The Development of Gluconeogenesis in Rat Liver

EFFECTS OF GLUCAGON AND ETHER

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1. Administration of glucagon to foetal rats produced a 10-15-fold increase in hepatic phosphoenolpyruvate carboxykinase activity together with a similar increase in the overall pathway of pyruvate conversion into glycogen in liver slices. 2. Glucagon was without effect on gluconeogenesis *in vivo*, which remained at approx. 0.1% of the incorporation as measured in newborn animals. 3. The apparent discrepancy between these results was due to the ether anaesthesia that was required for experimentation *in vivo*. Under conditions when minimal ether was used, the rates of labelling of glycogen from [3-¹⁴C]pyruvate *in vivo* were increased 10-20-fold and there was an additional stimulus by glucagon. 4. Ether anaesthesia produced a more reduced redox state of the foetal liver cytosol and lowered the ATP/ADP concentration ratio. 5. It is proposed that these effects are significant in the limitation of gluconeogenesis in the foetal rat liver, so that only with high phosphoenolpyruvate carboxykinase activity, high ATP concentration and a relatively oxidized cytosol redox state will a functional gluconeogenic pathway be present.

Experiments in which liver slices from rats of different ages are incubated with various gluconeogenic precursors have indicated the development of an active gluconeogenic pathway immediately after birth (Ballard & Oliver, 1963, 1965; Yeung & Oliver, 1967). Concomitant with this increasing activity of the complete pathway, there is a 25-fold increase in the activity of the hepatic cytosol phosphoenolpyruvate carboxykinase (EC 4.1.1.32) between term fetuses and newborn animals (Ballard & Hanson, 1967). Since all other enzymes involved in gluconeogenesis are already active in foetal liver, and do not increase substantially in activity in the newborn, we have concluded that the appearance of phosphoenolpyruvate carboxykinase is the initiating factor in the development of the complete pathway.

Experiments in which either substrate or tracer amounts of aspartate, lactate or pyruvate have been injected into intact fetuses and newborn rats suggest that the block in foetal gluconeogenesis is localized at the phosphoenolpyruvate carboxykinase step (Philippidis & Ballard, 1969). In the present paper we have examined this possibility by measuring the incorporation of labelled pyruvate

into liver glycogen after induction of phosphoenolpyruvate carboxykinase by glucagon in rat fetuses. The results indicate that foetal gluconeogenesis is prevented by the ether anaesthesia used as well as the low activity of phosphoenolpyruvate carboxykinase.

MATERIALS AND METHODS

Chemicals. [U-¹⁴C]Glucose, [3-¹⁴C]pyruvate and NaH¹⁴CO₃ were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. NAD⁺, NADH, ATP, ADP, AMP and lactate dehydrogenase were from P-L Biochemicals, Milwaukee, Wis., U.S.A. Pyruvate kinase, adenylate kinase, malate dehydrogenase and phosphoenolpyruvate were obtained from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. The highly purified glucagon used in these studies was a gift from Dr O. K. Behrens, Eli Lilly Laboratories, Indianapolis, Ind., U.S.A.

Animals. Pregnant Wistar albino rats were obtained from Carworth Farms Inc., New City, N.Y., U.S.A. The foetuses were used on the twenty-first day of gestation and all newborn rats were 1-2 days' old. The technique used to maintain the foetal rats in the saline bath was a modification (Philippidis & Ballard, 1969) of that described by Reade & Jenkin (1964). All compounds injected into the foetuses were dissolved in 0.9% NaCl. For the maintenance of foetuses *in utero*, a small incision was made in the abdominal wall of the pregnant rat while under ether

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anaesthesia. The uterus was partially removed and fetuses were injected intraperitoneally through the uterine wall with various compounds dissolved in 50 μ l of 0.9% NaCl. The uterus was put back into the animal and the incision closed: the peritoneum with thread, and the muscle and skin layers with wound clips. Usually ether anaesthesia was not continued and then the animals recovered from anaesthesia in less than 5 min. In those experiments where the effect of ether was being tested, the pregnant animal was kept under ether until the end of the experiment. In this case tracheotomy was performed on the pregnant rat and an ether + O₂ mixture was administered through the tracheal cannula.

