Factors Involved in Changes in Hepatic Lipogenesis During Development of the Rat

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1. Changes in the activities of acetyl-CoA carboxylase (EC 6.4.1.2), phosphofructokinase (EC 2.7.1.11), aldolase (EC 4.1.2.13), extramitochondrial aconitate hydratase (EC 4.2.1.3) and NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) have been measured in the livers of developing rats from late foetal life to maturity. 2. The effect of altering the weaning time on some enzymes associated with lipogenesis has been studied. Weaning rats at 15 days of age instead of 21 days results in an immediate increase in the activity of 'malic' enzyme (EC 1.1.1.40) whereas the activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and ATP citrate lyase (EC 4.1.3.8) did not increase until 4-5 days and acetyl-CoA carboxylase 2-3 days after early weaning. Weaning rats on to an artificial-milk diet led to complete repression of the rise in activity of hepatic enzymes associated with lipogenesis normally found on weaning, except for 'malic' enzyme, which increased in activity after 20 days of age. 3. The effect of intraperitoneal injections of glucagon, cortisol, growth hormone and thyroxine on the same hepatic enzymes has been investigated. Only thyroxine had any effect on enzyme activities and caused a 20-fold increase in 'malic' enzyme activity and a twofold increase in ATP citrate lyase activity. 4. The activities of hepatic glucose 6-phosphate dehydrogenase and 'malic' enzyme are higher in adult female than in adult male rats and it has been shown that this sex difference in enzyme activities is due to both male and female sex hormones. 5. Hepatic malate, citrate, pyruvate, glucose 6-phosphate and phosphoenolpyruvate concentrations have been measured throughout development. 6. The results are discussed in relation to the dietary and hormonal control of hepatic enzyme activities during development.

Marked changes in the conversion of carbohydrate into lipid in the liver occur during development of the laboratory rat (Ballard & Hanson, 1967; Taylor, Bailey & Bartley, 1967). In particular, at the weaning time when the milk diet is replaced by a high-carbohydrate-low-fat diet, there is a marked stimulation of lipogenesis. Hormonal changes are also occurring during development and so the developing rat provides a useful natural system for the study of hormonal and dietary effects on changes in enzyme activity.

Taylor *et al.* (1967) described 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 [^{14}C]acetate into lipid in the livers of rats throughout development. The present report describes the developmental patterns of two glycolytic enzymes, phosphofructokinase and aldolase, a key enzyme in lipogenesis, acetyl-CoA carboxylase, and two other enzymes that may be associated with lipogenesis, NADP-dependent iso-

citrate dehydrogenase and extramitochondrial aconitate hydratase. Further, the dietary and hormonal control of the observed developmental changes in enzymes associated with lipogenesis has been studied. Some hepatic metabolite concentrations have also been measured during development to determine if there is any correlation with changes in enzyme activity. The observation of Taylor *et al.* (1967) that the activities of hepatic glucose 6-phosphate dehydrogenase and 'malic' enzyme are higher in adult females than in adult male rats has been further investigated.

EXPERIMENTAL

Materials. All reagents were of analytical grade or the purest available. The following materials were obtained from C. F. Boehringer Corp. (London) Ltd., London W.5, U.K.: all enzymes, nucleotides and sugar phosphates, coenzyme A and phosphoenolpyruvate (tricyclohexylammonium salt). Sodium pyruvate, DL-isocitric acid, L-thyroxine and growth hormone (somatotrophin) were purchased from Sigma (London) Chemical Co. Ltd.,

London S.W.6, U.K. Glucagon was obtained from Calbiochem Ltd., London, W.1, U.K. and cortisol from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. $\text{NaH}^{14}\text{CO}_3$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NE220 liquid scintillator was obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K. Full-cream powdered cow's milk was manufactured by Cow & Gate Ltd., Guildford, Surrey, U.K. All other materials were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals and diets. Wistar rats from the Sheffield University Animal House colony were used throughout. Litters were normally removed from the mothers at 21 days of age and weaned on to a stock diet of Oxoid diet 86 [Herbert C. Styles (Bewdley) Ltd., Bewdley, Worcs., U.K.]. The animals were killed at about the same time of day (9–11 a.m.) by a blow on the head. Foetuses were removed from the mother near term (18–21 days); their age was estimated from foetal body weight (Lang, 1965).

