The Development of Gluconeogenesis in Rat Liver

CONTROLLING FACTORS IN THE NEWBORN

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1. Measurements in livers of rats delivered by Caesarian section show a rapid change in the relative proportion of adenine nucleotides. By 20 min the ATP/ADP ratio had increased from 1.76 to 8.7 and the value of the relationship $[ATP][AMP]/[ADP]^2$ increased from 1.0 to 4.4. These changes are dependent on the availability of oxygen to the animal. 2. The free $[NAD^+]/[NADH]$ ratio in the liver cytosol increases from 180 after delivery to reach a maximum of 1010 at 2 h, before falling to 540 in the 24 h-old animal. 3. The mitochondrial NAD redox potential also shows a sharp increase towards a more oxidized state in livers of delivered rats. 4. These results probably indicate that the foetal liver is hypoxic, with oxygenation occurring in the first hour after delivery. 5. Measurements in livers of naturally born rats 2 min after birth also suggest that this tissue is hypoxic with an ATP/ADP ratio of 1.83 and a free $[NAD^+]/[NADH]$ ratio of 117. 6. Concentrations of intermediates in the gluconeogenic pathway have been determined in livers of foetal, 1 h-old and 1-day-old rats. These experiments imply a facilitation of lactate dehydrogenase and glucose 6-phosphatase activities by 1 h after birth, and a stimulation of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase steps by 1 day after birth. 7. The appearance of gluconeogenesis in livers of newborn rats seems therefore to involve an oxygenation stage followed by an increase in phosphoenolpyruvate carboxykinase activity.

Measurements of the overall pathway of gluconeogenesis in liver slices and in the intact animal show that this sequence is absent in foetal rat liver and appears soon after birth (Ballard & Oliver, 1963; Philippidis & Ballard, 1969). Although a close developmental correlation between gluconeogenesis and phosphoenolpyruvate carboxykinase activity has been noted (Ballard & Hanson, 1967; Yeung & Oliver, 1967), it is likely that the liver cell must also become oxygenated before gluconeogenesis can occur at a substantial rate. The importance of this oxygenation was shown in foetal rats where a prolonged ether anaesthesia completely suppressed gluconeogenesis even after phosphoenolpyruvate carboxykinase had been induced by prior administration of glucagon (Philippidis & Ballard, 1970). Further, artificially produced hypoxic conditions in suckling rats caused a marked inhibition of the pathway and were accompanied by decreases in the free $[NAD^+]/[NADH]$ ratios in hepatic cytosol and mitochondrial compartments (Ballard, 1971).

For an understanding of the regulation of gluconeogenesis in the newborn, it is important to know whether liver oxygenation does occur after birth and

whether it coincides with the appearance of phosphoenolpyruvate carboxykinase activity. In the present work the contents of liver intermediates have been followed for several hours after birth. These measurements have been used to identify control sites in gluconeogenesis and to assess the relative timing of oxygenation and gluconeogenesis.

MATERIALS AND METHODS

Chemicals. L-[U- ^{14}C]Lactate and $NaH^{14}CO_3$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Enzymes and nucleotides required for the enzymic measurement of hepatic intermediates were from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany.

Animals. The rats used were hooded Wistar animals from the Divisional colony. Pregnant animals at days 21 or 22 of gestation were lightly anaesthetized with ether and the uterus was exposed by a longitudinal incision. Foetuses were carefully removed from the uterus without disturbing the umbilical blood supply. The livers of some foetuses were frozen *in situ* with Wollenberger clamps cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960) and the remaining foetuses were

delivered by Caesarian section and immediately transferred to a Humidicrib maintained at 35°C. Livers of these animals were freeze-clamped at various times after delivery. Preliminary measurements of hepatic intermediates and enzymes indicate a considerable variability between litters. Since this variability appeared to be due to differences in the weight of foetuses and the size of the livers, attempts were made to standardize these parameters. To accomplish this foetuses were used only if they weighed at least 5.0g and were from litters containing 10 or more animals. The weight range chosen was similar to that found in vaginally delivered rats. Animals used at times less than 5h after birth were not fed, whereas the 24h-old rats were from naturally delivered litters and were kept with their mothers until used.

Extraction of liver samples. Frozen livers were powdered while cooled by liquid N₂ and then weighed and extracted by homogenization in 2ml of 5% (v/v) HClO₄. After centrifugation the precipitate was re-extracted with 1ml of 5% (v/v) HClO₄ and adjusted to pH 6.5 with 2M-KHCO₃ and centrifuged at 0°C to remove KClO₄. The supernatants were used for the measurement of intermediates.

Measurements of liver metabolites. All compounds were measured spectrophotometrically by specific enzymic assays. When increased sensitivity was required several frozen livers were pooled before the preparation of extracts, and assays were performed in cuvettes with 5 cm light-paths and a volume of 2 ml.