Liver-slice experiments. Slices were cut from foetal and adult liver and incubated in 3 ml of a high-K⁺ medium containing 10 mM-KCl, 20 mM-MgCl₂, 40 mM-KHCO₃, 10 mM-CaCl₂, 60 mM-potassium pyruvate and 1 μ Ci of [3-¹⁴C]pyruvate (Ballard & Oliver, 1963). After incubation for 2 h at 37°C the slices were digested in 30% (w/v) KOH. Glycogen was isolated and the radioactivity determined as described by Cowgill & Pardee (1957). Rates of gluconeogenesis have been calculated according to the relationship:

$$\text{Incorporation } (\mu\text{mol}/2\text{ h/g}) = \frac{\text{total d.p.m. in glycogen}}{\text{d.p.m.}/\mu\text{mol of pyruvate}} \times \frac{1000}{\text{slice wt. (mg)}}$$

Preparation of tissue extracts. Livers were either removed from fetuses and newborn rats and frozen immediately in liquid dichlorodifluoromethane or were frozen *in situ* by clamping with tongs cooled in liquid N₂. Frozen livers were pounded in a mortar and extracted with 6% (w/v) HClO₄. Homogenates were centrifuged and the supernatants neutralized with 2 M-KHCO₃ to pH 6. The KClO₄ was removed by centrifugation.

For isolation of glycogen from whole livers, the livers frozen in dichlorodifluoromethane were digested in 60% (w/v) KOH, and the glycogen was purified as described by Cowgill & Pardee (1957).

Analysis of intermediates. Pyruvate, ADP and AMP were measured spectrophotometrically as described by Adam (1965) by the sequential addition of lactate dehydrogenase, pyruvate kinase and adenylate kinase. Lactate was measured by the method of Hohorst (1965). Phosphoenolpyruvate was determined as described by Czok & Eckert (1965). ATP was measured with hexokinase and glucose 6-phosphate dehydrogenase as described by Lamprecht & Trautschold (1965).

Phosphoenolpyruvate carboxykinase assay. This enzyme was measured as described by Chang & Lane (1966) with the slight modifications reported by Ballard & Hanson (1967). One unit of activity is defined as the incorporation of 1 μ mol of ¹⁴C-labelled bicarbonate/min at 37°C.

RESULTS

It has been shown that glucagon induces a number of enzymes in the foetal rat liver and that one of these is phosphoenolpyruvate carboxykinase (Greengard & Dewey, 1967; Yeung & Oliver, 1968). To establish time-response and dose-response curves for the glucagon stimulation of phospho-

Fig. 1. Induction of phosphoenolpyruvate carboxykinase in foetal rat liver. (a) Foetuses were injected *in utero* with either 50 μ g of glucagon in 0.9% NaCl (○) or with 0.9% NaCl alone (●). After the times indicated the animals were removed from the uterus and phosphoenolpyruvate carboxykinase activity was measured in liver extracts. (b) Foetuses were injected with various amounts of glucagon in 0.9% NaCl. After either 3 h (○) or 5 h (●) the foetuses were removed from the uterus and phosphoenolpyruvate carboxykinase activity was assayed in liver extracts.

enolpyruvate carboxykinase, the following experiments were performed. First, the foetal rats were injected with a dose of 50 μ g of glucagon and were killed 1, 3, 5, 7 and 11 h later. A maximum stimulation was observed at 3 h after the glucagon injection (Fig. 1a). Because the difference in stimulation between 3 and 5 h after glucagon injection was only slight, in the second experiment 50, 100, 250 or 500 μ g of glucagon was injected into each foetus and the foetuses were killed 3 and 5 h later. From the group killed 3 h after glucagon injection a maximum stimulation of phosphoenolpyruvate carboxykinase activity was observed with 100 μ g of glucagon, but those killed 5 h after

injection showed a peak with 250 μ g of glucagon and a 14-fold stimulation (Fig. 1b). It is therefore evident that a single injection of 250 μ g of glucagon per foetus produces a large increase in phosphoenolpyruvate carboxykinase activity after 5h, and we have therefore used this increase to follow any changes in gluconeogenesis that may accompany phosphoenolpyruvate carboxykinase induction.

