Diurnal Rhythms of Hepatic Carbohydrate Metabolism During Development of the Rat

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The '8+16' feeding schedule (8h feeding and 16h without food in each 24h cycle) was applied to nursing mother rats to study enzyme development in neonatal rats in the absence of solid food. A '16+8' suckling schedule (16h with the mother and 8h while the mother is fed in a separate cage) was used to show that the increases in pyruvate kinase, glucokinase and aldolase B activities that occur in the late suckling period of liver development do not require the intake of solid food at this time. Their activities may, however, be modulated by the composition of the diet at the time of weaning. Adaptation to the composition of the diet can occur within one feeding period, and to the periodicity of food provision in one or two feeding periods. In the early neonatal period, diurnal rhythms of tyrosine aminotransferase, liver glycogen and glucokinase are either greatly suppressed or absent, but develop rapidly after weaning. Food-dependent rhythms of glycogen and tyrosine aminotransferase were included in the late suckling period (day 14).

Developmental patterns of changes in activity of many hepatic enzymes have been described and reviewed (Walker, 1971; Greengard, 1971; Bailey & Lockwood, 1973). Many of these changes have been interpreted in terms of adaptation of the enzymes in response to, or anticipation of, the major changes in dietary status occurring at birth and at weaning. Thus food appears to be an important factor in determining the course of liver development, but the underlying mechanisms are still poorly understood.

In adult animals pronounced diurnal variations in some liver enzymes and in liver metabolism have been shown to occur (for examples see Watanabe *et al.*, 1968; Black & Axelrod, 1970; Walker & Potter, 1971) and similar diurnal rhythms have been shown to occur in neonatal animals, particularly with regard to tyrosine aminotransferase (Honova *et al.*, 1968; Ulrich & Yuwiler, 1971; Osawa *et al.*, 1972) and hydroxymethylglutaryl CoA reductase (McNamara *et al.*, 1972). There appears to be some dispute as to the nature of the amplitude and periodicity of the neonatal rhythms compared with adults, but the fact that they exist means that they must be taken into account in any further studies on liver development.

For several years, in this laboratory, we have used precisely controlled feeding schedules based on the natural diurnal variation in feeding activity of adult rats. One such regimen has been referred to as the

'8+16' schedule, the two numbers representing the number of hours with and without food respectively in each feeding cycle (Watanabe *et al.*, 1968). The present paper describes the application of controlled feeding schedules ('16+8' and '8+16' respectively) to suckling and weanling rats to better define the role of food in determining the phenotype of the liver during development. This work forms part of a programme to investigate the nature of the changes in enzymes that characterize the differentiated state in liver, and to study the relation between changes in the state of differentiation of liver and cell division within the tissue, particularly with regard to the parenchymal cells (Bonney *et al.*, 1973a,b; Hopkins *et al.*, 1973).

Experimental

Materials

All substrates, enzymes and cofactors used in the enzyme assays were obtained from the Boehringer (Mannheim) Corp., Ltd., New York, N.Y., U.S.A. Pelleted animal diets (Watanabe *et al.*, 1968) were obtained from General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.

Treatment of animals

Female rats, each with a litter of eight pups, were obtained on the day after giving birth from Sprague-Dawley Inc., Madison, Wis., U.S.A. The animals were housed in a windowless room subject to controlled temperature and humidity. Lighting was regulated to provide 12h of light and 12h of darkness.

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Food consisted of a pelleted diet containing 61% carbohydrate (30% protein) as described previously (Watanabe *et al.*, 1968). Food was available *ad libitum* to all the mothers for the first seven days after giving birth. Preliminary experiments indicated that the use of specially designed spill-proof containers, normally used to measure food consumption, served to allow free access to food for the mothers, but generally prevented the pups from obtaining solid food. These containers were always used for feeding *ad libitum*. In some experiments feeding *ad libitum* was continued until weaning. In other experiments the mother was removed from the cage during the first 8 h of the dark period on day 8 and allowed to feed in a separate cage. The female was then returned to the pups for the remaining 16 h of the 24 h cycle. No food was available in the cage containing the pups, but water was available *ad libitum* in both cages. This protocol was then maintained until weaning. Thus the mothers were maintained on the '8+16' feeding schedule and the pups on a new '16+8' schedule. This protocol could be started successfully no sooner than 8 days after birth, but did serve to ensure that the pups had no access to solid food after that time and before weaning.