Tissue intermediates were measured by the following methods: lactate (Hohorst, 1965, p. 266), pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate (Czok & Eckert, 1965), dihydroxyacetone phosphate and fructose 1,6-diphosphate (Bücher & Hohorst, 1965), glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1965, p. 134), oxaloacetate and aspartate (Pfleiderer, 1965), citrate (Dagley, 1965), malate (Hohorst, 1965, p. 328), acetoacetate (Mellanby & Williamson, 1965), β -hydroxybutyrate (Williamson & Mellanby, 1965), ATP (Lamprecht & Trautschold, 1965); ADP and AMP (Adam, 1965); α -oxoglutarate (Bergmeyer & Bernt, 1965); ammonia (Haeckel & Haeckel, 1970) and glutamate (Bernt & Bergmeyer, 1965). For ammonia measurements all solutions were prepared in water that had been deionized by passage through a mixed-bed ion-exchange resin. Nevertheless, a blank corresponding to

the oxidation of approx. 5nmol of NADH was always found. A slightly larger value was obtained when measurements were made on a supernatant prepared by centrifuging a solution of 5% (v/v) HClO₄ that had been neutralized with 2M-KHCO₃. These values for ammonia in 'blank extracts' usually represented 10–30% of the total NADH oxidation in the assay and were subtracted from the test measurements.

Inorganic phosphate was determined colorimetrically as described by Taussky & Shorr (1953).

Enzyme assays. Glutamate dehydrogenase activity was measured in the direction of NADH oxidation in extracts of freeze-dried liver homogenates by using the incubation conditions given by Haeckel & Haeckel (1970) for ammonia estimation but including 0.15M-NH₄Cl. One unit of activity is defined as the oxidation of 1 μ mol of NADH/min at 25°C. Phosphoenolpyruvate carboxykinase was measured as described by Chang & Lane (1966) with slight modifications (Ballard & Hanson, 1967). One unit of activity is defined as the incorporation of 1 μ mol of NaH¹⁴C₃/min at 37°C. Phosphoenolpyruvate carboxykinase was assayed in supernatants prepared by centrifuging 10% (w/v) homogenates of liver in 0.25M-sucrose at 100000g for 15 min.

Gluconeogenesis measurements. These were carried out in whole animals after injection of 50 μ mol (0.5 μ Ci) of sodium L-[U-¹⁴C]lactate. Gluconeogenic rates are expressed as the percentage of injected radioactivity recovered in glucose plus glycogen at 15 min after radioisotope administration. Appropriate corrections were made for uncharged radioactive impurities (Ballard, 1971).

RESULTS

When foetal rats are delivered by Caesarian section, the relative proportions of hepatic adenine nucleotides change rapidly towards a more phosphorylated state. The first of these changes occurs within 10 min of delivery, when the ADP concentration has fallen (Table 1). Neither ATP, AMP nor P_i concentrations are altered at this time. Consequently the [ATP]/[ADP] ratio increases from 1.76 in foetal liver to 2.59 at 10 min after delivery, and the proportion of 'high-energy' bonds in the adenine nucleotide pool (the adenylate charge,

Table 1. Concentrations of adenine nucleotides and P_i in livers of foetal and delivered rats

All concentrations are expressed as μ mol/g ($\pm$ S.E.M.) with the number of determinations in parentheses. Other details are given in the Materials and Methods section. All four measurements were made on each liver sample.

Time after delivery	ATP	ADP	AMP	P _i
0	0.995 $\pm$ 0.086 (9)	0.564 $\pm$ 0.021	0.359 $\pm$ 0.037	4.61 $\pm$ 0.20
10 min	0.987 $\pm$ 0.200 (7)	0.380 $\pm$ 0.039	0.384 $\pm$ 0.082	4.75 $\pm$ 0.36
20 min	1.35 $\pm$ 0.14 (8)	0.156 $\pm$ 0.019	0.076 $\pm$ 0.014	4.29 $\pm$ 0.30
30 min	1.62 $\pm$ 0.08 (8)	0.234 $\pm$ 0.014	0.124 $\pm$ 0.020	4.72 $\pm$ 0.67
1 h	1.70 $\pm$ 0.09 (8)	0.232 $\pm$ 0.010	0.108 $\pm$ 0.010	3.38 $\pm$ 0.25
2 h	1.98 $\pm$ 0.03 (6)	0.297 $\pm$ 0.016	0.096 $\pm$ 0.009	3.79 $\pm$ 0.38
3 h	1.85 $\pm$ 0.15 (4)	0.243 $\pm$ 0.018	0.091 $\pm$ 0.016	3.83 $\pm$ 0.38
24 h	2.08 $\pm$ 0.09 (8)	0.336 $\pm$ 0.015	0.109 $\pm$ 0.008	3.85 $\pm$ 0.20

Fig. 1. (a) Apparent equilibrium constant for the adenylate kinase reaction ($K = \frac{[ATP][AMP]}{[ADP]^2}$). (b) Adenylate charge ratio ($\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$). Both measurements were calculated from the measurements on livers from individual animals that are summarized in Table 1. Values shown are the means $\pm$ S.E.M. for the number of animals shown for each age in Table 1.