To establish any correlation between phosphoenolpyruvate carboxykinase activity and the overall pathway of gluconeogenesis, the conversion of [3- 14 C]pyruvate into glycogen was studied in glucagon-treated and in control animals. Term foetuses were injected with 250 μ g of glucagon intraperitoneally through the uterine wall and controls were injected with 0.9% sodium chloride. Then 5h later the animals were killed, liver slices were incubated with [3- 14 C]pyruvate and the incorporation of 14 C from [3- 14 C]pyruvate into glycogen was measured. In this experiment the slices were incubated for 2h with 60mM-potassium pyruvate. Hepatic phosphoenolpyruvate carboxykinase activity was measured at the same time in both glucagon-treated and control animals. When the activity of this enzyme was increased about 15-fold, the incorporation of 14 C from [3- 14 C]pyruvate into glycogen was similarly increased (Table 1). Both phosphoenolpyruvate carboxykinase activities and incorporation rates in the induced animals are approximately half those of comparable values in adult animals (Table 1). The experiments clearly show that phosphoenolpyruvate carboxykinase increases in parallel to gluconeogenesis.

To correlate these changes with the situation *in vivo*, two types of experiments were carried out. In the first type of experiment glucagon was injected into the foetuses through the uterine wall and the pregnant rat left for 5h. After this time the uterus was opened while the mother was partly immersed in the saline bath (Philippidis & Ballard, 1969) and the foetuses were removed from the uterus but left connected to their placentas via umbilical vessels. This procedure did not result in separation of placentas from uterus so long as the dissection was carried out with the uterus immersed in the saline bath and the foetuses were on the twenty-first day of gestation. The procedure was not usually successful if attempted on the twenty-second day of gestation. At this later stage the injection of glucagon into foetuses *in utero* often induced parturition, or alternatively resulted in the spontaneous release of placentas in the saline bath. [3- 14 C]Pyruvate (2 μ Ci) was injected into the foetuses and the radioactivity in liver glycogen determined after they had been killed 1h later. In the second technique used, 5h after glucagon administration the foetuses were injected with [3- 14 C]pyruvate through the uterine wall while the foetuses remained in the uterus.

Table 1. *Effect of prior injection of glucagon on the incorporation of 14 C from [3- 14 C]pyruvate into glycogen by liver slices of foetal rats*

Age	Phosphoenolpyruvate carboxykinase activity (units/g of liver)	Incorporation of 14 C from [3- 14 C]pyruvate into glycogen (μ mol/g of liver)
Foetus	0.060 $\pm$ 0.010	1.08 $\pm$ 0.18
Glucagon-treated foetus	1.06 $\pm$ 0.01	12.7 $\pm$ 1.0
Adult	2.5 $\pm$ 0.3	24.9 $\pm$ 6.5

After an additional 1h radioactivity in liver glycogen was determined as before. Two advantages of the first preparation are: (a) the foetuses are relatively isolated from the mother so that any leakage of radioactive precursor from the site of injection cannot directly get into the maternal circulation where it could be converted into other compounds and pass back into the foetus; (b) it was possible by having the foetuses in the saline bath to kill them at various times after injection. On the other hand, a disadvantage of this technique is that the mother must be maintained under light ether anaesthesia, and this may cause hypoxic conditions even if the ether is administered as an ether + oxygen mixture. In the preparation *in utero* it is not essential for the mother to be kept under ether anaesthesia for the complete experiment.

With the saline-bath technique the incorporation of 14 C from [3- 14 C]pyruvate into glycogen was extremely low in both glucagon-treated and untreated foetuses (Table 2). The values shown in Table 2 were obtained from animals 1h after the injection of [3- 14 C]pyruvate, but similarly low values have been obtained when the animals are killed 15, 30 or 45min after injection. Although phosphoenolpyruvate carboxykinase activity is increased by glucagon, no effect on the incorporation of 14 C from [3- 14 C]pyruvate was evident. The incorporation of 14 C from [3- 14 C]pyruvate into liver glycogen of 2-day-old rats was approx. 1000-fold that in the foetus.

To study the possibility that the ether anaesthesia used in the previous experiments caused a reduced environment in the liver cytosol, which in turn could be unfavourable for gluconeogenesis, experiments were designed in which anaesthesia was administered for minimum times. The foetuses

Table 2. *Incorporation of [3-¹⁴C]pyruvate into liver glycogen in vivo*

Foetuses were injected with glucagon (250 µg) or saline as described in the Materials and Methods section. The newborn animals had no injection. After 5 h one of three procedures was carried out. In the first type of experiment the uterus was opened and the foetuses were removed from the uterus into a saline bath, but left connected to the uterus via their placentas (Philippidis & Ballard, 1969). Alternatively the foetuses remained *in utero*. In all experiments 2 µCi (1 µmol) of [3-¹⁴C]pyruvate was injected into each foetus. In some experiments the mothers were kept under anaesthesia throughout the period of incorporation of ¹⁴C from [3-¹⁴C]pyruvate by administration of an ether + O₂ mixture by tracheal cannulation. At 1 h after [3-¹⁴C]pyruvate injection the animals were killed and the liver glycogen was isolated for determination of radioactivity. Phosphoenolpyruvate carboxykinase activity was also measured in samples of liver. Values are the means ± s.e.m. for the numbers of animals given in parentheses.

