Changes in Hepatic Lipogenesis during Development of the Rat

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1. Changes in the activities of ATP citrate lyase, 'malic' enzyme, glucose 6-phosphate dehydrogenase, pyruvate kinase and fructose 1,6-diphosphatase, and in the ability to incorporate [1-¹⁴C]acetate into lipid have been measured in the livers of developing rats between late foetal life and maturity. 2. In male rats the activities of those systems directly or indirectly concerned in lipogenesis (acetate incorporation into lipid, ATP citrate lyase and glucose 6-phosphate dehydrogenase) fall after birth and are maintained at a low value until weaning. After weaning these activities rise to a maximum between 30 and 40 days and then decline, reaching adult values at about 60 days. 'Malic' enzyme activity follows a similar course, except that none could be detected in the foetal liver. Pyruvate kinase activity is lower in foetal than in adult livers and rises to slightly higher than the adult value in the post-weaning period. Fructose 1,6-diphosphatase activity rises from a very low foetal value to reach a maximum at about 10 days but falls rapidly after weaning to reach adult values at about 30 days. 3. Weaning rats on to a high-fat diet caused the low activities of acetate incorporation, ATP citrate lyase, glucose 6-phosphate dehydrogenase and pyruvate kinase, characteristic of the suckling period, to persist. 'Malic' enzyme and fructose 1,6-diphosphatase activities were not altered appreciably. 4. No differences could be detected in hepatic enzyme activities between males and females up to 35 days, but after this time female rats gave higher values for acetate incorporation, glucose 6-phosphate dehydrogenase activity and 'malic' enzyme activity. 5. The results are discussed in relation to changes in alimentionation and hormonal influences.

Although changes in the metabolism of protein and carbohydrate during development have been widely studied (Herrmann & Tootle, 1964; Dawkins, 1966) less attention has been paid to lipid metabolism. It is well established that hepatic lipogenesis is suppressed immediately after birth and rises after weaning (Villem & Hagerman, 1958; Carroll, 1964); however, no information is available for the time-course of changes occurring after 30 days. The work reported here provides data on lipogenesis and on the activities of several enzymes associated with lipogenesis, from foetal life to maturity in the normal laboratory rat.

A preliminary account of these results has already been published (Bailey, Taylor & Bartley, 1967). Since the completion of the work described in the present paper, Ballard & Hanson (1967) have reported changes in hepatic activities of 'malic' enzyme and ATP citrate lyase in early development that are essentially in agreement with our results.

EXPERIMENTAL

Materials. All reagents used were of analytical grade or the purest available. The following chemicals were obtained from C. F. Boehringer Corp. (London) Ltd., London, W. 5: ADP, ATP, NADH, NADP⁺, NADPH, phosphoenolpyruvate (tricyclohexylammonium salt), fructose 1,6-diphosphate and lactate dehydrogenase. Sodium [1-¹⁴C]acetate and [U-¹⁴C]palmitic acid were obtained from The Radiochemical Centre, Amersham, Bucks. Except where stated, other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset.

Animals and diets. Wistar rats from the Sheffield University Animal House Colony were used throughout. The animals were killed, all at about the same time of day, by a blow on the head. Foetuses were removed from the mother near term, i.e. at 19–20 days as estimated by dated matings. The weight range of the foetuses used was 2.5–5.0 g. (cf. Lang, 1965). Litters were kept with their mothers, who had access to food cubes *ad libitum*. The young were weaned at 21 days, but from about 14 days onwards they could reach the food containers and consumed a mixed diet of food cubes and milk.

The food cubes were Oxoid diet 86 [Herbert C. Styles (Bewdley) Ltd., Bewdley, Worcs.], the composition of which is given by Bartley, Dean, Taylor & Bailey (1967), except in the experiment referred to in Table 1, where the 'high-fat diet' consisted of 30% of butter and 70% of diet 86. This mixture was compressed in a pelleting machine and fed *ad libitum* to mother and litter from 14 days *post partum*.

Measurement of acetate incorporation into lipid of liver slices. Liver slices were incubated for 90 min. at 37° in 25 ml. conical flasks with 1.9 ml. of Krebs-Ringer bicarbonate saline (Krebs & Henseleit, 1932) and 10 μ moles (1 μ C) of sodium [14 C]acetate in a final volume of 2.0 ml. Each flask contained 200 mg. wet wt. of liver slices (0.5 mm. thick) and the gas phase was O₂ + CO₂ (95:5). The reaction was stopped after 90 min. by adding 3.0 ml. of 10% (w/v) KOH. The total lipid was extracted, washed free of acetate and counted in a Nuclear-Chicago 725 liquid-scintillation counter as described by Bartley *et al.* (1967). The amount of 14 C incorporated into lipid increased throughout the incubation period.

