The Development of Ketogenesis at Birth in the Rat

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In the suckling newborn rat, blood ketone bodies begin to increase slowly 4 h after birth and then rise sharply between 12 and 16 h, whereas the major increase in plasma non-esterified fatty acids and liver carnitine occurs during the first 2 h of life, parallel with the onset of suckling. In the starved newborn rat, which shows no increase in liver carnitine unless it is fed with a carnitine solution, the developmental pattern of the ketogenic capacity (tested by feeding a triacylglycerol emulsion, which increases plasma non-esterified fatty acids by 3-fold) is the same as in the suckling animal. This suggests that the increases in plasma non-esterified fatty acids and liver carnitine seen 2 h after birth in the suckling animal are not the predominant factors inducing the switch-on of ketogenesis. Injection of butyrate to starved newborn pups resulted in a pattern of blood ketone bodies which was similar to that found after administration of triacylglycerols, but, at all time points studied, the hyperketonaemia was more pronounced with butyrate. It is suggested that, even if the entry of long-chain fatty acids into the mitochondria is a rate-limiting step, it is not the only factor controlling ketogenesis after birth in the rat. As in the adult rat, there is a reciprocal correlation between the liver glycogen content and the concentration of ketone bodies in the blood.

In the foetal rat, the main oxidizable substrate is glucose provided by the mother via the placenta. Non-esterified fatty acids cannot readily cross the placental barrier (Koren & Shafrir, 1964), and the capacity of the liver to oxidize non-esterified fatty acids and to form ketone bodies is very low *in utero* (Drahota *et al.*, 1964; Augenfeld & Fritz, 1970). At birth, when suckling commences, the previous high-carbohydrate diet is replaced by the high-fat–low-carbohydrate diet of the milk (Luckey *et al.*, 1954). The concentration of blood ketone bodies increases from around 0.2 mM at birth to 2 mM 24 h later (Snell & Walker, 1973; Wapnir *et al.*, 1973; Foster & Bailey, 1976a; Yeh & Zee, 1976). A key question to our understanding of the regulation of hepatic metabolism is what brings about this apparent increase in the rate of ketogenesis during the first 24 h of life.

In the adult rat, a number of mechanisms have been proposed to explain the increased rate of ketogenesis during starvation. These include an increased delivery of fatty acids to the liver, an increase in the activity of carnitine acyltransferase (EC 2.3.1.21), which transfers long-chain acyl-CoA from the cytosol to the mitochondria, together with an increase in hepatic carnitine, the obligatory cofactor of this enzyme (cf. McGarry & Foster,

1977, for a review). McGarry *et al.* (1977) have shown *in vitro* that malonyl-CoA, a metabolite of lipogenesis, inhibits the activity of the carnitine acyltransferase I, so establishing a link between carbohydrate and fat metabolism in the liver. Intramitochondrial control of ketogenesis has also been suggested by the experiments of McGarry & Foster (1971), using octanoate, and Whitelaw & Williamson (1977), using butyrate; the control point is, however, unknown.

Most of the information at present available on the onset of ketogenesis at birth allows a comparison between the late-foetal period or immediately after birth with rats which are 24 h old, a time when oxidation of non-esterified fatty acids and ketone bodies is already well-developed. After birth, apart from an increased delivery of non-esterified fatty acids to the liver, there is an increase in the activity of the enzymes concerned with β -oxidation in the liver, and particularly in the activity of the carnitine acyltransferase system (Augenfeld & Fritz, 1970; Foster & Bailey, 1976b). Carnitine also increases in concentration in liver after birth and it has been suggested that this is an important factor in the regulation of fatty acid oxidation and ketogenesis at this time (Robles-Valdes *et al.*, 1976). The activities of the enzymes of the hydroxymethylglutaryl-CoA pathway (responsible for the synthesis of acetoacetate from acetyl-CoA) also increase during the

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first 24 h of life (Shah & Bailey, 1977). However, there appear to have been no systematic studies of the time course of the alteration of blood ketone-body concentrations after birth in relation to possible factors that may regulate the rate of ketogenesis in the adult animal.

In the present work we report the relationships between the time course of the increase in blood ketone bodies after birth and the changes in plasma non-esterified fatty acids, hepatic concentrations of carnitine and glycogen, and activity of hydroxymethylglutaryl-CoA synthase. In addition, the effects on the concentrations of blood ketone bodies of administration of triacylglycerol (free from carnitine) or butyrate to rat pups starved from birth for various times have been studied.