Experimental conditions	Glucagon	Phosphoenolpyruvate carboxykinase activity (units/g of liver)	Incorporation of ¹⁴ C from [3- ¹⁴ C]pyruvate into glycogen (d.p.m./100 mg of liver)
Foetus, saline bath	—	0.084 ± 0.015 (6)	15 ± 4 (6)
	+	1.03 ± 0.13 (7)	15 ± 2 (7)
Foetus, <i>in utero</i> , no ether	—	—	300 ± 60 (19)
	+	—	1340 ± 120 (20)
Foetus, <i>in utero</i> , ether	—	0.056 ± 0.004 (14)	37 ± 5 (14)
	+	1.09 ± 0.04 (14)	26 ± 6 (14)
Newborn	—	2.55 ± 0.1 (8)	17 300 ± 600 (8)

were injected with [3-¹⁴C]pyruvate while the pregnant rat was under light ether anaesthesia, after which the mother was permitted to recover from the anaesthesia. The recovery usually took 2–5 min. As shown in Table 2, there was substantial incorporation of ¹⁴C from [3-¹⁴C]pyruvate into glycogen under these conditions. Glucagon stimulated this incorporation approximately fourfold as compared with the 10–15-fold increase in phosphoenolpyruvate carboxykinase activity. It should also be noted that the incorporation of ¹⁴C from [3-¹⁴C]pyruvate into glycogen in the absence of ether and in the presence of glucagon (1340 d.p.m./100 mg of liver) is considerably lower than the value of 17 300 d.p.m./100 mg of liver found in newborn animals (Table 2). In parallel experiments in which the mothers were maintained under ether anaesthesia throughout the incorporation period, only small amounts of ¹⁴C appeared in the glycogen, in agreement with the saline-bath experiments. Glucagon had no effect if anaesthesia was maintained.

One technical difficulty of the preparation in which the mother is dissected in the saline bath is that leakage of label from the foetus to the saline bath undoubtedly occurs. It has been shown by the following experiments that this leakage is not serious and does not markedly influence the conclusions of the experiments. First, experiments in the saline bath and experiments *in utero* give similar results when the animals are maintained under ether anaesthesia (Table 2). Secondly, the radioactivity calculated to be distributed throughout the extracellular plus intracellular fluids of the mother

was never greater than 20% of the injected dose in experiments *in utero*. In the saline-bath technique the radioactivity that could be accounted for in the maternal fluid was approx. 8% of the injected dose. These experiments were carried out by obtaining blood samples by heart puncture from the mother at 15, 45 and 60 min after injection of the foetuses.

In the experiments described above the incorporation of ¹⁴C from [3-¹⁴C]pyruvate into glycogen was measured. This conversion involves two metabolic phases, the gluconeogenic sequence and the pathway of glycogen synthesis. If oxygen is limiting under some experimental conditions it may affect both or only one of these two enzyme sequences. It is known that at this stage of gestation foetal rats have a high hepatic glycogen concentration as well as all enzymes required to catalyse the synthesis of glycogen from glucose (Shelley, 1961; Ballard & Oliver, 1963). To distinguish glycogen synthesis from gluconeogenesis, [U-¹⁴C]glucose was injected into foetal rats and the time-course of incorporation of ¹⁴C into glycogen was followed. Radioactivity in glycogen was high from 10 min through to 45 min after injection. The results obtained at 30 min show (Table 3) that ether inhibits the incorporation of [U-¹⁴C]glucose into glycogen, especially in the glucagon-treated animals. However, this effect is much less than the one of ether on the incorporation of ¹⁴C from [3-¹⁴C]pyruvate into glycogen (Table 2). It is also apparent that glucagon lowers the incorporation of [U-¹⁴C]glucose into glycogen in all types of experiments, presumably owing to a glycogenolytic action of glucagon.