In premature-weaning experiments rats were removed from the mother at 15 days of age and fed with a crumbled form of the stock diet. In late-weaning experiments the rats were removed from the mother at 16 days of age and fed on artificial rat-milk diet (Pinto & Bartley, 1969). The animals continued to thrive and gain weight at the normal rate after these weaning procedures.

Hormone injections and operations. The hormones glucagon, cortisol, growth hormone and thyroxine were injected intraperitoneally as a suspension or solution in 0.9% NaCl. The following doses were given separately to different litters of rats, daily from 14 to 17 days of age while the animals remained with their mothers: cortisol, 50 μg ; growth hormone, 50 μg ; thyroxine, 10 μg and glucagon, 60 μg . Control litter mates were injected at the same time with the same volume of 0.9% NaCl. Animals were killed at 18 days of age.

Operations to remove testes and ovaries from developing rats were performed under sterile conditions. Half the number of males and females from litters were castrated or ovariectomized at 21 days of age. The animals were also weaned at this time. They recovered quickly from the operations and gained weight at a similar rate to the control litter mates on which sham operations were performed. The animals were killed at 50 days of age.

Measurement of enzyme activities. Different liver homogenates were prepared for the various enzymes assayed. For 'malic' enzyme, pyruvate kinase, phosphofructokinase, aldolase, isocitrate dehydrogenase and acetyl-CoA carboxylase assays, 0.5 g of liver was homogenized in 4.5 ml of ice-cold 50 mM-tris-HCl buffer, pH 7.3, containing 1 mM-EDTA. For ATP citrate lyase assay, 0.5 g of liver was homogenized in 4.5 ml of ice-cold 0.4 M-KCl in aqueous 20% (v/v) ethanol. For glucose 6-phosphate dehydrogenase assay 0.5 g of liver was homogenized in 9.5 ml of ice-cold 0.9% NaCl containing 0.66 mM-EDTA. For extramitochondrial aconitate hydratase assay 0.5 g of liver was homogenized in 4.5 ml of ice-cold 0.25 M-sucrose adjusted to pH 7.3 with 1 M-NaOH. All homogenates were made from fresh tissue except those for isocitrate dehydrogenase and aldolase, for which liver that had been deep frozen (-20°C) for 24 h was used. For aconitate hydratase assay crude homogenates were centrifuged at 150 000g for 30 min as described by Guarriera-Bobyleva & Buffa (1969). Other homogenates

were centrifuged at 100 000g for 15 min. The supernatant solutions were used for the enzyme assays.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49), ATP citrate lyase [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8], pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) and 'malic' enzyme [L-malate-NADP oxidoreductase (decarboxylating), EC 1.1.1.40] were assayed as described by Taylor *et al.* (1967). Phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was assayed as described by Underwood & Newsholme (1965) and the same assay procedure was used for aldolase (fructose 1,6-diphosphate-D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) except that aldolase was omitted from the assay mixture and 1.7 mM-fructose 1,6-diphosphate replaced the fructose 6-phosphate. NADP-dependent isocitrate dehydrogenase [DL-isocitrate-NADP oxidoreductase (decarboxylating), EC 1.1.1.42] was assayed by the method of Kornberg (1955) and extramitochondrial aconitate hydratase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] was assayed by the same method except that 1 mM-trisodium citrate was used as substrate instead of DL-isocitric acid. All spectrophotometric assays were carried out at 25°C and the change in E_{340} was followed, by using a Beckman DB spectrophotometer coupled to a Sargent chart recorder, after preincubation of reagents at 25°C for 5 min.