Materials and Methods

Animals

The rats were from an albino Wistar strain; gestational age was determined as described previously (Girard *et al.*, 1973). The time of delivery of the pups born naturally was determined by carefully checking the appearance of the first and the last pup of the litter in the cage. They were then left with the mother at room temperature (20–22°C). Suckling commenced between 1 and 2 h after birth and was checked by the presence of milk in the stomach of the pups. Repeated observations have shown that in our colony of rats suckling does not commence until after the birth of the last pup of the litter. Only litters with eight to ten pups were used. In the experiments with starved pups, they were delivered by caesarian section on day 22 of gestation (normal term in this strain of rats is 22 days) and transferred to a Humidicrib (Jouan, Paris, France) in which the temperature was maintained at 37°C and the relative humidity at 70%.

Experimental procedures

Triacylglycerol feeding. Starved newborn rats were fed, by using a flexible catheter (0.5 mm diameter; Intramedic PE 10; Clay Adams, NJ, U.S.A.) connected to a syringe, with either 100 μ l of NaCl (0.9%, w/v) for control experiments or 100 μ l of a 10% (w/v) triacylglycerol emulsion free from glycerol (Vitrum A.B., Stockholm, Sweden). The composition of the emulsion determined by g.l.c. was as follows: linoleic acid, 47%; oleic acid, 31%; palmitic acid, 11%; linolenic acid, 6%; stearic acid, 5%; palmitoleic acid, traces. After the feed, the pups were returned to a Humidicrib and blood was collected 1, 2 or 3 h later. In certain experiments (–)-carnitine was added to the triacylglycerol emulsion to give a final concentration of 300 μ M.

Sodium butyrate injection. Sodium butyrate (200 mg) was dissolved in 1 ml of 0.9% (w/v) NaCl and the pH was adjusted to 7.4. Starved newborn rats were given a subcutaneous injection of 50 μ l of 0.9% (w/v) NaCl or 50 μ l of the sodium butyrate solution (20 mmol/kg body wt.). They were then returned to the Humidicrib and blood was collected 1 h later. This time was chosen since, in preliminary experiments, it was shown that the peak in blood [ketone bodies] occurs 1 h after butyrate injection at any time tested after birth.

Collection of blood. The techniques used for collection of blood samples and the treatment of blood or plasma for assay of metabolites have been described previously (Girard *et al.*, 1973).

Analytical methods. Plasma non-esterified fatty acids were determined with radioactive nickel by the method of Ho (1970). Acetoacetate and 3-hydroxybutyrate were measured with a fluorimetric adaptation (Girard *et al.*, 1973) of the method of Williamson *et al.* (1962). For the determination of glycogen, the liver was frozen in liquid N₂ and then homogenized in water [2.5% (w/v) homogenate] at 4°C. The assay was carried out by the method of Roehrig & Allred (1974) on 50 μ l of the liver homogenate. Carnitine and acylcarnitine were determined by the method of Pearson *et al.* (1974) with 5,5'-dithiobis-(2-nitrobenzoate). A double-beam spectrophotometer (model 24; Beckman, Irvine, CA, U.S.A.) was used to overcome the problem of high blanks caused by the large concentrations of thiols contained in liver, and internal standards of (–)-carnitine were used. The assay of β -hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5) in liver of starved newborn rats was carried out by the method of Williamson *et al.* (1968).

Results are expressed as mean values $\pm$ s.e.m. Statistical analysis was performed by the Student's *t* test.

Special chemicals. Enzymes and coenzymes were obtained from Boehringer Corp., 75006 Paris, France. ⁶³Ni was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium butyrate was purchased from BDH Chemicals, Poole, Dorset, U.K., and (–)-carnitine from Sigma, St. Louis, MO, U.S.A.

Results and Discussion

Non-esterified fatty acid availability

In the newborn rats left with their mother, plasma non-esterified fatty acids increase 2-fold in the first hour after birth, before suckling begins (Fig. 1). This is related to the cold-exposure of the newborn rat after delivery, since this increase in plasma non-esterified fatty acids does not occur in pups maintained at thermoneutrality (37°C) immediately after

Fig. 1. Plasma concentrations of non-esterified fatty acids during the first 24h after birth in the suckling rat

The results are expressed as mM and each point is the mean $\pm$ S.E.M. for six to ten determinations.