We have attempted to relate these various effects

of glucagon and ether to changes in the relative proportions of adenine nucleotides in foetal liver, since it is likely that if hypoxia was caused by the ether

anaesthesia it would be reflected in a lower degree of phosphorylation of hepatic adenine nucleotides. Table 4 shows that ATP concentrations in foetal liver are about half those found in livers from adult and newborn animals. ADP concentrations in the adult are twice as high as in the newborn or the foetus whereas the AMP concentration does not change with development. As a result of these changes in ATP and ADP concentrations the ATP/ADP concentration ratio changes at birth from 3.0 to 7.0 and falls to 3.3 in the adult. The change from foetus to newborn is considered to indicate a transfer from a relatively hypoxic condition to a situation where oxygen is available. The suggestion that ether causes hypoxia is tested in Table 4. The ATP/ADP concentration ratios in foetuses maintained either in the saline bath or *in utero* with ether administered to the mother are approx. 2.2. In foetuses that are exposed to ether for a minimal time the higher ATP/ADP concentration ratio 3.0 is found. This presumably indicates a relatively hypoxic condition under ether anaesthesia. We also noted that prior administration of glucagon had no effect on the proportions of adenine nucleotide in foetal liver, but these values are not shown in Table 4.

Table 3. *Incorporation of [U-¹⁴C]glucose into liver glycogen by foetal rats*

Foetuses were injected with glucagon or saline as described in the Materials and Methods section. A second injection of 2 μ Ci of [¹⁴C]glucose (specific radioactivity 3 μ Ci/ μ mol) was given 5 h later. The different foetal preparations used are described in Table 2. Glycogen was isolated and the radioactivity determined 30 min after injection of glucose. Values are the means $\pm$ S.E.M. for four determinations.

Foetal preparation	Glucagon	Incorporation of [U- ¹⁴ C]glucose into glycogen (d.p.m./100 mg of liver)
Saline bath	—	1420 $\pm$ 170
	+	560 $\pm$ 130
<i>In utero</i> , no ether	—	1040 $\pm$ 220
	+	610 $\pm$ 160
<i>In utero</i> , ether	—	740 $\pm$ 170
	+	240 $\pm$ 20

Table 4. *Concentrations of adenine nucleotides in rat liver*

Adenine nucleotides were determined in freeze-clamped livers as described in the Materials and Methods section. Values are the means $\pm$ S.E.M. for the numbers of determinations given in parentheses.

Experimental conditions	[ATP] (μ mol/g of liver)	[ADP] (μ mol/g of liver)	[AMP] (μ mol/g of liver)	[ATP]/[ADP] ratio
Adult	2.60 $\pm$ 0.15 (8)	0.79 $\pm$ 0.08 (8)	0.23 $\pm$ 0.02 (8)	3.3
Newborn	2.52 $\pm$ 0.13 (6)	0.36 $\pm$ 0.04 (6)	0.25 $\pm$ 0.01 (6)	7.0
Foetus, saline bath	1.10 $\pm$ 0.06 (6)	0.50 $\pm$ 0.02 (6)	0.12 $\pm$ 0.02 (6)	2.2
Foetus, <i>in utero</i> , no ether	1.25 $\pm$ 0.07 (6)	0.41 $\pm$ 0.04 (6)	0.20 $\pm$ 0.03 (6)	3.0
Foetus, <i>in utero</i> , ether	1.03 $\pm$ 0.03 (4)	0.48 $\pm$ 0.03 (4)	—	2.2

Table 5. *Lactate/pyruvate concentration ratios and NAD⁺/NADH concentration ratios in foetal and newborn liver*

Lactate and pyruvate were measured in livers frozen in dichlorodifluoromethane as described in the Materials and Methods section. Values are the means $\pm$ S.E.M. for the numbers of determinations in parentheses. The NAD⁺/NADH concentration ratios were calculated as described by Williamson, Lund & Krebs (1967). Values for foetuses in the saline bath and for newborn rats are from Philippidis & Ballard (1969).