Weaning was carried out artificially at 21 days after birth. Pups were housed three to a cage and were weaned to the '8+16' feeding schedule, i.e. food was available for only the first 8 h of the dark period on day 21 and then the first 8 h of each subsequent dark period. In most experiments the diet was the 61% carbohydrate (30% protein) diet, but in some experiments the animals were weaned to diets containing 79% carbohydrate (12% protein) or 31% carbohydrate (60% protein). In all three diets the carbohydrate was available as free glucose and the fat content was 5%.

Experimental protocol

Three groups of females plus litters were used to carry out the experiments described below.

Group 1. Females were fed *ad libitum* and kept in the cages with the pups until weaning. The light period commenced at 08.30 h and finished at 20.30 h. Male or female pups were removed at random from the litters at 08.30, 12.30, 16.30 and 20.30 h. Three animals were taken at each time-point and care was taken not to deplete any one litter below six pups/litter. Pups were transferred between litters to maintain a litter size of 6–8 pups at all times.

Group 2. Animals in this group were treated as the animals in group 1 except that they were subject to an inverted lighting schedule with the dark period commencing at 08.30 h. Animals were removed at the same clock times as those described above. Thus between the two groups it was possible to obtain seven different time-points in any one 24 h period. On day

21 animals in this group were weaned to the '8+16' feeding schedule with the 61% carbohydrate diet.

Group 3. The '16+8' feeding schedule was used for the animals in this group commencing on day 8. On day 14 animals were killed at the clock times indicated in Fig. 2 on the inverted lighting schedule. On day 21 animals were either weaned to one of the three experimental diets on the '8+16' feeding schedule, not weaned or starved and killed at the times indicated in Table 1.

Preparation of homogenates

Livers were rapidly removed from the animals into ice-cold 0.9% NaCl. The tissue was blotted, weighed and homogenized in a Potter–Elvehjem homogenizer in an equal volume of a buffer consisting of 75 mM-Tris-HCl, 0.25 M-sucrose and 0.5 mM-dithiothreitol at pH 7.4. A sample of this homogenate containing 100 mg of tissue was taken and used for the determination of glycogen. The remainder of the homogenate was centrifuged at 105 000 g for 60 min in a Beckmann L2 centrifuge at 4°C. The resulting supernatant was taken and used for the enzyme assays.

Enzyme assays

The following enzymes were assayed with fresh supernatants. Pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase; EC 2.7.1.40) was assayed by the method of Bücher & Pfeleiderer (1955) in the presence of fructose diphosphate to ensure maximal activity of the enzyme. Hexokinase (ATP-D-hexose 6-phosphotransferase; EC 2.7.1.1) and glucokinase (ATP-D-glucose 6-phosphotransferase; EC 2.7.1.2) were assayed by the method described by Walker & Parry (1966). Aldolase (D-fructose 1,6-diphosphate-D-glyceraldehyde 3-phosphate lyase; EC 4.1.2.13) was assayed by the method of Blostein & Rutter (1963) with fructose diphosphate and fructose 1-phosphate as substrates. The contributions of aldolases A and B to the total aldolase activity in a mixture were calculated from the fructose diphosphate/fructose 1-phosphate activity ratio as described by Endo *et al.* (1970). All assays were carried out at pH 7.4 and 25°C. The change in E_{340} was measured continuously in a Gilford model 220 spectrophotometer and chart recorder after preincubation of the reaction mixtures at 25°C for 5 min. Endogenous rates were determined by using suitable controls. A unit of enzyme activity is defined as the amount of enzyme required to metabolize 1 μ mol of substrate/min at 25°C.

Tyrosine aminotransferase (L-tyrosine-2-oxoglutarate aminotransferase; EC 2.6.1.5) was assayed for in supernatants previously stored at -20°C by the method of Diamondstone (1966). Tyrosine aminotransferase activity is expressed as units/g of liver at 37°C.