Measurement of the oxidation of palmitate by liver slices. Reactions were carried out at 37° for 130 min. in double-side-armed Warburg respirometers filled with air. The reaction mixture contained 200 mg. wet wt. of liver slices (0.5 mm. thick), 2.5 μ moles (1 μ C) of potassium [14 C]-palmitate, 0.5 μ mole of fat-free bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and 2.0 ml. of Krebs-Ringer phosphate saline (Krebs, 1933) in a total volume of 3.0 ml. Fatty acids were removed from the bovine plasma albumin by the method of Garland, Newsholme & Randle (1964). [14 C]Palmitic acid was converted into potassium [14 C]palmitate as described by Björntop (1966). The reactions were terminated by adding 0.3 ml. of 50% (v/v) HClO₄. Evolved 14 CO₂ was absorbed in 0.2 ml. of 20% (w/v) NaOH contained in the centre well of the Warburg flask. The Na₂¹⁴CO₃ formed was converted into Ba¹⁴CO₃, which was suspended in 10 ml. of NE221 gel scintillator [Nuclear Enterprises (G.B.) Ltd., Edinburgh, 11] and counted in a Nuclear-Chicago 725 liquid-scintillation counter. The Warburg flask contents were washed out into centrifuge tubes with 2.0 ml. of distilled water and the precipitated protein present was sedimented by centrifugation (maximum speed in an MSE Super Minor centrifuge for 15 min.). A portion (0.1 ml.) of the supernatant ('acid-soluble material') was added to 10 ml. of NE220 liquid scintillator (Nuclear Enterprises) and counted as before.

Measurement of enzyme activities. The assay methods for the activities of the following enzymes were as described by Bartley *et al.* (1967): glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49), fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) and ATP citrate lyase [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8]. Pyruvate kinase activity (ATP-pyruvate phosphotransferase, EC 2.7.1.40) was estimated as described by Taylor & Bailey (1967), except that the final concentration of phosphoenolpyruvate was 1 mM and that fructose 1,6-diphosphate (0.5 mM) was added to ensure full allosteric activation. 'Malic' enzyme activity [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40] was determined by a modification of the method of Ochoa (1955) in which the glycylglycine buffer used by that author was

replaced by tris buffer (final concn. 33.3 mM), pH 7.4. In our experience tris buffer gave higher values for enzyme activity and much lower control rates of NADP⁺ reduction in the absence of malate than did glycylglycine buffer.

Assays of enzyme activity were carried out in random order, two or more age-groups being included in any one batch of assays, with the supernatants from the centrifugation of liver homogenates at 20000g for 30 min. The homogenates were made as follows: for pyruvate kinase and 'malic' enzyme, 1 g. of liver in 39 ml. of ice-cold distilled water; for ATP citrate lyase, 1 g. of liver in 9.0 ml. of ice-cold 0.4 M-KCl made in aq. 20% (v/v) ethanol; for glucose 6-phosphate dehydrogenase and fructose 1,6-diphosphatase, 1 g. of liver in 39 ml. of ice-cold 0.9% NaCl containing 0.66 mM-EDTA. For the fructose 1,6-diphosphatase assays livers that had been stored at -20° were used; for all other assays fresh livers were used.

Units of enzyme activity. All enzyme activities are expressed as units/g. wet wt. of liver, 1 unit being defined as 1 μ mole of substrate utilized/min. at 25°.

RESULTS

[14 C]Acetate incorporation into lipid of liver slices (Fig. 1). Under the conditions of the experiment, foetal liver slices incorporated 2-6% of the acetate present into lipid. Incorporation fell to about 1% at 3 days after birth and remained at a low value until 20 days. Thereafter it rose to reach a maximum of about 12% at 30-35 days. For male rats the incorporation values then fell steadily towards the adult value of 1-2%. However, even at 65 days, incorporation was greater than in liver slices from adult rats, aged 100-130 days, taken from stock. No clear differences were noted between the values for male and female rats up to 40 days, but in more mature animals the acetate incorporation was greater in the females than in the males.