$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$; Atkinson & Walton, 1967) is not affected (Fig. 1). The relationship based on an equilibrium between adenine nucleotides catalysed by adenylate kinase ($K = \frac{[ATP][AMP]}{[ADP]^2}$) is also increased significantly at this time (Fig. 1). From 10 min to 20 min after birth there is a marked increase in the phosphorylation of adenine nucleotides with ATP increasing from 0.987 to 1.35 μ mol/g of tissue (Table 1). The adenylate charge increases to 0.90 at this time and remains at this value at least until 3 h after birth (Fig. 1). The apparent equilibrium constant for the adenylate kinase reaction is further displaced from its expected value of 1 to reach 4.4 by 20 min after birth before gradually decreasing with age to 2.0 by 24 h. There are no marked changes in P_i concentration (Table 1) but a slight decrease

Table 2. Effect of pH on the adenylate kinase equilibrium constant

Tubes containing 2 μ mol of ATP, 0.3 μ mol of AMP 2 μ mol of $MgSO_4$, 1 unit of crystalline adenylate kinase from muscle and 100 μ mol of imidazole buffer adjusted to the required pH with HCl were incubated for 15, 30 and 90 min at 37°C. At these times $HClO_4$ was added and after neutralization with $KHCO_3$ and centrifugation to remove $KClO_4$, the concentrations of individual adenine nucleotides were measured enzymically. Values are the means of two separate experiments. In each experiment the three time-periods were averaged, as equilibrium had been attained by 15 min. The equilibrium constant (K) was determined from the relationship $K = \frac{[ATP][AMP]}{[ADP]^2}$ and the adenylate charge from $\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$.

pH	Adenylate charge	K
6.0	0.839	0.96
6.5	0.845	1.14
7.0	0.846	1.24
7.5	0.842	1.28
8.0	0.837	1.27

occurs as the sum of all adenine nucleotides increases.

It is possible that the pH value of the liver cell may fall after birth as a result of the changes in adenine nucleotides and other anions. A more acidic environment might explain the increase in the apparent equilibrium constant for the adenylate kinase reaction. To test this possibility the equilibrium constant was measured as a function of pH by using crystalline adenylate kinase from muscle. The results indicate a slight fall in the equilibrium constant at a pH below 7 (Table 2) but nothing that could explain the alterations observed *in vivo*. The results also agree with calculations of the equilibrium constant at various concentrations of Mg^{2+} and H^+ ions that were computed from the dissociation constants for Mg^{2+} , H^+ and K^+ complexes of AMP, ADP and ATP (Rose, 1968).

A consequence of birth is the ready availability of oxygen to the tissues. In the foetus oxygen must traverse the placenta so that its supply to the foetal liver may be less than in the newborn. If the displacement of the apparent equilibrium constant for adenylate kinase is dependent on this increase in oxygen, young rats that are exposed to a hypoxic atmosphere would not be expected to show similar changes to those found in normally oxygenated animals. Animals from a single litter were delivered and half were left in a normal atmosphere and half in an atmosphere containing 12% air (Ballard, 1971). Measurements of hepatic adenine nucleotides showed that only in those rats exposed to air was the adenylate charge increased

Table 3. *Effect of the oxygen content of the atmosphere on the relationship $[ATP][AMP]/[ADP]^2$ in liver of newborn rats*

Foetal rats were delivered from a single litter and immediately exposed to the appropriate atmosphere. After 15 min the livers were freeze-clamped and adenine nucleotides measured. Values are the means ($\pm$ S.E.M.) for six animals in each group.

Treatment of animals	Adenylate charge	$\frac{[ATP][AMP]}{[ADP]^2}$
100% air atmosphere, 15 min	0.770 ± 0.057	2.65 ± 0.32
12% air atmosphere, 15 min	0.386 ± 0.011	0.71 ± 0.04

Table 4. *Metabolite concentrations in livers of foetal and delivered rats*

Concentrations are expressed as $\mu\text{mol/g}$ ($\pm$ S.E.M.) with the number of determinations shown in parentheses. Other details are given in the Materials and Methods section.