Acetyl-CoA carboxylase activity [acetyl-CoA- CO_2 ligase (ADP), EC 6.4.1.2] was determined by the method of Vagelos, Alberts & Martin (1963) as modified by Greenspan & Lowenstein (1967) and Gregolin, Ryder & Lane (1968). The enzyme preparation was preincubated at 25°C for 30 min in the following mixture with the final concentrations indicated: tris-HCl buffer, pH 7.5, 80 mM; 2-mercaptoethanol, 6.7 mM; tripotassium citrate, 8 mM; MgCl_2 , 10 mM; in a volume of 0.6 ml. The following reagents were then added to give the final concentrations indicated and a final volume of 1.0 ml: ATP, 8 mM; acetyl-CoA, 0.2 mM; MgCl_2 , 10 mM; $\text{NaH}^{14}\text{CO}_3$, 40 mM (1 μCi). The reaction was terminated after 30 min of incubation at 25°C by the addition of 0.05 ml of 5 M- HClO_4 . Excess of $^{14}\text{CO}_2$ was removed under vacuum and an 0.2 ml sample was taken for assay of radioactivity in a vial containing 10 ml of scintillator fluid (NE220). The radioactivity of the samples was counted in a Nuclear-Chicago 725 liquid-scintillation counter. Malonyl-CoA decarboxylase activity was negligible in the enzyme preparations.

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

Measurement of metabolite concentrations. The animals were lightly anaesthetized with ether. The liver was exposed and a lobe was immediately clamped *in situ* in aluminium tongs that had been precooled in liquid nitrogen. The tissue was ground to a powder on a mortar at -195°C . A homogenate was made in ice-cold 6% (v/v) HClO_4 and centrifuged. The precipitated protein was washed with ice-cold 3% (v/v) HClO_4 and the combined supernatants were neutralized with 2 M- KHCO_3 . After being kept for 1 h at 0°C the precipitated KClO_4 was removed by centrifugation. The supernatant was used for determination of metabolite concentrations. Malate was determined by the method of Holzer & Söling (1963),

pyruvate and phosphoenolpyruvate by the method of McQuate & Utter (1963), citrate by the method of Gruber & Moellering (1966) and glucose 6-phosphate by the method of Hohorst (1963).

RESULTS

Acetyl-CoA carboxylase activity (Fig. 1). The activity of acetyl-CoA carboxylase is very low in the foetal liver. The low enzyme activity is maintained after birth throughout the suckling period. There is a rapid increase in activity on weaning

Fig. 1. Activities of hepatic acetyl-CoA carboxylase during development of the rat. After weaning the activities given are those of male rats. The incorporation of $^{14}\text{CO}_2$ into malonyl-CoA was measured as described in the Experimental section. F, Foetal rats; S, adult stock rats. The results are the mean of five to seven determinations $\pm$ S.E.M.

Fig. 2. Activities of hepatic phosphofructokinase (●) and aldolase (○) during development of the rat. After weaning the activities given are those of male rats. Enzyme activities were measured as described in the Experimental section. F, Foetal rats; S, adult stock rats. The results are the mean of five determinations $\pm$ S.E.M.

(21 days) and a peak of activity is reached between 40 and 50 days of age. After this time the activity declines to adult values.

Phosphofructokinase and aldolase activities (Fig. 2). The decrease in activity of phosphofructokinase after birth is in good agreement with the results of Burch *et al.* (1963). There is no increase in activity immediately after weaning, although there is a slight peak of activity between 40 and 50 days after which activity declines to adult values. There is little change in aldolase activity from birth until 20 days, as was also found by Burch *et al.* (1963). On weaning activity increases to reach adult values by 50 days.

Extramitochondrial aconitate hydratase and NADP-dependent isocitrate dehydrogenase activities (Fig. 3). Extramitochondrial aconitate hydratase activity is low in foetal liver and then rises steadily to give a peak at 20 days. On weaning the activity falls until 30 days and then increases to a maximum at 40–50 days, with adult activities being similar to these values. The activity of NADP-dependent isocitrate dehydrogenase is fairly constant from foetal life to about 10 days and then the developmental pattern is similar to that of aconitate hydratase. In contrast with these results for isocitrate dehydrogenase Vernon & Walker (1968) found a reasonably constant activity from birth until 30 days of age.