Fig. 1. Incorporation of [14 C]acetate into lipid of liver slices from male (●) and female (○) rats. Details of the method are in the Experimental section. On the abscissa scale, 'F' denotes foetal rats and 'S' denotes adult stock rats. The line is drawn by eye through the points for male rats.

Fig. 2. Activities of hepatic ATP citrate lyase during development in male (●) and female (○) rats. Enzyme activity was measured spectrophotometrically as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats and 'S' denotes adult stock rats. The line is drawn by eye through the points for male rats.

Fig. 3. Activities of hepatic 'malic' enzyme during development in male (●) and female (○) rats. Enzyme activity was measured spectrophotometrically as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats and 'S' denotes adult stock rats. The line is drawn by eye through the points for male rats.

ATP citrate lyase activity (Fig. 2). The ATP citrate lyase activity values for foetal livers were not significantly different from those of the adult stock rats, but during the 5 days following birth activity progressively fell to a very low value. From about 20 days, activity increased to reach a maximum coincident with that for acetate incorporation, i.e. at 30–40 days. The decline to adult values was complete by about 70 days. No differences were apparent between the results for male and female rats in this series.

'Malic' enzyme activity (Fig. 3). The activity of this enzyme also rose from a very low value at 20 days to reach a peak at about 30 days, falling thereafter to reach adult values at 70 days. Unlike ATP citrate lyase, however, 'malic' enzyme was scarcely detectable in the foetus or the neonatal

Fig. 4. Activities of hepatic glucose 6-phosphate dehydrogenase during development in male (●) and female (○) rats. Enzyme activity was measured spectrophotometrically as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats and 'S' denotes adult stock rats. The line is drawn by eye through the points for male rats.

Fig. 5. Activities of hepatic pyruvate kinase during development in male (●) and female (○) rats. Enzyme activity was measured spectrophotometrically as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats and 'S' denotes adult stock rats. The line is drawn by eye through the points for male rats.

rat. Special care was taken to exclude the possibility that apparent absence of enzyme might be due to inhibition of the enzyme in the assay mixture. In eight samples of foetal liver tested, activity was never higher than 0.1 unit/g. wet wt. of liver. That these low values were not due to enzyme inhibition was demonstrated by the fact that the assay mixture, including foetal enzyme, responded fully to the addition of a sample of 'malic' enzyme from a 30–40-day-old rat. Again no difference could be detected between the values for male and female rats until 40 days, after which females gave rather higher values than males.

Glucose 6-phosphate dehydrogenase activity (Fig. 4). The activity of this enzyme fell after birth, but neither as rapidly nor to the same extent as did ATP citrate lyase activity or acetate incorporation into lipids. A brief maximum occurred at about 34 days in male rats, the activity falling to the adult value by about 40–45 days. There was no apparent difference in the liver glucose 6-phosphate dehydrogenase activity of male and female rats up to about 35 days. Beyond this time the females were characterized by higher values of activity.

Pyruvate kinase activity (Fig. 5). Enzyme activity in the foetal liver was about half that in the adult rat. During the first 20 days of life there was no marked change in activity, but after this a threefold increase took place steadily, the values attained at 70–80 days being slightly higher than those found for adult stock rats. Males and females gave similar results throughout.

Fructose 1,6-diphosphatase activity (Fig. 6). Activities of this enzyme in foetal liver were extremely low, less than 1 unit/g. wet wt. of liver, as previously reported by Ballard & Oliver (1962) and Burch (1965). Immediately after birth activity rose rapidly, attaining a maximum value of 10 units/g. wet wt. of liver at 8–10 days. Thereafter activity fell to reach the adult value at 25–30 days.

Utilization of palmitate. In an attempt to determine changes in the liver's capacity for lipid catabolism, the formation of carbon dioxide (Fig. 7) and acid-soluble products (Fig. 8) from [U- 14 C]-palmitate was measured in tissue slices. The production of $^{14}\text{CO}_2$ was rather greater 1 day after birth than in the foetus but fell progressively to reach a steady value at 10 days. Production of labelled acid-soluble material increased similarly after birth but, in contrast with carbon dioxide production, it was maintained at a high value for 15–20 days and did not reach a final steady low value until about 30 days.