Time after delivery	Lactate	Pyruvate	Ammonia	α -Oxoglutarate	Glutamate
0	5.25 ± 0.46 (10)	0.094 ± 0.011 (10)	0.327 ± 0.033 (6)	0.052 ± 0.008 (6)	1.65 ± 0.17 (6)
10 min	6.40 ± 0.67 (6)	0.184 ± 0.061 (6)	0.335 ± 0.030 (8)	0.061 ± 0.013 (8)	1.86 ± 0.18 (8)
20 min	—	—	0.412 ± 0.030 (8)	0.093 ± 0.013 (8)	1.49 ± 0.17 (8)
30 min	5.33 ± 0.26 (14)	0.330 ± 0.031 (14)	0.345 ± 0.047 (8)	0.148 ± 0.024 (8)	1.20 ± 0.10 (8)
1 h	3.93 ± 0.54 (10)	0.297 ± 0.046 (10)	0.269 ± 0.043 (8)	0.219 ± 0.036 (8)	1.37 ± 0.07 (8)
2 h	1.67 ± 0.36 (7)	0.135 ± 0.022 (7)	0.223 ± 0.014 (6)	0.263 ± 0.047 (6)	1.66 ± 0.23 (6)
5 h	—	—	0.301 ± 0.053 (6)	0.155 ± 0.021 (6)	1.81 ± 0.33 (6)
24 h	0.54 ± 0.04 (15)	0.030 ± 0.003 (15)	0.291 ± 0.023 (7)	0.051 ± 0.004 (7)	1.49 ± 0.25 (7)

above the values found in foetal liver and the apparent equilibrium constant for adenylate kinase displaced beyond the theoretical value of approx. 1 (Table 3).

The concentrations of lactate, pyruvate, ammonia, α -oxoglutarate and glutamate were measured in livers of young rats (Table 4) to determine the NAD^+/NADH concentration ratios in cytosol and mitochondrial compartments. Both pyruvate and α -oxoglutarate increase markedly after delivery; by 24 h after birth the amounts of these keto acids have fallen to values that are as low as those found in the foetus. Ammonia and glutamate concentrations do not vary significantly during the 24 h after birth, whereas the lactate concentration begins to decrease about 30 min after delivery and continues until a content of $0.54 \mu\text{mol/g}$ is attained in the 24 h-old animals.

Free NAD^+/NADH ratios in the cell cytosol were calculated from lactate and pyruvate concentrations and the equilibrium constant for the lactate dehydrogenase reaction (Williamson, Lund & Krebs, 1967a). This ratio increases from 180 in foetal liver to a maximum of 1010 by 2 h after birth and then falls to 540 in the livers of 1-day-old animals (Fig. 2). The more oxidized environment suggested from the present results with foetal rats, compared with the results from an earlier study (Philippidis & Ballard, 1970) probably reflects a change in technique from rapid excision of livers

and freezing in dichlorodifluoromethane to freeze clamping *in situ*. The mitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio calculated from the components of the glutamate dehydrogenase reaction (Williamson *et al.* 1967a) indicates a change from a relatively reduced situation in foetal liver to a more oxidized one in the newborn. The $[\text{NAD}^+]/[\text{NADH}]$ ratio then falls from 8.9 at 1 h after birth to 2.6 in the 1-day-old animal.

One condition that is necessary for the validity of these calculations of $[\text{NAD}^+]/[\text{NADH}]$ ratios is that the activities of the respective dehydrogenases are sufficient to maintain equilibrium between the reaction constituents. The comprehensive studies of Veech, Eggleston & Krebs (1969) indicate that most, if not all, cellular dehydrogenases do maintain such an equilibrium and that similar $[\text{NAD}^+]/[\text{NADH}]$ ratios can be obtained from the reactants of several dehydrogenases in the same hepatic cell compartment. Therefore the calculations made in the present work are probably justified if the activities of lactate dehydrogenase and glutamate dehydrogenase in livers of foetal and newborn rats are comparable with the activities found in liver from adult animals. Burch *et al.* (1963) have shown that lactate dehydrogenase activity in foetal liver is similar to that found in the adult, whereas glutamate dehydrogenase assays (Table 5) indicate that this enzyme is present in foetal liver at 40% of the activity found in adult liver.

Fig. 2. Free [NAD⁺]/[NADH] ratios calculated for (a) the mitochondrial fraction of liver, from the [glutamate]/[α-oxoglutarate][ammonia] ratio, and (b) the cytosol fraction of liver, from the [lactate]/[pyruvate] ratio. Each ratio was calculated from measurements on an individual liver so that the values plotted are the means±S.E.M. of these ratios. The number of animals used at each age is shown in Table 4.

Table 5. *Glutamate dehydrogenase activities in livers of developing rats*

Activity was measured as described in the Materials and Methods section and is expressed as the mean±S.E.M. for five determinations at each age.