Early-weaning experiments (Figs. 4 and 5). Some animals, as described in the Experimental section, were weaned early at 15 days instead of the normal 21 days of age. If the increases in the activities of the lipogenic enzymes normally found

Fig. 3. Activities of hepatic NADP-dependent isocitrate dehydrogenase (○) and extramitochondrial aconitate hydratase (●) during development of the rat. After weaning the activities given are those of male rats. Enzyme activities were measured as described in the Experimental section. F, Foetal rats; S, adult stock rats. The results are the mean of five determinations $\pm$ S.E.M.

Fig. 4. Effect of early weaning on the activities of hepatic 'malic' enzyme (a) and ATP citrate lyase (b). Rats were weaned on to the normal diet at 15 days of age (●). Controls were weaned at 21 days of age (○). Enzyme activities were measured as described in the Experimental section. The results for the early-weaned animals are the mean of five rats and for the controls the mean of two rats.

Fig. 5. Effect of early weaning on the activities of hepatic acetyl-CoA carboxylase (a) and glucose 6-phosphate dehydrogenase (b). Rats were weaned on to the normal diet at 15 days of age (●). Controls were weaned at 21 days of age (○). Enzyme activities were determined as described in the Experimental section. The results for the early-weaned animals are the mean of five rats and for the control the mean of two rats.

at weaning are due to the change in diet, then it should be possible to advance the rises in enzyme activity by early weaning. Fig. 4(a) shows the results obtained for hepatic 'malic' enzyme activity. In the normally weaned animals there is no detectable 'malic' enzyme activity before 21 days. However, early weaning produced an almost immediate appearance of 'malic' enzyme activity, which then increases with time. Fig. 4(b) shows the results for ATP citrate lyase. In this case a rise in enzyme activity occurs 4 days after early weaning. Fig. 5 shows that results similar to those for ATP citrate lyase are obtained for acetyl-CoA carboxylase and for glucose 6-phosphate dehydrogenase. Therefore of the lipogenic enzymes studied only 'malic' enzyme shows an immediate response to a dietary change that necessitates an increase in the lipogenic capacity of the liver.

Late-weaning experiments (Table 1). Late-weaning experiments were carried out as described in the Experimental section. The results in Table 1 show that for ATP citrate lyase, acetyl-CoA carboxylase, pyruvate kinase and glucose 6-phosphate dehydrogenase the usual sharp rise in

activity that occurs on weaning at 21 days on to the stock diet is not observed when animals are fed with an artificial milk diet continuously from 15 days of age. Indeed, the enzyme activities tend to decrease from 20 to 60 days of age at which time they are lower than normally found for adult livers. For 'malic' enzyme, however, enzyme activity still increases at 20 days, but there is no peak of enzyme activity that, in normally weaned animals, occurs at about 35 days of age.

Hormonal control of enzyme activity. Hormone injections were carried out as described in the Experimental section. Cortisol, growth hormone and glucagon were without effect on 'malic' enzyme, ATP citrate lyase, glucose 6-phosphate dehydrogenase and pyruvate kinase activities. The effect of thyroxine injections on these enzymes is shown in Table 2. Treatment with thyroxine causes a 20-fold increase in 'malic' enzyme activity and a doubling of ATP citrate lyase activity. The hormone has less effect on the other enzymes. The effect of thyroxine on 'malic' enzyme can be detected as early as 3 days of age and the

Table 1. *Effect on hepatic enzyme activities of weaning rats on to an artificial-rat-milk diet*

Rats were removed from their mothers at 16 days of age and fed on an artificial-rat-milk diet (see the Experimental section). For each result the mean $\pm$ s.e.m. of five animals from different litters is given. The activities of 'malic' enzyme, ATP citrate lyase, pyruvate kinase and glucose 6-phosphate dehydrogenase after normal weaning are taken from the results of Taylor *et al.* (1967). All enzyme activities are expressed as μ mol of substrate transformed/min per g wet wt. of liver at 25°C.