Effect on hepatic enzyme activities of weaning on to a high-fat diet. The rise after weaning in the activity of enzymes concerned with lipogenesis could be ascribed to a change in the fat content of the diet at this time. To test this possibility a group of rats was weaned on to a diet of butter + diet 86 (3:7, w/w), and the activities of the enzymes were measured at 35 days. This time was chosen because it corresponds to the peak of activity for most of the enzymes concerned. The controls for this experiment were a similar group of rats weaned on to diet 86. The results are shown in Table 1. Acetate incorporation into lipid and the activities of ATP citrate lyase, glucose 6-phosphate dehydrogenase and pyruvate kinase were all lower by 50–75% on the high-fat diet than on the control diet. Fructose 1,6-diphosphatase activity was

Fig. 6. Activities of hepatic fructose 1,6-diphosphatase during development in male rats. Enzyme activity was measured as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats.

Fig. 7. Production of $^{14}\text{CO}_2$ from [U- ^{14}C]palmitate by liver slices from male rats during development. The details are as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats.

Fig. 8. Production of ^{14}C -labelled acid-soluble products from [U- ^{14}C]palmitate by liver slices from male rats during development. The details are as described in the Experimental section. On the abscissa scale, 'F' denotes foetal rats.

unaffected and that of 'malic' enzyme fell by only 15% on the high-fat diet, in contrast with the fall of 55% in the activity of ATP citrate lyase.

Table 1. *Influence of diet on hepatic enzyme activities in 35-day-old rats*

Stock rats had been weaned on to diet 86 and high-fat rats on to a diet consisting of butter + diet 86 (3:7, w/w). Acetate incorporation is expressed as the percentage of acetate initially present incorporated into the lipid of liver slices under standard conditions (see the Experimental section). All the enzyme activities are expressed as μ moles of substrate transformed/min./g. wet wt. of liver at 25°. For each result the mean $\pm$ s.e.m. is given, and the number of animals used is indicated in parenthesis.

	Acetate incorporation (%)	ATP citrate lyase	'Malic' enzyme	Glucose 6-phosphate dehydrogenase	Pyruvate kinase	Fructose 1,6-diphos- phatase
Stock rats	6.23 $\pm$ 0.77 (6)	1.97 $\pm$ 0.11 (9)	5.90 $\pm$ 0.54 (6)	6.45 $\pm$ 0.88 (6)	20.2 $\pm$ 0.71 (6)	4.01 $\pm$ 0.11 (6)
High-fat rats	2.31 $\pm$ 0.51 (6)	0.88 $\pm$ 0.12 (9)	5.00 $\pm$ 0.12 (6)	1.85 $\pm$ 0.21 (6)	8.52 $\pm$ 0.58 (6)	4.24 $\pm$ 0.25 (6)
Ratio high-fat/stock	2.70	2.24	1.18	3.49	2.37	0.95

DISCUSSION

Three periods of change in lipogenesis during the life of the laboratory rat may be distinguished: the fall immediately after birth, the sharp rise after 20 days and the steady decline from about 35 days to maturity. The period of depressed lipogenesis is coincidental with the suckling period, so that the influence of nutritional factors may be suspected rather than the implementation of a genetically determined programme. Nutritionally the early life of the rat is characterized by two changes: a sudden one, when transplacental alimentation, predominantly carbohydrate, is replaced by a high-fat diet of milk; and a more gradual one, when milk is replaced by solid food at weaning. The change at birth is physiologically defined in that, given an adequate maternal diet, the availability of nutrient to the foetus and the composition of the milk are essentially invariable. In contrast, the change at weaning is not strictly defined, since the animals may be weaned on to any of a wide range of diets, all of which may be regarded as normal. Thus the results found for changes in enzyme activities at birth are probably valid for rats in general, whereas those occurring at weaning are valid only insofar as the weaning diet is specified. Diet 86, which we have used in these experiments, and which is in general use in Britain for feeding laboratory rats, has a relatively low fat content (about 3%). Thus the rapid increase in lipogenesis at weaning is probably due to the change from a high- to a low-fat diet. The third period of change in lipogenesis, occurring after 40 days, is unlikely to be dependent on diet, since after weaning this is constant.

The activities of ATP citrate lyase, 'malic' enzyme and glucose 6-phosphate dehydrogenase in liver during development follow qualitatively the pattern of lipogenesis, with the important exception that 'malic' enzyme is absent from the foetus. If, as suggested by Kornacker & Ball (1965), ATP citrate lyase and 'malic' enzyme constitute part

of a sequence that provides extramitochondrial acetyl-CoA and NADPH for fatty acid biosynthesis, then some other system must supply the NADPH in the foetal liver. This function could be fulfilled by glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase (EC 1.1.1.43), the activities of which are similar in foetal and adult livers.

