr

& Randall (1951). Crystalline bovine serum albumin was used as the standard.

*Identification of keto acids formed by liver from the <sup>14</sup>C-labelled protein hydrolysate.* Liver homogenates were prepared in 10 vol. of 0.25 M-sucrose solution and diluted with 9 vol. of 0.05 M-potassium phosphate buffer (pH 7.4) immediately before use.

A 0.2 μC sample of <sup>14</sup>C-labelled algal-protein hydrolysate was incubated with 0.5 ml. of diluted homogenate and 0.5 ml. of 0.1 M-oxoglutarate. The tubes were shaken at 37° for 20 min. and the reaction was stopped by the addition of 0.5 ml. of 2% (w/v) tungstic acid solution. The tubes then received 0.5 ml. of 2,4-dinitrophenylhydrazine (0.1% in 2 N-HCl) and were incubated at 25° for 15 min. The hydrazones were extracted and purified by the method of Tauber (1955) and separated on Whatman no. 3MM paper by ascending chromatography for 16 hr. in butan-1-ol-ethanol-0.5 N-NH<sub>3</sub> (7:1:3, by vol.) at room temperature (Haway & Thompson, 1953). After drying in a current of cold air, the chromatograms were scanned for radioactivity in the Nuclear-Chicago Corp. Actigraph II 4π gas-flow chromatogram scanner (speed, 6 in./hr.; time constant, 10 sec.; slit width, ½ in.; Micromil windows removed).

The <sup>14</sup>C-labelled algal-protein hydrolysate was also tested for keto acids by repeating the procedure outlined above in the absence of homogenate. Amino acid extraction by the procedure was also tested and in neither case was a significant amount of radioactivity recovered on the chromatograms. The details of the preparation supplied by The Radiochemical Centre make it unlikely that radioactive carbohydrate would be a significant contaminant.

## RESULTS

Fig. 1 shows the incorporation of <sup>14</sup>C from amino acids into liver-slice glycogen as a function of age of the donor animal. Incorporation was first

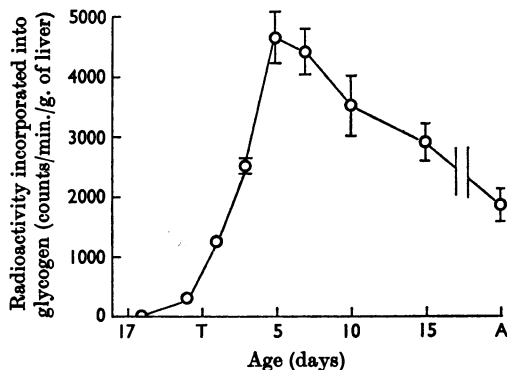


Fig. 1. Development of glyconeogenesis from amino acids in neonatal rat liver. Liver slices were incubated with <sup>14</sup>C-labelled algal-protein hydrolysate for 2 hr. as described in the text. Each point represents the mean of determinations on four to six animals in the postnatal region. Prenatal values were obtained on pooled liver slices from foetal animals. The vertical bars represent the s.e.m. values where they are large enough to be shown. T, Term; A, adult.

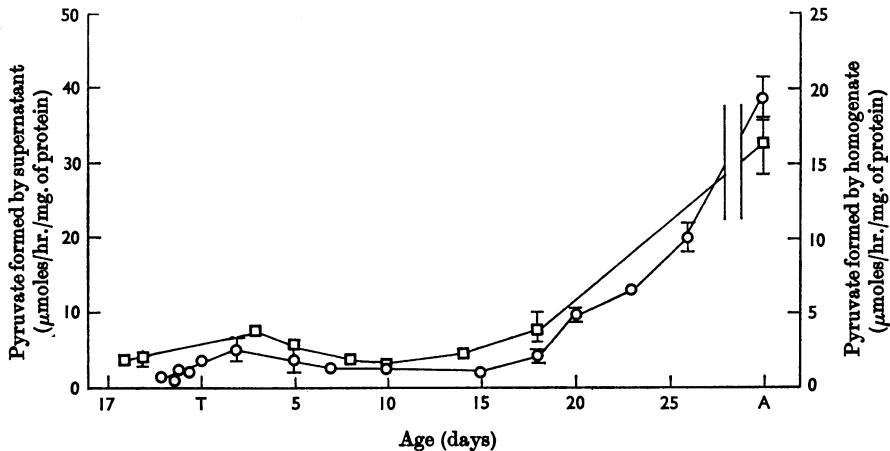


Fig. 2. Specific activity of alanine transaminase during development of neonatal rat liver. Each point represents the mean of determinations on four to six animals. Prenatal values for the supernatant enzyme were obtained on extracts of pooled livers from foetal animals. The vertical bars represent the s.e.m. values where they are large enough to be shown. T, Term; A, adult.  $\square$ , Homogenate activity;  $\circ$ , soluble supernatant activity.

detected at the twentyfirst foetal day and then rose rapidly, reaching a maximum at the fifth postnatal day. A gradual decline to the adult value followed.