Age (days)	'Malic' enzyme		ATP citrate lyase		Acetyl-CoA carboxylase		Pyruvate kinase		Glucose 6-phosphate dehydrogenase	
	Artificial milk diet	Normal diet	Artificial milk diet	Normal diet	Artificial milk diet	Normal diet	Artificial milk diet	Normal diet	Artificial milk diet	Normal diet
16	0	0	0.27 $\pm$ 0.02	0.1	0.05 $\pm$ 0.005	0.03	11.7 $\pm$ 0.5	11	1.3 $\pm$ 0.2	1.2
21	0.70 $\pm$ 0.08	0.1	0.5 $\pm$ 0.05	0.1	0.035 $\pm$ 0.006	0.05	8.7 $\pm$ 0.6	11	1.05 $\pm$ 0.05	0.9
26	0.87 $\pm$ 0.19	1.5	0.34 $\pm$ 0.05	0.9	0.04 $\pm$ 0.007	0.11	9.0 $\pm$ 0.14	21	1.1 $\pm$ 0.04	1.5
32	1.32 $\pm$ 0.38	8.2	0.4 $\pm$ 0.2	1.8	0.04 $\pm$ 0.007	0.14	10.8 $\pm$ 0.5	33	1.7 $\pm$ 0.35	6.0
39	1.16 $\pm$ 0.13	5.5	0.24 $\pm$ 0.05	2.0	0.06 $\pm$ 0.02	0.22	6.1 $\pm$ 0.3	36	1.2 $\pm$ 0.06	3.1
45	1.08 $\pm$ 0.1	4.0	0.08 $\pm$ 0.01	1.9	0.03 $\pm$ 0.003	0.20	3.6 $\pm$ 0.46	40	1.0 $\pm$ 0.06	2.5
51	2.05 $\pm$ 0.14	3.5	0.22 $\pm$ 0.01	1.8	0.028 $\pm$ 0.002	0.20	2.9 $\pm$ 0.1	42	0.9 $\pm$ 0.06	2.6
58	1.6 $\pm$ 0.05	2.0	0.27 $\pm$ 0.03	1.6	0.025 $\pm$ 0.002	0.18	4.0 $\pm$ 0.21	42	1.1 $\pm$ 0.04	2.6

response of the enzyme activity to hormone injection increases with age up to the weaning time (E. A. Lockwood & E. Bailey, unpublished work). These results agree well with those of Hemon (1968).

Sex difference in activities of glucose 6-phosphate dehydrogenase and 'malic' enzyme (Table 3). The influence of sex hormones on glucose 6-phosphate dehydrogenase and 'malic' enzyme activities was investigated by castrating or ovariectomizing animals at 21 days of age and measuring enzyme activities at 50 days of age. The results show that, although the absolute activities vary from litter to litter, castration causes a statistically significant increase in glucose 6-phosphate dehydrogenase activity (about 80%) whereas ovariectomy leads to a statistically significant decrease in the enzyme activity (about 40%). Similar results were obtained for 'malic' enzyme.

Metabolite concentrations during development (Fig. 6). The variation in malate, citrate, pyruvate, phosphoenolpyruvate and glucose 6-phosphate concentrations in the liver throughout development are shown in Fig. 6. The concentrations of pyruvate remain constant until weaning when they increase to reach maximum values at 40 days and thereafter fall to adult values. Concentrations of phosphoenolpyruvate do not vary during the period of development studied. Glucose 6-phosphate concentrations increase steadily from 4 days to reach adult values at 30 days of age. Malate concentrations increase sharply between 4 and 10 days and then decrease after 20 days to adult values. The concentrations of citrate show a complex series of variations.