Chemical assays

For the determination of liver glycogen, samples of crude homogenates (containing 100mg of tissue) were precipitated in 10% (w/v) (final concn.) trichloroacetic acid and rehomogenized with a Polytron homogenizer (Kinematica G.m.b.H., Lucerne, Switzerland) to ensure complete cell breakage. Glycogen was extracted by the method of Roe *et al.* (1961) and measured with the anthrone reagent (Ashwell, 1957). This acid extraction has been compared with the alkaline extraction method described by Roe & Dailey (1966) and shown to yield equivalent results (Bonney *et al.*, 1973a). Blood glucose concentrations were determined by using the semimicro method described in the Glucostat assay kit (Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Results

Diurnal patterns in suckling and weanling animals

Fig. 1 shows a composite of data obtained at different times of day between 6 and 30 days after birth by using animals from groups 1 and 2. Stomach weights indicated that at 6 and 8 days after birth the neonates suckled predominantly in the light period. At 14 and 16 days there was little observable difference in stomach weight in relation to time of day. However, on day 20 (the day before weaning) there was a pronounced increase in stomach weight during the dark period. After weaning to the '8+16' feeding schedule pronounced changes in the diurnal pattern of stomach weight were apparent as early as the feeding period on day 22.

Fig. 1. Diurnal rhythms of stomach weight (a), liver glycogen (b), glucokinase (c), and tyrosine aminotransferase (d)

Each point is the mean of three determinations $\pm$ S.E.M. ●, Animals maintained on the inverted lighting (group 2) schedule; ○, animals on the normal lighting schedule (group 1). Animals were found to first open their eye-lids on day 15 and were weaned at the start of the dark period on day 21. The dark period is shown by the hatched area and the feeding period (stippled area) is indicated below it on days 22, 24 and 30.

In general the changes in liver glycogen content and tyrosine aminotransferase activity are similar to the changes in stomach weight. Diurnal variation before day 20 is small, whereas after weaning the patterns rapidly become similar to those of the adult animals (Walker & Potter, 1971).

Although Shatton *et al.* (1969) and Sigman *et al.* (1972) provided electrophoretic evidence for the presence of glucokinase in foetal liver no activity could be detected, in the present study, until 8 days after birth. At 8 days a trace of activity (0.02–0.03 unit/g of liver) was detected that increased to 0.1 unit/g by 10 and 12 days after birth. Thus glucokinase activity was detected in neonatal livers earlier than that described by Walker & Holland (1965) or by Jamdar & Green-gard (1970), but later than that reported by Adelman & Freeman (1972). The low activity of the enzyme during this period precluded the detection of diurnal rhythms. On days 22 and 24 there was a rapid increase in enzyme activity during the dark period to give the adult values.

Diurnal variation in enzyme activity for pyruvate kinase, aldolase or hexokinase was not detected at any of the developmental periods studied (data not shown). Blood glucose concentrations increased from approx. 80mg/ml of blood at 6 days after birth to approx. 120mg/ml by 12 days and then remained relatively constant. No rhythm of blood glucose concentration was detected at any of the stages studied; thus the neonatal animals appeared to be able to control their blood glucose concentration within a very narrow range. There was also no diurnal rhythm of liver weight before weaning. After weaning there was a pronounced increase in liver wt./100g body wt. during the dark period. For example on day 20 the weight increased from 3.47g to 5.00g during the dark period and then returned to the basal value during the light period in the respective groups.

Studies with the '16+8' suckling schedule

No evidence of solid food was found in the stomachs of the neonatal animals used in the experiment described in Fig. 1 where the spill-proof containers were used. Thus it appeared feasible that the observed increase in glucokinase in the late suckling period may not be correlated with the gradual self-weaning of young animals resulting in increased ingestion of solid food as suggested by Vernon & Walker (1968). To make absolutely certain that neonatal animals were prevented from eating solid food before day 21 the '16+8' suckling schedule was used as described in the Experimental section. There was little or no increase in body weight of the pups for the 2 day period after the start of this protocol, but after day 10 the growth rate of the animals was essentially the same as animals fed *ad libitum*. The appearance of glucokinase activity at day 12 after birth in the livers of these animals confirmed the results obtained with the spill-proof food containers. There were also increases in pyruvate kinase and aldolase B activities before weaning (P. R. Walker, R. J. Bonney & V. R. Potter, unpublished work).