Fig. 2. Whole-blood concentrations of ketone bodies (acetoacetate plus 3-hydroxybutyrate) during the first 24h after birth in the suckling rat

The results are expressed as mM and each point is the mean $\pm$ S.E.M. for 8–20 determinations.

birth (Girard *et al.*, 1973; Snell & Walker, 1973). The delivery of the whole litter in the rat lasts 1–2h. Until the last pup of the litter is born the mother leaves the newborn on the floor of the cage at room temperature (20°C). This cold-exposure stimulates non-shivering thermogenesis and results in lipolysis in brown adipose tissue (see Hull & Hardman, 1970, for a review), which explains the increase in plasma non-esterified fatty acids observed in these conditions. However, when the newborn rats are placed, at the end of the delivery period, in the nest, which is again at thermoneutrality, milk triacylglycerols are essential to maintain circulating concentrations of non-esterified fatty acids, as newborn rats have no white adipose tissue at birth (Hahn & Novak, 1975). The blood concentration of ketone bodies (acetoacetate plus 3-hydroxybutyrate) determined in the same groups of pups showed a different pattern (Fig. 2); there was a gradual increase in the first 10–12h after birth and then a steep rise to reach a plateau at 16h. Wapnir *et al.* (1973) have also measured the changes in contents of blood ketone bodies during the first 24h after birth and found that the major increase occurred between 6 and 12h. However, few time points were studied, there was no difference in ketonaemia between suckled and unfed and cold-exposed (30°C) pups, and a non-enzymic method was used for the determination of ketone bodies. The pattern of change in blood ketone-body concentrations with time in the unfed pups (see Fig. 5) differs from that previously published by us (Girard *et al.*, 1973). Ketone-body concentrations are higher for the first 6h after birth in the present study, but are lower at 16h. The explanation may lie in the different strains of rat used in the two studies. In the present work, the differences between the time course

for the concentrations of plasma non-esterified fatty acids and blood ketone bodies indicate that availability of substrate is not the reason for slow onset of ketogenesis after birth. This of course assumes that the blood concentrations of ketone bodies are a true reflection of the rate of ketogenesis by the liver. The activities of the enzymes of ketone-body utilization in peripheral tissues increase after birth (see Bailey & Lockwood, 1973, for a review), which implies that increased blood ketone-body concentration reflect increased production rather than a failure to utilize them.

Hepatic glycogen

There is considerable evidence that in the adult rat there is a reciprocal relationship between the amount of liver glycogen and the capacity to form ketone bodies (Robles-Valdes *et al.*, 1976). This inverse relationship also holds in the newborn rat, the main increase in blood ketone bodies occurring when liver glycogen is virtually completely depleted (compare Figs. 2 and 3). From a physiological standpoint it is clearly an advantage to suppress ketogenesis as long as hepatic glycogen is available to maintain blood glucose. It is unlikely that the glycogen itself inhibits ketogenesis; for instance, in some types of glycogen-storage disease (see Williamson & Whitelaw, 1978) ketogenesis does occur, although high concentrations of glycogen are present in the liver. The onset of ketogenesis may, however, be linked to metabolic events accompanying the decreased availability of hepatic carbohydrate after birth, such as decreased lipogenesis (Ballard & Hanson, 1967; Taylor *et al.*,

Fig. 3. Concentrations of liver glycogen in suckling rats during the first 24 h after birth

The results are expressed as mg/g fresh wt. of liver and each point is the mean $\pm$ s.e.m. for six to ten determinations.

Fig. 4. Concentrations of total and free carnitine in the liver of suckling rats during the first 24 h after birth

The results for total (●) and free carnitine (○) are expressed as nmol/g fresh wt. of liver and each point is the mean $\pm$ s.e.m. for six to ten determinations.

1967) or increased gluconeogenesis (Ballard, 1971; Girard *et al.*, 1975). The pattern of depletion of glycogen in the suckling newborn pup is similar to that in the pup unfed from birth (Girard *et al.*, 1973). This suggests that either the hypoglycaemia of starvation is not a potent additional stimulus to glycogenolysis in the immediate postnatal period or that the rate is already maximal.

Hepatic carnitine

It has been suggested that the hepatic carnitine is a key factor in the regulation of ketogenesis in the newborn rat and that carnitine can be supplied to the suckling pup via the mother's milk (Robles-Valdes *et al.*, 1976). The liver content of free and total carnitine (free plus acylcarnitine) increased rapidly in the first 2 h after birth, but only in pups that had suckled, and then the rise was very gradual over the next 22 h (Fig. 4). The differences between the time courses of the concentrations of liver carnitine and blood ketone bodies (compare Figs. 2 and 4) suggests that the increase in liver carnitine is not a key factor in determining the time of onset of ketogenesis after birth.