Fig. 2 presents the specific activity of alanine transaminase in rat liver homogenates and in the supernatant fraction as a function of age. The enzyme activity showed no significant increase until after the twentieth postnatal day. The enzyme activity extracted from perinatal or adult rat liver was unchanged by preincubation with pyridoxal phosphate.

Fig. 3 shows the specific activity of aspartate transaminase in whole-liver homogenates and in the supernatant fraction. The enzyme is readily detectable in late foetal liver and showed a postnatal rise in specific activity of approximately twofold in whole homogenates. The activity determined in the supernatant rose about fourfold in postnatal life, reaching maximum activity at the tenth day. Most of the postnatal increase in total activity is thus due to the soluble enzyme. The shapes of the curves in both Figs. 2 and 3 are essentially the same when expressed on the basis of enzyme activity/g. fresh wt. of liver.

The experiments on radioactive keto acid production from the  $^{14}\text{C}$ -labelled algal-protein hydrolysate showed that in 10-day postnatal liver the major product of transamination was oxaloacetate. The chromatograms of the dinitrophenylhydrazones showed that the total radioactivity was distributed as follows: 67% in oxaloacetate, 15% in pyruvate, 12% in  $\alpha$ -oxoglutarate and 6% in an unidentified

spot travelling behind  $\alpha$ -oxoglutarate. The algal-protein hydrolysate contained alanine, aspartate and glutamate in similar concentrations (5, 6 and 7% respectively).

## DISCUSSION

Previous work on the postnatal development of glucose 6-phosphatase (Weber & Cantero, 1957; Kretchmer, 1959; Coquoin-Carnot & Roux, 1960; Dawkins, 1960; Burch *et al.* 1963), hexose diphosphatase (Ballard & Oliver, 1962), the conversion of malate into phosphoenolpyruvate (Ballard & Oliver, 1963) and the incorporation of [ $^{14}\text{C}$ ]pyruvate into glycogen (Ballard & Oliver, 1963) and glucose (Ballard, 1965; Ballard & Oliver, 1965) by liver slices indicated the postnatal development of this system in rat liver. The results on liver slices further indicated that the development of the specific enzymes was mirrored by functional development of the overall metabolic pathway.

In this work, the utilization of amino acids for glycogen synthesis follows the developmental pattern predicted from the earlier results. It appears unlikely that a postnatal change in permeability of liver slices to amino acids could explain the observed results, and Wise & Oliver (1967) showed that the size of the amino acid pool in neonatal rat liver, expressed as leucine, varies only between 4.5 and 5.5 mg./g. fresh wt. of tissue. However, the utilization of amino acids for the synthesis of carbohydrate is initiated by the transaminases and other enzymic systems that convert