Thus the increases in the activity of these enzymes are programmed to take place at a given stage of neonatal development before weaning although the changes in activity may be modulated by the nature of the diet used to wean the rats. This is shown more clearly in Table 1. In this experiment five groups of rats were maintained on the '16+8' schedule until day 21. The animals were then allowed to continue suckling or weaned to one of the three diets indicated or starved. The results for day 22 (i.e. after one 8 h feeding period and a 16h fast in the case of weaned animals) show that glucokinase activity increases in all five groups, particularly in the animals weaned to the 61% and 79% carbohydrate diets. The high con-

Table 1. *Effect of the composition of the diet on liver enzymes after weaning*

Animals from group 3 were either not weaned, or weaned on to the diet indicated, or starved on day 21 and assays carried out on the days indicated. All the animals were killed at the end of the light period.

Age (days)	Diet	Liver weight (g)	Enzyme activity (units/g of liver)			
			Total pyruvate kinase	Aldolase B	Hexokinase	Glucokinase
20	Suckling	1.63 ± 0.04	24.1 ± 0.27	4.17 ± 0.17	0.10 ± 0.01	0.35 ± 0.01
22	Starvation	1.52 ± 0.09	29.7 ± 2.1	6.59 ± 0.32	0.19 ± 0.01	0.62 ± 0.02
22	Suckling	2.02 ± 0.03	28.9 ± 2.4	6.35 ± 0.68	0.20 ± 0.01	0.80 ± 0.03
22	79% Carbohydrate	1.72 ± 0.07	61.8 ± 6.2	6.51 ± 0.24	0.21 ± 0.02	1.37 ± 0.05
22	61% Carbohydrate	1.77 ± 0.08	35.3 ± 2.8	6.10 ± 0.21	0.21 ± 0.01	0.86 ± 0.07
22	31% Carbohydrate	2.01 ± 0.07	26.5 ± 1.4	5.46 ± 0.42	0.24 ± 0.008	0.52 ± 0.03
25	Starvation	1.07 ± 0.13	19.3 ± 2.7	6.02 ± 0.13	0.15 ± 0.02	0.15 ± 0.02
28	Suckling	2.26 ± 0.04	24.9 ± 0.8	5.46 ± 0.16	0.22 ± 0.01	0.55 ± 0.02
28	79% Carbohydrate	2.56 ± 0.12	91.6 ± 10.0	7.95 ± 0.13	0.25 ± 0.01	1.77 ± 0.15
28	61% Carbohydrate	2.78 ± 0.08	102.9 ± 15.3	6.75 ± 0.13	0.25 ± 0.02	0.89 ± 0.05
28	31% Carbohydrate	3.32 ± 0.24	34.5 ± 5.8	5.22 ± 0.21	0.18 ± 0.01	0.52 ± 0.02

Fig. 2. Changes in stomach weight (a) liver weight (b), glycogen (c), glucokinase (d) and tyrosine aminotransferase (e) in liver in 14-day-old animals on the '16+8' suckling schedule

Animals from group 3 were killed at 4h intervals as shown. Each point is the mean of 3 determinations $\pm$ s.e.m. The 16h suckling period (stippled area) commences, as indicated by the arrow, at 16.30h and lasts for the final hours of the dark period and the whole of the light period.

centration of protein in the 31% carbohydrate (60% protein) diet appeared to inhibit the rate of increase of glucokinase activity. Similarly, the increases in aldolase B and total pyruvate kinase activity were lowest on the high-protein diet, although hexokinase had the highest increase. There was little difference in aldolase B activity in the other groups whereas pyruvate kinase induction was greatest in the diet containing the most carbohydrate (+79% on the 79% carbohydrate diet). All the enzymes showed increased activities during starvation. It is noteworthy that the liver is capable of the synthesis of approx. 1 unit of glucokinase and 35 units of pyruvate kinase in a single feeding period. Five days was found to be the maximum period that the starved animals could survive and enzyme analysis at this stage showed that there was little or no change in hexokinase, aldolase B and total pyruvate kinase activity during this period, which is in agreement with Walker & Holland (1965).