To test directly whether exogenous carnitine does indeed promote ketogenesis in the first 24 h of life the effects of feeding triacylglycerol [with and without added (–)-carnitine] on the ketonaemia of 13 h-old starved pups were studied (Table 1). Rats fed with the triacylglycerol emulsion alone have an increase in blood ketone bodies similar to that in pups of the same age, suckled naturally, although they have half the content of carnitine in the liver (Table 1). Exogenous (–)-carnitine was then added to the triacylglycerol emulsion to give a final concentration

of 300 μ M, which approximates to that found in rat milk (Robles-Valdes *et al.*, 1976). Rats fed with this supplemented triacylglycerol emulsion have a hepatic content of free carnitine more than twice that in comparable control rats, unfed or fed with triacylglycerol alone (Table 1). These experiments suggest that the increase in carnitine in the normally suckled rat is mainly due to transfer from the mother's milk rather than synthesis *de novo*. Despite this increase in carnitine there was no significant difference between the amounts of blood ketone bodies in the pups fed with triacylglycerol alone and those fed with triacylglycerol plus (–)-carnitine (Table 1).

Comparison of long-chain fatty acids and butyrate

In pups suckled by the mother the major increase in blood ketone bodies occurred in a relatively short interval between 12 and 16 h after birth (Fig. 2). It was decided to see whether a similar time course occurred if starved pups were either fed with triacylglycerol or injected with sodium butyrate at various intervals after birth. The formation of ketone bodies from butyrate does not involve transport into the mitochondria via carnitine acyltransferase, because it is converted to butyryl-CoA within the mitochondrial matrix (Aas & Bremer, 1968). When starved pups were fed with triacylglycerol (without carnitine), the largest increase in blood ketone bodies occurred between 13 and 16 h after birth (Fig. 5), as in the suckling pups. This was not due to a difference in triacylglycerol absorption and hydrolysis related

Table 1. Relationship between hepatic carnitine and blood ketone bodies in newborn rats

Newborn rats, kept at 37°C, and starved from birth for 13 h were fed with either 100mg of a 10% (w/v) triacylglycerol emulsion or the same emulsion containing carnitine (300 μM). Another group of rats was allowed to suckle naturally. All rats were killed at 16 h after birth. The carnitine concentrations are expressed as nmol/g fresh wt. of liver and the blood ketone bodies as mM and are mean values ± S.E.M. with the numbers of observations in parentheses. Values that are statistically different (Student's *t* test) from those of the starved group are shown by **P* < 0.01 and values for the group fed with triacylglycerol plus carnitine that are statistically different from those of the group fed with triacylglycerol alone are shown by †*P* < 0.01.

State of rats	Liver concentration (nmol/g)			Blood concentration (mM)		
	Free carnitine	Acyl-carnitine	Total carnitine	Acetoacetate	3-Hydroxy-butyrate	Total ketone bodies
Unfed (14)	132 ± 4	29 ± 3	164 ± 4	0.05 ± 0.01	0.09 ± 0.01	0.14 ± 0.01
Unfed + triacylglycerol (6)	129 ± 4	27 ± 2	156 ± 6	0.60 ± 0.04*	0.6 ± 0.11*	1.2 ± 0.2*
Unfed + triacylglycerol + carnitine (6)	300 ± 23*†	71 ± 5*†	370 ± 21*†	0.40 ± 0.04*	0.8 ± 0.04*	1.2 ± 0.06*
Suckling (6)	270 ± 31*	70 ± 4*	340 ± 27*	0.45 ± 0.03*	1 ± 0.13*	1.45 ± 0.15*

Fig. 5. Effects of triacylglycerol feeding on the blood concentrations of ketone bodies in starved newborn rats. Newborn rats, kept at 37°C, were starved from birth and then fed either 100 μl of 0.9% (w/v) NaCl (○) or 100 μl of a 10% (w/v) triacylglycerol emulsion (●). For further details see the Materials and Methods section. Arrows indicate the time of feeding for each group of rats. The results are expressed as mM and each point is the mean ± S.E.M. for 12 determinations.