Age	Activity (μmol/min per g of liver)
21 day foetus	34.3±2.9
1 day	60.8±7.3
7 days	75.5±11.2
Adult	86.9±10.1

The fall in the [NAD⁺]/[NADH] ratio by 24h after birth is more pronounced in the mitochondrial compartment than in the cytosol compartment.

Table 6. *Measurement of mitochondrial NAD redox state*

The [NAD⁺]/[NADH] ratio has been calculated from measurements of β-hydroxybutyrate and acetoacetate or of glutamate, ammonia and α-oxoglutarate (Williamson *et al.* 1967a). Values are the means±S.E.M. of determinations on eight 1-day-old animals.

β-Hydroxybutyrate (μmol/g)	0.62 ± 0.06
Acetoacetate (μmol/g)	0.23 ± 0.02
[NAD ⁺]/[NADH]	7.4
Glutamate (μmol/g)	1.48 ± 0.09
α-Oxoglutarate (μmol/g)	0.042 ± 0.005
Ammonia (μmol/g)	0.43 ± 0.04
[NAD ⁺]/[NADH]	3.2

Table 7. *Metabolite concentrations in livers of normally born rats*

All measurements were made on freeze-clamped livers of rats 2min after natural birth. Values are the means±S.E.M. for determinations on eight animals from two litters.

Measurement	Value
ATP (μmol/g wet wt.)	1.21 ± 0.11
ADP (μmol/g wet wt.)	0.663±0.060
AMP (μmol/g wet wt.)	0.348±0.047
Adenylate charge	0.693±0.033
[ATP][AMP]/[ADP] ²	1.09 ± 0.23
Lactate (μmol/g wet wt.)	5.07 ± 0.61
Pyruvate (μmol/g wet wt.)	0.066±0.006

This difference may be a true result, caused perhaps by oxidation of lipid at this age, or could be an artifact induced if the components of the glutamate dehydrogenase reaction are not at equilibrium, or if they are distributed unevenly throughout the cell. This could perhaps be caused by the substantial protein degradation and ammonia production that is associated with a rapid rate of gluconeogenesis. By 24h after birth ketogenesis has developed (Hahn, 1970) and β-hydroxybutyrate dehydrogenase is active, so that the concentrations of acetoacetate and β-hydroxybutyrate can be used as an independent measure of the [NAD⁺]/[NADH] ratio in mitochondria. In Table 6 are presented measurements of acetoacetate, β-hydroxybutyrate, ammonia, α-oxoglutarate and glutamate in livers of 1-day-old rats. Although the effects of animal variation have been minimized by assaying all intermediates in each liver, the [NAD⁺]/[NADH] ratio calculated from the β-hydroxybutyrate dehydrogenase reaction indicates a more oxidized pool of NAD than is obtained by measuring the glutamate dehydrogenase reactants.

Fig. 3. Crossover plot comparing the concentrations of gluconeogenic intermediates in livers of 1h-old rats (●) with the respective compounds in livers of foetal rats (○). Values shown are the means $\pm$ S.E.M. for eight to ten determinations. Abbreviations used and the concentrations of each intermediate in foetal liver are: lactate (Lac), $5.74 \mu\text{mol/g}$; pyruvate (Pyr), $0.074 \mu\text{mol/g}$; oxaloacetate (OAA), $0.0075 \mu\text{mol/g}$; phosphoenolpyruvate (PEP), $0.033 \mu\text{mol/g}$; 2-phosphoglycerate (2PGA), $0.012 \mu\text{mol/g}$; 3-phosphoglycerate (3PGA), $0.082 \mu\text{mol/g}$; dihydroxyacetone phosphate (DAP), $0.012 \mu\text{mol/g}$; fructose 1,6-diphosphate (FDP), $0.0059 \mu\text{mol/g}$; fructose 6-phosphate (F6P), $0.113 \mu\text{mol/g}$; glucose 6-phosphate (G6P), $0.406 \mu\text{mol/g}$.

Concentrations of adenine nucleotides and of pyruvate and lactate were measured in the livers of naturally born rats at approx. 2min after birth (Table 7). The adenylate charge ratio, the adenylate kinase equilibrium relation ($[\text{ATP}][\text{AMP}]/[\text{ADP}]^2$), the concentrations of individual adenine nucleotides and of lactate are all similar to values determined in livers of foetal rats. The pyruvate concentration ($0.066 \mu\text{mol/g}$) in normally delivered animals was 70% of that observed in foetal rats and only 36% of that found in livers of rats 10 min after Caesarian delivery.