The correlation between the activity of ATP citrate lyase and that of 'malic' enzyme in our experiments is not as close as that found by Kornacker & Ball (1965) or Baldwin, Ronning, Radanovics & Plange (1966) for changes induced by diet. We find, in agreement with Ballard & Hanson (1967), that ATP citrate lyase activity in the foetal liver is considerable, despite the absence of 'malic' enzyme, and, although the changes in the activities of these two enzymes between weaning and maturity are qualitatively similar, the ratio between the activities varies appreciably (cf. Figs. 2 and 3). Moreover, the response of the two enzymes to weaning on to a high-fat diet is different (see below).

The suppression of hepatic lipogenesis and ATP citrate lyase activity in the young during suckling is accompanied by a rise in the activities of these systems in the mammary gland of the mother over this period. The very rapid fall in lipogenesis in the mammary gland immediately after weaning is thought by Levy (1964) to be due to accumulation of milk in the gland.

The results of the experiments in which rats were weaned on to a high-fat diet are consistent with the view that the low activities of lipogenesis, ATP citrate lyase and glucose 6-phosphate dehydrogenase in the livers of suckling rats are due in part to the high fat content of the milk. However, the hepatic enzyme activities obtained on the high-fat diet are not as low as those found during suckling over the period 10–20 days after birth, so that it is likely that factors other than the fat content of the diet are involved. Moreover, the very slight effect of the high-fat diet on 'malic' enzyme

activity makes it unlikely that the lipid content of the diet could be responsible for the low activity of this enzyme during the suckling period.

If the production of labelled acid-soluble material from [^{14}C]palmitate is accepted as an index of fatty acid utilization then this, in contrast with lipogenesis, is elevated during the suckling period when the diet contains a large amount of fat. A similar finding has been reported by Drahota, Hahn & Honova (1966), who demonstrated an increased accumulation of acetoacetate in liver slices during the period 1–10 days after birth.

The activity of fructose 1,6-diphosphatase, a key enzyme in gluconeogenesis, was negligible in the foetal liver, as previously reported by Ballard & Oliver (1962) and Burch (1965). The activity of this enzyme rose during the suckling period and declined after weaning, in contrast with lipogenesis. Glycogen synthesis from pyruvate or galactose is known to follow a similar course to fructose 1,6-diphosphatase activity (Ballard & Oliver, 1963, 1964).

The significance of the changes in hepatic pyruvate kinase activity is not clear. There is little change in activity immediately after birth but a threefold rise occurs after weaning on to a stock diet but not after weaning on to a high-fat diet. Hepatic pyruvate kinase activities are known to be affected by the quantity, proportion and quality of carbohydrate in the diet (Krebs & Eggleston, 1965; Bartley *et al.* 1967; E. Bailey & C. B. Taylor, unpublished work). It is likely that the change after weaning is due to dietary factors.

The fluctuations during development in the activity of enzyme systems associated with lipogenesis are in marked contrast with the behaviour of the enzymes of the tricarboxylic acid cycle and the electron-transport chain. The activities of the latter enzymes rise rapidly after birth, attaining adult values in the first few days of life (Dawkins, 1966), presumably reflecting the constant physiological need for oxidative processes after the relatively anoxic metabolic phase associated with birth.

The enzyme changes found during the first 30 days of life and the directions in which these changes occur show that the liver adapts in such a way as to enable it to deal most efficiently with alterations in the form of nutrient available to the organism. In particular, hepatic lipogenesis varies inversely with the exogenous supply of lipid, although fat is unlikely to be the sole controlling factor concerned.

The changes that occur after 40 days in lipogenesis and ATP citrate lyase, 'malic' enzyme and glucose 6-phosphate dehydrogenase activities have not previously been reported. Since the diet is

constant from weaning to maturity, a change in nutrient cannot be invoked to explain these changes. Their magnitude and their time-course are not consistent with a simple overshoot in enzyme synthesis. The period during which the enzyme activities are falling coincides with the onset of sexual maturity in the male (testes weights, as a percentage of body weight, rise over this period to reach a plateau value at 50 days). Thus it is possible that the fall in enzyme activities could be due to the influence of sex hormones, an idea that is supported by the finding that, in general, enzyme activities in male and female animals diverge only after 40 days. Irrespective of the cause of these changes, it is important to note that, in the normal laboratory rat given a commonly used diet, some hepatic enzymes do not reach a steady activity until 60 days after birth.

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