Experimental conditions	[Lactate] (μ mol/g of liver)	[Pyruvate] (μ mol/g of liver)	[Lactate]/[pyruvate] ratio	[NAD ⁺]/[NADH] ratio
Newborn	1.0 $\pm$ 0.2 (6)	0.074 $\pm$ 0.017 (6)	13.5	667
Foetus, saline bath	11.8 $\pm$ 2.2 (6)	0.053 $\pm$ 0.006 (6)	213	42
Foetus, <i>in utero</i> , no ether	7.0 $\pm$ 0.8 (4)	0.060 $\pm$ 0.008 (4)	116	78
Foetus, <i>in utero</i> , ether	14.6 $\pm$ 1.5 (6)	0.036 $\pm$ 0.005 (6)	405	22
Glucagon-treated foetus, <i>in utero</i> , no ether	4.5 (2)	0.040 (2)	112	89
Glucagon-treated foetus, <i>in utero</i> , ether	14.6 $\pm$ 1.5 (6)	0.040 $\pm$ 0.008 (6)	365	25

In addition to these effects of ether on adenine nucleotides, hypoxic conditions could influence the redox state of the liver cytosol. If ether anaesthesia makes the foetuses hypoxic, a decrease in the NAD^+/NADH concentration ratio may be observed. When ether was administered to pregnant rats, the foetal hepatic lactate concentrations were threefold those when ether was not administered (Table 5). As a result the lactate/pyruvate concentration ratio increased, thus implying a shift in the redox state of the liver cytosol to a more reduced state. This effect of ether on the NAD^+/NADH concentration ratio is also found in the saline-bath experiments. The change in the ratio that occurs at birth is in the opposite direction. There is a shift from the NAD^+/NADH concentration ratio 78 in the foetus with no ether to 667 in the newborn. Injection of glucagon did not alter the concentrations of lactate and pyruvate in foetal liver.

DISCUSSION

If hepatic gluconeogenesis is limited in the foetal rat by a low activity of cytosol phosphoenolpyruvate carboxykinase, and by this alone, induction of this enzyme should be accompanied by an increase in the complete pathway. Both glucagon and cyclic 3':5'-AMP are effective in producing a large increase in phosphoenolpyruvate carboxykinase activity in foetal rat liver, although the effect is not limited to this enzyme (Greengard & Dewey, 1967; Yeung & Oliver, 1968). We have used glucagon in the present studies and find that the resultant 10–15-fold increase in phosphoenolpyruvate carboxykinase activity is accompanied by a similar increase in the incorporation of ^{14}C from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ into the glycogen of liver slices. When we attempted to confirm these slice results *in vivo* we found minimal labelling of glycogen in control foetuses, and this labelling was not increased by glucagon.

For all experiments on foetal animals some anaesthesia is necessary. We have used ether for this purpose since this method of anaesthesia is both easy to control and conveniently performed. Notwithstanding these advantages, hypoxic conditions may be produced if the mother has less effective breathing. In an attempt to overcome this problem we have varied our experimental technique so that three foetal preparations were obtained: (1) saline-bath experiments in which anaesthesia must be maintained (Philippidis & Ballard, 1969); (2) experiments *in utero* in which the mother was kept under anaesthesia for the complete experiment; (3) experiments *in utero* in which anaesthesia was used only for the surgery. Measurements of incorporation of ^{14}C from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ into foetal liver glycogen with these three techniques showed substantial incorporation only in (3), and this pre-

paration was the only one in which a glucagon stimulation was demonstrable. We interpret these differences to the variable anaesthesia and to the probable variation in hypoxia.

Hypoxia and changes in cytosol redox state. This hypoxia is presumably the cause of the relatively reduced environment in the cytosol that is indicated by the elevated lactate/pyruvate concentration ratios reported by us previously (Philippidis & Ballard, 1969) and by Hornbrook, Burch & Lowry (1965). In the present paper we report NAD^+/NADH concentration ratios 42 and 22 for the two types of foetal preparations in which anaesthesia was maintained, 78 for the third technique and 667 for the newborn liver. These changes in the redox state of the liver cytosol correlate well with the increases in gluconeogenesis, but they are apparently in conflict with the results of Williamson *et al.* (1967), who found a decrease in the NAD^+/NADH concentration ratio from 725 to 208 in a comparison between liver from fed rats and livers from rats treated with alloxan, a condition that stimulates gluconeogenesis (Friedmann, Goodman & Weinhouse, 1965).