Fig. 6. Plasma non-esterified fatty acids in starved newborn rats fed with NaCl or a triacylglycerol emulsion. Newborn rats, kept at 37°C, and starved from birth were fed 100 μl of 0.9% (w/v) NaCl (○) or 100 μl of a 10% (w/v) triacylglycerol emulsion (●) at different times after birth. Plasma was sampled 1, 2 or 3 h after the feeding. The results are expressed as mM and each point is the mean ± S.E.M. for five or six determinations.

to the postnatal age: at any time studied after birth triacylglycerol feeding was associated, within 1h, with an increase in plasma non-esterified fatty acids

from 0.2mM to approx. 0.7mM (Fig. 6), so that plasma concentrations of non-esterified fatty acids achieved 3h after triacylglycerol feeding were the same at different times after birth (Fig. 6), whereas blood ketone-body concentrations were very different (Fig. 5).

Similarly, 1h after injection of butyrate the ketonaemia was most pronounced between 12 and 16h (Fig. 7). However, at any time interval the concentration of blood ketone bodies after butyrate injection was substantially higher than with triacylglycerol feeding. Although the same amount of carbon was provided in both cases, a strict quantitative comparison was not possible because of the difference in the mode of administration of fatty acids (oral or subcutaneous). Nevertheless these experiments do suggest that a limitation of metabolism of long-chain fatty acids exists before their entry into the mitochondria as acyl-CoA derivatives. The increase in ketonaemia between 12 and 16h with both long-chain fatty acids and butyrate may indicate a second site of regulation of ketogenesis which is probably located within the mitochondria. This assumes that it is not an increase in the butyryl-CoA synthetase activity which is responsible for the increased ketogenesis from butyrate after 12h. An argument in favour of this assumption is that there is no significant increase in the mitochondrial-matrix decanoyl synthetase activity between birth

Fig. 7. Effects of sodium butyrate injection on the blood concentrations of ketone bodies in starved newborn rats. Newborn rats, kept at 37°C, were starved from birth and then given a subcutaneous injection of either 50 μ l of 0.9% (w/v) NaCl (○) or of 10mg of sodium butyrate in 50 μ l of 0.9% (w/v) NaCl (●). For further details see the Materials and Methods section. Arrows indicate the time of injection for each group of rats. The results are expressed as mm and each point is the mean $\pm$ S.E.M. for six to eight determinations.

and 1 day after birth in the rat (Foster & Bailey, 1976b).

Conclusions

In many respects the time course of development of ketogenesis after birth resembles that which occurs on transition from the fed to the starved state in the adult rat (McGarry *et al.*, 1973). An increased delivery of non-esterified fatty acid is not by itself sufficient to increase the concentration of ketone bodies; the major increase occurs when hepatic glycogen has been depleted. As in the adult rat (McGarry & Foster, 1971; Williamson & Whitelaw, 1978), short-chain fatty acids, in this case butyrate, are more effective precursors of ketone bodies than long-chain fatty acids when appreciable amounts of glycogen are present.

Two sites could be regarded as the intramitochondrial limiting step: (1) the disposal of acetyl-CoA linked to the activity of citrate synthase, the fate of citrate and the provision of oxaloacetate and (2) the activity of the enzymes of the hydroxymethylglutaryl-CoA cycle. Hipolito-Reis *et al.* (1974) have indeed shown a 3-fold increase in the activity of hydroxymethylglutaryl-CoA synthase, the limiting enzyme of the pathway, between birth and 1 day after birth. However, in the starved newborn rat, which develops normally its capacity to form ketone bodies between 12 and 16h after birth, there is only a 60% increase in the activity of the hydroxymethylglutaryl-CoA synthase between birth and 12h and then no increase until 16h (Fig. 8). This fits well with

Fig. 8. Activity of hydroxymethylglutaryl-CoA synthase in livers of starved newborn rats. Newborn rats, kept at 37°C, were starved from birth. The results are for cytosolic (●), particulate (○) and total (□) activity expressed as units of activity (μ mol/min per g fresh wt. of liver) and each point is the mean $\pm$ S.E.M. for four to six determinations.

the observation of Hipolito-Reis *et al.* (1974), showing that the postnatal increase in the activity of the mitochondrial hydroxymethylglutaryl-CoA synthase is prevented by starvation.

In the newborn, a rise in the content of liver carnitine is not necessary for an increased ketogenic capacity, a fact that does not fit with the hypothesis developed by McGarry *et al.* (1975) in the adult rat. As an alteration in the rate of entry of acyl-CoA inside the mitochondria only partially explains the slow onset of ketogenesis at birth, and as an increase in the hydroxymethylglutaryl-CoA pathway does not appear to be a prerequisite for an increased ketogenic capacity, it is suggested that an important control point could be the fate of acetyl-CoA inside the mitochondria.

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