DISCUSSION

The developmental pattern of acetyl-CoA carboxylase activity after birth is similar to that of other hepatic enzymes associated with lipogenesis and to hepatic lipogenesis measured by acetate-incorporation experiments (Taylor *et al.* 1967). In particular acetyl-CoA carboxylase shows a marked increase in activity at weaning. However, only very low activity is detected in the foetal liver whereas lipogenesis, measured by acetate and pyruvate incorporations into lipid (Taylor *et al.* 1967; Vilee & Hagerman, 1958), is as high in late foetal tissue as in adult liver. Similarly ATP citrate lyase is also as active in foetal as in adult liver. Therefore either the low concentrations of acetyl-CoA carboxylase are sufficient to maintain the necessary rate of lipogenesis or the foetal enzyme activity is decreased by the preparative procedure. However, the possibility of a significant contribution by a pathway of fatty acid biosynthesis not involving malonyl-CoA cannot be excluded.

The changes in activity of the two glycolytic

Table 2. *Effect of thyroxine injections on hepatic enzyme activities in the weanling rat*

Intraperitoneal injections of thyroxine (10 µg) were given to rats daily from 14 to 17 days of age while they remained with the mother. Control litter mates were injected with the same volume of 0.9% NaCl. All enzyme activities (determined at 18 days of age) are expressed as µmol of substrate transformed/min per g wet wt. of liver at 25°C. The results are the mean of five determinations ± s.e.m.

	'Malic' enzyme	ATP citrate lyase	Glucose 6-phosphate dehydrogenase	Pyruvate kinase
Thyroxine-treated	5.4 ± 0.1*	0.3 ± 0.01*	0.98 ± 0.05†	9.0 ± 0.5
Controls	0.2 ± 0.05	0.15 ± 0.01	0.62 ± 0.02	7.4 ± 0.3

* Difference between thyroxine-treated and control animals is statistically significant, $P < 0.01$.

† Difference between thyroxine-treated and control animals is statistically significant, $P < 0.05$.

Table 3. *Effect of castration and ovariectomy on hepatic enzyme activities*

Half the number of male and female rats from litters were castrated or ovariectomized at 21 days of age. Sham operations were carried out on control litter mates. All animals were weaned at 21 days and killed at 50 days of age. In any litter when two results are available both are given, otherwise the results are the mean of three determinations ± s.e.m. Means for the total number of animals treated are given ± s.e.m. Enzyme activities are expressed as µmol of substrate transformed/min per g wet wt. of liver at 25°C.

Litter	Glucose 6-phosphate dehydrogenase				'Malic' enzyme		
	1	2	3	Mean	1	2	Mean
Castrated	3.2, 5.4	2.4 ± 0.2	4.0 ± 0.7	3.6 ± 0.7*	5.4, 5.4	4.1 ± 0.2	4.6 ± 0.3*
Sham-castrated	2.4, 2.4	1.5, 1.1	1.5, 1.5	1.7 ± 0.2	3.6, 4.2	2.9, 2.1	3.2 ± 0.4
Ovariectomized	3.6 ± 0.4	3.9, 3.9		3.8 ± 0.3†	3.4 ± 0.34	5.0, 4.4	3.9 ± 0.4†
Sham-ovariectomized	7.5, 5.1	4.8, 5.8		5.8 ± 0.5	5.0, 5.4	5.1, 5.3	5.2 ± 0.1

* Difference between castrated and sham-castrated is statistically significant, $P < 0.05$.

† Difference between ovariectomized and sham-ovariectomized is statistically significant, $P < 0.05$.

enzymes, phosphofructokinase and aldolase, may be compared with the changes found for pyruvate kinase. Increases in pyruvate kinase activity after weaning, when there is a pronounced increase in the conversion of carbohydrate into lipid, are more immediate and much greater than the changes in activity of phosphofructokinase and aldolase, although the activity of pyruvate kinase *in vitro* is at all ages higher than these other glycolytic enzymes. These developmental patterns suggest that pyruvate kinase plays an important part in the control of hepatic glycolysis. Thus the activity of an enzyme, as measured *in vitro*, may be less important as a criterion for a regulatory function than the response of the enzyme to physiological changes such as development and diet and to physiological activators and inhibitors.