On day 28 the enzyme activities showed similar differences between the four groups as shown on day 22. A glucokinase activity of 1.77 units/g and pyruvate

kinase activities approaching 100 units/g are much higher than the adult activities of these enzymes, but this kind of 'overshoot' phenomenon has been described previously (Taylor *et al.*, 1967). Rat milk contains approx. 35% protein, 50% fat and 10% carbohydrate on a dry weight basis (Dymysz *et al.*, 1964); thus inspection of the data would suggest that fat represses these enzyme activities before weaning and that the increases are generally proportional to the carbohydrate content of the diet, although high protein concentrations may be inhibitory. The values for the liver weights of the animals on these different diets show that there is little difference between the groups and no correlation between the size of the liver and the enzyme activities.

Diurnal rhythms in livers of animals on the '16+8' suckling schedule

Since the feeding period after weaning appeared important in determining the amplitude of the diurnal rhythms the effect of the '16+8' suckling schedule

was investigated in 14-day-old animals. Animals from group 4 were killed on day 14 since this allowed the maximum period on the schedule, but took place before 'eye-opening'. The results are shown in Fig. 2. The stomachs of the neonates were almost empty at the time the mother returned to the cage after her 8 h feeding period. Suckling commenced immediately and the stomach contained food throughout the suckling period and decreased during the 8 h in which the mother was absent from the cage. Glycogen concentrations and tyrosine aminotransferase activity showed pronounced rhythms in response to this feeding schedule whereas glucokinase showed relatively little change during this period.

Discussion

The use of the '16+8' suckling schedule has clearly demonstrated that the changes in the enzymes of carbohydrate metabolism measured in the present paper precede the intake of solid food that occurs at weaning. It appears therefore that the increases in enzyme activity are triggered by changes in the hormonal status of the neonate as suggested by Greengard (1971) or as a more remote possibility, by changes in the composition of the maternal milk supply. When weaning occurs, particularly to a diet with a very high carbohydrate content, enzymic adaptation can take place within a single feeding period (Table 1) yielding enzyme activities that can be twice those found in adult animals on the same diet. When weaned on day 21 the animals can also adapt to the periodic nature of the food supply almost immediately (Fig. 1, day 22).

The appearance of glucokinase activity in the late neonatal period may be part of the development of the capacity for diurnal storage of glycogen. It is noteworthy that the increase in glucokinase activity on days 22 and 24 occurs during the time when the animal is increasing its glycogen reserve during the feeding period.

The data presented in the present paper provide some evidence for the existence of diurnal rhythms in neonatal animals that are greatly decreased in amplitude compared with the adult rhythm for the same parameter. The rhythms appear to be greatly influenced by the supply of milk and further work is required to establish whether these rhythms are intrinsic to the animal or to the parenchymal cells and are merely modulated by the changing pattern of food supply. Light and food may act as 'Zietgebers' to reinforce and fix the periodicity and amplitude of these rhythms in relation to the time of day. It is clear that enzymic changes in the liver of the neonatal animal reflect not only the changes in the composition of the diet (e.g. the high-fat/low-carbohydrate to high-carbohydrate/low-fat transition at weaning), but also the changes in periodicity of food availability (Walker & Potter, 1971).

The neonatal system with the '16+8' suckling schedule may provide a useful tool for investigating the relation between endogenous and external stimuli that results in the pattern of diurnal rhythms characteristic of the adult animal. The schedule may be especially useful for studies involving the inducibility of various enzymes by insulin, cortisol, glucagon and adrenocorticotrophic hormone (Adelman & Freeman, 1972) when the control values may be made more secure and the injections can be made at various times in the 24 h period. It may be that the observations on insulin inducibility of glucokinase in the period 4–8 days (Adelman & Freeman, 1972) were related to the fact, noted above, that the '16+8' schedule cannot be instituted earlier than 8 days, when a continual suckling seems mandatory.

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