A consideration of the reactions involved in gluconeogenesis shows three possible steps where the cytosol redox state is directly involved. In the reaction catalysed by lactate dehydrogenase:

a relatively reduced environment will hinder the formation of pyruvate. Similarly, a reduced redox state of the cytosol will lower the concentration of oxaloacetic if this is in equilibrium with malate via malate dehydrogenase:

A lower oxaloacetate concentration may depress phosphoenolpyruvate formation since the Michaelis constant for oxaloacetate in the reaction catalysed by phosphoenolpyruvate carboxykinase is much larger than the concentrations of oxaloacetate normally observed *in vivo* (Williamson, Browning, Scholz, Kreisberg & Fritz, 1968; Chang, Maruyama, Miller & Lane, 1966). We therefore suggest that the low NAD^+/NADH concentration ratio of foetal rat liver, particularly in the hypoxic conditions produced by ether, provides a much less favourable situation for the conversion of lactate into phosphoenolpyruvate than in the liver of the newborn animal. In addition to reactions (1) and (2), the cytosol redox state directly affects the synthesis of glucose from lactate at the glyceraldehyde phosphate dehydrogenase step:

In this case a low NAD^+/NADH concentration

ratio would seem to favour 3-phosphoglyceraldehyde synthesis and thus the formation of glucose from lactate, and it is because of this reaction that Williamson *et al.* (1967) have suggested that a more reduced cytosol redox state would favour gluconeogenesis. The conflict between these ideas will only be resolved if it is possible to determine the rate-limiting steps in gluconeogenesis from lactate.

In this regard, most cross-over studies performed with liver perfusions in adult rats suggest that the rate-limiting step in gluconeogenesis is before phosphoenolpyruvate (Williamson, Browning & Scholz, 1969; Exton & Park, 1966; Veneziale, Walter, Kneer & Lardy, 1967; Williamson *et al.* 1968). However, with pyruvate as substrate, Exton & Park (1968) found that ethanol increased gluconeogenesis. This was interpreted as resulting from the stimulus of glyceraldehyde phosphate dehydrogenase by the NADH generated by ethanol oxidation. The high pyruvate concentration used would severely depress the concentration of NADH in the cytosol to a point where insufficient reduced nucleotide was available for reaction (3) unless more NADH could be formed. It is likely that this control by NADH availability occurs only under such conditions of high pyruvate concentration.

In addition to the ether-anaesthesia experiments, our previous work (Philippidis & Ballard, 1969) supports the suggestion that a relatively high NAD^+/NADH concentration ratio in the liver cytosol is important for phosphoenolpyruvate synthesis. These studies showed that the removal of radioactive lactate is slower in foetal liver than in the newborn, possibly owing to the more reduced redox state of foetal liver. This inference is supported by experiments in which radioactive pyruvate was injected. Pyruvate gave rise to large amounts of radioactive lactate in foetal liver but not in the liver of the newborn rat. Other experiments showed that phosphoenolpyruvate concentrations were substantially increased after pyruvate was injected into newborn rats, but lactate caused a much lower stimulation. These high pyruvate concentrations would be expected to cause NADH oxidation via lactate dehydrogenase, and this in turn could result in higher oxaloacetate concentrations by displacement of the malate dehydrogenase equilibrium. In foetal but not in newborn liver the injection of $[^{14}\text{C}]$ aspartate caused considerable labelling of the malate pool. Again this suggests that a more reduced redox state in the cytosol of foetal liver favours a displacement of the malate dehydrogenase equilibrium towards malate. This would lower the cellular oxaloacetate concentration and thus restrict phosphoenolpyruvate synthesis.

Hypoxia and changes in adenine nucleotides. According to Atkinson (1966), changes in the relative proportion of adenine nucleotides can alter

the direction of cellular pathways. The shift towards a high ATP/ADP concentration ratio that accompanies birth or is found in the least hypoxic foetal preparation may play an important role in the initiation of gluconeogenesis. Gluconeogenesis requires nucleotide triphosphates for three key steps: ATP for pyruvate carboxylase in the mitochondria, and GTP for phosphoenolpyruvate carboxykinase and ATP for phosphoglycerate kinase in the cytosol. The overall pathway will be favoured in the newborn where ATP concentration is highest as compared with the foetal preparation where anaesthesia is minimal, and that in turn will be favoured over the foetal preparation in which continual anaesthesia was accompanied by the lowest ATP/ADP concentration ratios (see also Mäenpää, 1967). It is not possible to determine whether the mitochondrial pool of adenine nucleotides is changing more significantly than the cytosol pool when hypoxia is initiated, but it is reasonable to assume that the initial fall in ATP concentration occurs in the mitochondria at the site of oxidative phosphorylation.

It appears that the development of a functional gluconeogenic sequence at birth in rat liver is regulated by at least three controls: the synthesis of cytosol phosphoenolpyruvate carboxykinase, the change in cytosol redox state and the increase in the degree of phosphorylation of adenine nucleotides.

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