A comparison of hepatic concentrations of gluconeogenic intermediates was made between foetal rats and animals 1h after Caesarian delivery. In Fig. 3 the concentration of each compound in 1h-old animals is expressed as a percentage of that in foetal rats, and the metabolites are arranged for graphical representation in sequence from lactate to glucose 6-phosphate. The concentrations of lactate and pyruvate in livers of 1h-old rats are 52 and 292% of the respective foetal values,

Fig. 4. Crossover plot comparing the concentrations of gluconeogenic intermediates in livers of 1day-old rats (●) with those of the respective compounds in livers from rats 1h after delivery (○). Values are the means $\pm$ S.E.M. for eight to ten determinations. Abbreviations used are the same as in Fig. 3. The concentrations of each intermediate in livers from 1h-old rats are: lactate, $2.96 \mu\text{mol/g}$; pyruvate, $0.216 \mu\text{mol/g}$; oxaloacetate, $0.0162 \mu\text{mol/g}$; phosphoenolpyruvate, $0.037 \mu\text{mol/g}$; 2-phosphoglycerate, $0.013 \mu\text{mol/g}$; 3-phosphoglycerate, $0.091 \mu\text{mol/g}$; dihydroxyacetone phosphate, $0.016 \mu\text{mol/g}$; fructose 1,6-diphosphate, $0.0052 \mu\text{mol/g}$; fructose 6-phosphate, $0.037 \mu\text{mol/g}$; glucose 6-phosphate, $0.145 \mu\text{mol/g}$.

indicating a forward crossover between these compounds. Oxaloacetate is also increased in the livers of 1h-old animals, whereas phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate and dihydroxyacetone phosphate show slight increases from the concentrations in foetal liver. Fructose 6-phosphate and glucose 6-phosphate concentrations are much lower than those found in foetal liver.

An analogous comparison between metabolite concentrations in 1-day-old animals and 1h-old animals was made to resolve sites of regulation in the developing pathway of gluconeogenesis. This study (Fig. 4) shows a fall in lactate, pyruvate and oxaloacetate concentrations by 1 day after birth, with increases in the tissue contents of phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, dihydroxyacetone phosphate and fructose 1,6-diphosphate at this age. This pattern indicates a forward crossover between oxaloacetate and phosphoenolpyruvate, at the phosphoenolpyruvate carboxykinase reaction. Fructose 6-phosphate and glucose 6-phosphate show a further fall in concentration in 1-day-old animals, and maintain a constant proportion to each other.

For a forward crossover to indicate a site of activation in a crossover plot, it must be established that flux through the complete sequence is increased. Rats of the various ages used in the

crossover experiments were injected with 50 μmol of L-[U- ^{14}C]lactate and the rate of appearance of label in glucose+glycogen of the whole animal was measured (Ballard, 1971). These experiments indicate that the rate of gluconeogenesis is extremely low in foetal rats, with a slight but statistically significant increase occurring by 1 h after birth (Table 8). The rate of gluconeogenesis in 1-day-old animals is approx. 350-fold higher than in foetal animals and 70-fold higher than in rats 1 h after birth. It is recognized that the magnitude of this increase in gluconeogenesis is somewhat overestimated as a result of the diminishing lactate pool in the liver and blood of the young rat. Further, it is assumed that the gluconeogenesis measured has occurred in liver, but the kidney may contribute. Nevertheless, the results strongly suggest an increase in gluconeogenic flux from the foetal stage to 1 h after birth and from 1 h to 1 day after birth. Phosphoenolpyruvate carboxykinase meas-

urements in livers of foetal, 1h-old and 1-day-old animals show no increase in activity in the first hour after delivery and a 35-fold increase from the 1h-old animal to the 1-day-old animal (Table 8).

Concentrations of malate and aspartate in livers of foetal, 1h-old and 1-day-old animals are shown in Table 9. These intermediates have not been included in the crossover plots, since their specific role in gluconeogenesis in young rats is uncertain. Both malate and aspartate concentrations increase from the foetus to 1 h after delivery, and although the malate concentration has fallen by 24 h after birth, the hepatic concentration of aspartate shows a further increase. Oxaloacetate concentrations were calculated by assuming that malate dehydrogenase and lactate dehydrogenase are at equilibrium with the same pool of NAD in the cytosol fraction of the liver cell. By this method of calculation oxaloacetate increases fivefold from the foetus to 1 h after delivery, with a 50% decrease by 1 day

Table 8. *Gluconeogenesis and phosphoenolpyruvate carboxykinase activity in foetal and neonatal rats*

Phosphoenolpyruvate carboxykinase activity has been measured in the cytosol fraction of developing liver. Gluconeogenesis is expressed as the percentage incorporation of label into glucose+glycogen 15 min after an injection of 50 μmol (0.5 μCi) of L-[U- ^{14}C]lactate. Other details are given in the Materials and Methods section. Values are the means $\pm$ S.E.M. with the number of determinations in parentheses.