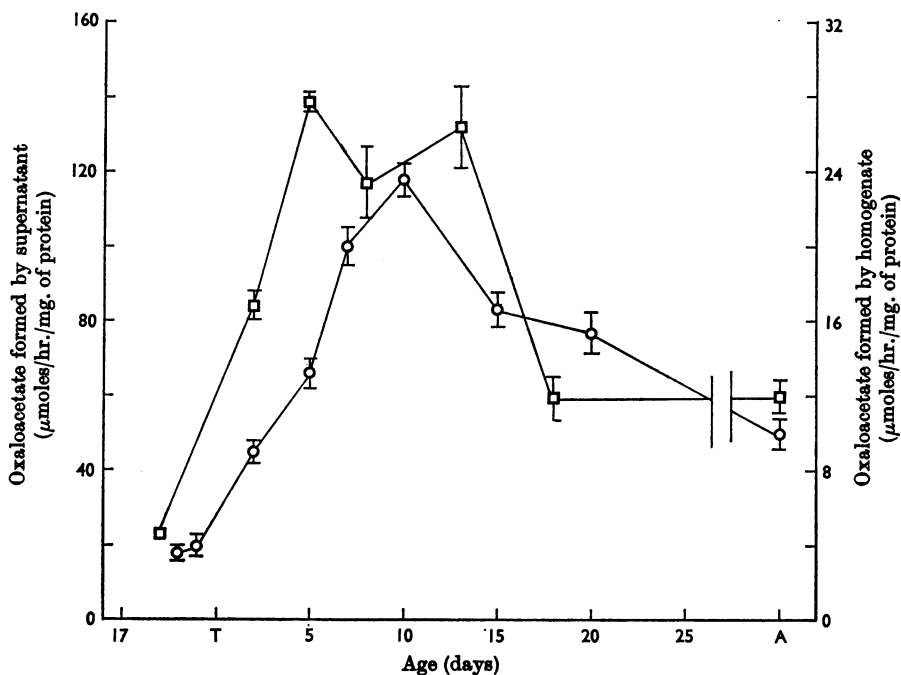


Fig. 3. Specific activity of aspartate transaminase during development of neonatal rat liver. Each point represents the mean of determinations on six animals. The vertical bars represent the s.e.m. values where they are large enough to be shown. T, Term; A, adult. □, Homogenate activity; ○, soluble supernatant activity.

amino acids into intermediates of carbohydrate metabolism. The overall process may thus be controlled by enzymes operating at the initial steps of degradation. For example, various transaminases have been reported to develop activity in rat liver at the time of birth, including tyrosine transaminase (Auerbach & Waisman, 1959; Kretchmer, Levine, McNamara & Barnett, 1956; Kretchmer & McNamara, 1956), phenylalanine transaminase (Kenney & Kretchmer, 1959), tryptophan transaminase (Trappit, 1964) and histidine transaminase (Makoff & Baldrige, 1964).

After short-term incubation of neonatal rat liver homogenates with  $\alpha$ -oxoglutarate and the  $^{14}\text{C}$ -labelled amino acid mixture, oxaloacetate, pyruvate and  $\alpha$ -oxoglutarate together accounted for 94% of the radioactive keto acids that were formed. Although aspartate, alanine and glutamate are the direct precursors of these keto acids, other amino acids give rise to them during their metabolism. Under the conditions of the experiment keto acids directly derived from the other amino acids might be expected to accumulate unless their rate of formation is slow, their subsequent metabolism is very rapid or the initial metabolic transformation occurs by pathways other than transamination. Little quantitative information about the relative

rates of alternative degradative pathways of amino acids is available, but transamination appears to play a significant physiological role (Meister, 1965). The results of Awapara & Seal (1952) indicate that in rat liver alanine and aspartate are most rapidly deaminated through this pathway, leucine and phenylalanine are deaminated to a smaller extent and the other amino acids are only slowly metabolized. It seems likely that the major contribution to the observed gluconeogenesis is made by aspartate, alanine and glutamate. A marked postnatal increase in soluble aspartate-transaminase activity was observed, whereas alanine transaminase, although weakly active in neonatal rat liver, increased significantly only in late postnatal life.

The late postnatal development of alanine transaminase reported here is somewhat different from the results of Kafer & Pollak (1961). These authors, measuring glutamate synthesis, reported enzyme activities to be higher in foetal rat liver homogenates than in adult liver. However, when alanine production was measured the activities were higher in adult liver than in the foetus, and the results are thus difficult to interpret. In guinea pig, which is considerably more mature at birth than is the rat, the liver enzyme shows a rapid increase in activity at term and reaches adult

values soon afterwards, at 10 days *post partum* (Uusitalo, 1966). Nakata, Suematsu & Sakamoto (1964) have also reported postnatal increases in the activities of the soluble forms of both alanine transaminase and aspartate transaminase in rat liver, but their results show parallel development of both the enzymes. The mitochondrial enzymes showed no postnatal increase in activity. Our own results show non-parallel development of the two transaminases: aspartate transaminase reaches maximum activity well before alanine transaminase, and the difference in the time of development was consistently observed with each of the different assay procedures employed.

It is noteworthy that Lardy, Paetkau & Walter (1965) have implicated aspartate transaminases, both intra- and extra-mitochondrial, as essential enzymes for gluconeogenesis from pyruvate via oxaloacetate in rat liver. The correlation between the postnatal development of the transaminase, the other obligatory enzymes of gluconeogenesis and the utilization of amino acids and pyruvate for carbohydrate synthesis reported in this and other papers is in accord with the suggestions of Lardy *et al.* (1965).

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