Time after birth or delivery	Phosphoenolpyruvate carboxykinase activity (units/g of liver)	Gluconeogenesis
0	0.112 $\pm$ 0.043 (5)	0.04 $\pm$ 0.01 (7)
1 h	0.111 $\pm$ 0.048 (6)	0.20 $\pm$ 0.06 (8)
24 h	3.89 $\pm$ 0.20 (6)	14.3 $\pm$ 3.0 (7)

Table 9. *Malate, aspartate and oxaloacetate concentrations in developing liver*

Values are the means $\pm$ S.E.M. for the numbers of determinations in parentheses. Oxaloacetate is calculated in method 1 from the relationship:

$$\text{Oxaloacetate} = \frac{[\text{malate}][\text{pyruvate}] K_{\text{MDH}}}{[\text{lactate}] K_{\text{LDH}}}$$

where K_{MDH} is the equilibrium constant for the malate dehydrogenase reaction at pH 7.0 (2.78×10^{-5} ; Krebs & Veech, 1969), and K_{LDH} is the equilibrium constant for the lactate dehydrogenase reaction at pH 7.0 (1.11×10^{-4} ; Krebs & Veech, 1969). Concentrations of pyruvate and lactate are the mean values in Table 4. Oxaloacetate is calculated in method 2 from the relationship:

$$\text{Oxaloacetate} = \frac{[\alpha\text{-oxoglutarate}][\text{aspartate}]}{[\text{glutamate}] K_{\text{AAT}}}$$

where K_{AAT} represents the equilibrium constant for the aspartate aminotransferase reaction at pH 7.0 (6.61; Krebs & Veech, 1969), and α -oxoglutarate and glutamate concentrations are the mean values from measurements in Table 4.

Time after birth or delivery ... Measurement	Concentration ($\mu\text{mol/g}$)		
	0	1 h	24 h
Malate	284 $\pm$ 26 (8)	334 $\pm$ 21 (10)	223 $\pm$ 12 (8)
Aspartate	254 $\pm$ 18 (8)	441 $\pm$ 34 (10)	660 $\pm$ 102 (8)
Oxaloacetate, direct measurement	7.5 $\pm$ 0.6 (6)	16.2 $\pm$ 2.2 (6)	8.3 $\pm$ 0.4 (5)
Oxaloacetate, method 1	1.3	6.3	3.1
Oxaloacetate, method 2	1.2	10.7	3.4

after birth (Table 9). This type of calculation should give oxaloacetate concentrations in the cytosol fraction if malate, lactate and pyruvate are evenly distributed throughout the cell. Oxaloacetate concentrations may also be calculated from the components of the aspartate aminotransferase reaction and the equilibrium constant. This method may estimate oxaloacetate concentration in the total liver, since aspartate aminotransferase occurs at considerable activity in the cytosol and mitochondrial compartments of foetal liver (Hanson & Ballard, 1968). Calculations of oxaloacetate content based on the aspartate aminotransferase reaction show a ninefold increase to 10.7 nmol/g, from the foetus to the 1 h-old animal and a decrease to 3.4 nmol/g in the liver of 1-day-old animals. Direct measurements of oxaloacetate show less change with development, but the direction of change is similar to those derived by calculation.

DISCUSSION

Hepatic oxygenation in the newborn. Foetal liver shows a pattern of metabolite concentrations that are typical of hypoxia (Ballard, 1971). This includes a low degree of phosphorylation of adenine nucleotides and a highly reduced NAD redox potential in both cytosol and mitochondria. Whether there is in fact a low oxygen concentration in foetal rat liver has not been established, but the maintenance of low ATP and high AMP concentrations may well reflect either a limiting availability of oxygen or alternatively a greater utilization of ATP. The second possibility is unlikely, since gluconeogenesis and urea synthesis utilize much of the ATP generated in liver (Williamson, Scholz, Browning, Thurman & Fukami, 1969b; Williamson, Scholz & Browning, 1969a), whereas both pathways are limited in foetal rat liver by the absence or near absence of at least one enzyme (Ballard & Hanson, 1967; Charbonneau, Roberge & Berlinguet, 1967).

The NAD and adenine nucleotide pools are linked by several series of reactions (Krebs & Veech, 1969), but especially since NADH is generated in the tricarboxylic acid cycle, where its oxidation is coupled to ATP synthesis. A limitation of ATP synthesis by oxygen lack would thus explain the relatively low $[NAD^+]/[NADH]$ ratios that are found in foetal rat liver.

Anaesthesia is always a problem in studies on foetal tissues. It is necessary to use anaesthesia of some type in order to obtain samples from foetuses, and this anaesthesia may itself cause changes in concentrations of foetal metabolites. Certainly a prolonged ether anaesthesia lowers the degree of phosphorylation of the hepatic adenine nucleotide pool and also decreases the free $[NAD^+]/[NADH]$

ratio in the cell cytosol (Philippidis & Ballard, 1970). As it would be difficult to determine whether the apparent hypoxic state of foetal liver is real or due to the minimal ether anaesthesia, some measurements were undertaken in livers of normally delivered rats that had just been born. Under these conditions the pattern of adenine nucleotides and of lactate and pyruvate was similar to that found in the foetal liver samples, suggesting that the values observed in the latter are indeed representative of true concentrations *in vivo*. Actually the lactate/pyruvate ratio of 77 found in livers of newborn rats (Table 7) was higher than values of 56 found in foetal animals (Table 4). This could imply a change during vaginal delivery towards an increased NADH/NAD⁺ concentration ratio in the cell cytosol. The potential for hypoxia during parturition in the rat is high. The foetuses are often born with membranes intact so that the placenta is detached from the uterus and is delivered simultaneously with the young (Barclay, Franklin & Prichard, 1944; Parkes, 1952). Such a situation would mean that the foetus is without an external source of blood oxygenation during delivery.

Adenylate kinase is localized in the cytosol or outer mitochondrial compartment of the liver cell, with very little activity within the inner compartment of mitochondria (Adelman, Lo & Weinhouse, 1968; Heldt & Schwalbach, 1967). Although the activity is probably sufficient to maintain equilibrium between ATP, ADP and AMP in the cell cytosol, it is unlikely to do so within the atracyloside-sensitive barrier of mitochondria. Presumably the displacement of the apparent equilibrium constant that occurs in livers of oxygenated newborn rats (Fig. 1) indicates a lowering of the ADP concentration within the mitochondria. This is probably caused by an increase in oxidative phosphorylation and implies that the rate of oxidative phosphorylation is more rapid than nucleotide translocation and equilibration via adenylate kinase. Increases in the apparent equilibrium constant for adenylate kinase have been noted in studies with isolated mitochondria (Williamson, Olson, Herczeg & Coles, 1967b), in livers perfused with oleate and ethanol (Williamson *et al.* 1969b) and in livers perfused with fructose (Woods, Eggleston & Krebs, 1970).

Development of gluconeogenesis. To obtain a substantial rate of gluconeogenesis, the liver cell must have all relevant enzymes present, a continual supply of energy in the forms of ATP and GTP, adequate amounts of precursor, and reasonably high concentrations of substrates for those enzymes that may be rate-limiting. When the liver of newborn rats becomes oxygenated all but one of these requirements are met. ATP concentrations are high and the ATP/ADP ratio is much greater

than that found in livers of adult rats (Philippidis & Ballard, 1970). Lactate concentrations in liver and blood remain elevated at the foetal value. Pyruvate concentrations are increased 3.5-fold, to a maximum of $0.33 \mu\text{mol/g}$, and the oxaloacetate concentration is also increased. All three methods for estimating oxaloacetate that have been used in the present study indicate a liver content of about 10 nmol/g by 1 h after birth, a value that is close to the Michaelis constant for oxaloacetate in the phosphoenolpyruvate carboxykinase reaction ($25 \mu\text{M}$; Ballard, 1970a), when allowance is made for the extracellular fluid of liver. Clearly, by 1 h after birth the changes that have occurred are favourable for an active pathway of gluconeogenesis. However, phosphoenolpyruvate carboxykinase activity is still very low and is probably rate-limiting. Additional evidence for a proposed rate-limiting role for phosphoenolpyruvate carboxykinase comes from the crossover plot in Fig. 4. This result is analogous to the changes reported in perfusion studies when liver from starved rats is compared with the liver of fed rats (Exton & Park, 1969).

The facilitation of a step in gluconeogenesis beyond glucose 6-phosphate is indicated by the crossover studies in Figs. 3 and 4. Under conditions of increased flux it would be expected that the final intermediates in the pathway would be increased rather than decreased as noted. It is possible that glucose 6-phosphate concentrations are lowered in the newborn by an increase in glucose 6-phosphatase activity, but this enzyme does not increase rapidly until several hours after birth (Dawkins, 1963). An increase in glycolysis or glycogen synthesis could depress glucose 6-phosphate, but both pathways would have lower activities in livers of newborns as compared with the foetus (Ballard, 1970b). The hexokinase activity is not lowered in the livers of newborn rats (Ballard, 1970b) and the only known allosteric modifier of this enzyme is an inhibition by glucose 6-phosphate.

Decreases in glucose 6-phosphate concentrations have been observed previously in livers of newborn rats (Dawkins, 1963) and in starved rats as compared with fed animals (Start & Newsholme, 1968). In starved rats the rate of gluconeogenesis would be more rapid than in the fed animal (Von Holt, Schmidt, Feldman & Hallmann, 1961), so that the glucose 6-phosphate concentration would also be expected to increase. Possibly these findings are indicative of an unknown regulatory action on hexokinase or glucose 6-phosphatase.

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