In the early-weaning experiments malic enzyme activity increases immediately in response to the change in diet whereas the other enzymes associated with lipogenesis show a delayed response. These results indicate that 'malic' enzyme plays an important role in the hepatic conversion of carbohydrate into fat. The suggestion of Kornacker & Ball (1965) that 'malic' enzyme functions to provide

NADPH for lipogenesis does not seem likely in this case since both glucose 6-phosphate dehydrogenase and NADP-dependent isocitrate dehydrogenase, which are both much higher in activity, may provide reduced cofactor. However, if the role of 'malic' enzyme is to form malate from pyruvate as suggested by Wada, Maruyama, Shibayama & Sakamoto (1968) for rat liver, and by Walker & Bailey (1969) for the desert locust fat-body, then an immediate increase in malic enzyme activity on weaning would be expected. With the change to a high-carbohydrate diet the glycolytic rate would be increased. A proportion of the pyruvate formed would then be directed into the synthesis of fatty acids by the following sequence of reactions: pyruvate to mitochondrial acetyl-CoA; condensation of acetyl-CoA and oxaloacetate to give citrate; citrate passes out of the mitochondria and is metabolized by ATP citrate lyase to regenerate acetyl-CoA outside the mitochondria. Acetyl-CoA then leads directly to the synthesis of fatty acids. For this link-up between glycolysis and lipogenesis to occur a supply of mitochondrial oxaloacetate is necessary to condense with the acetyl-CoA. Malic enzyme may convert pyruvate into malate which, after

Fig. 6. Changes in hepatic metabolite concentrations during development of the rat. Metabolite concentrations were determined in freeze-clamped tissue as described in the Experimental section. S, Adult stock rats. Metabolites are: malate, ●; citrate, ○; pyruvate, □; phosphoenolpyruvate, ▲; glucose 6-phosphate, △. The results are the means of at least five determinations $\pm$ S.E.M.

transport into the mitochondrion, would be converted into oxaloacetate by malate dehydrogenase. Extramitochondrial citrate can also be metabolized by aconitate hydratase and NADP-dependent isocitrate dehydrogenase, thus providing NADPH for lipid biosynthesis. It is noteworthy that on normal weaning, when ATP citrate lyase increases rapidly, aconitate hydratase and NADP-dependent isocitrate dehydrogenase activities decrease slightly. This is probably due to competition for substrate between the two citrate-utilizing enzymes. This suggestion is supported by the changes in the concentration of citrate at this time.

The effect of prolonging the high-fat diet past the normal weaning time by feeding an artificial-milk diet is to suppress the activities of most of the enzymes. This experiment, together with the early weaning experiments, indicates that diet is the controlling factor in the changes in activity of pyruvate kinase, glucose 6-phosphate dehydrogenase, ATP citrate lyase and acetyl-CoA carboxylase at the weaning time. However, with 'malic' enzyme other factors must also be involved, since the enzyme activity is not completely suppressed by the high-fat diet. The effect of thyroxine treatment on hepatic 'malic' enzyme activity suggests that this hormone is another factor controlling the activity of the enzyme. Beltz & Reineke (1968)

have shown that the thyroid secretion rate abruptly increases when the neonatal rat body weight increases above 22g, corresponding to 12 or 13 days of age. Also Hommes, Wilmink & Richters (1969) have found that there are very low concentrations of active thyroid hormones in the blood plasma during the first 14 days after birth and the concentration of the hormones increases to the adult value during the next 2 weeks. These findings suggest that physiologically thyroxine may well be involved in the increase in 'malic' enzyme activity at weaning.

Although only 'malic' enzyme is affected at the weaning time by both dietary and hormonal factors the results with castrated and ovariectomized animals demonstrate that on maturation the female and male sex hormones have a controlling influence on glucose 6-phosphate dehydrogenase and 'malic' enzyme activities. Tepperman & Tepperman (1964) reported that treatment with oestrogen caused an increase in activity of glucose 6-phosphate dehydrogenase and, to a lesser extent, in activity of 'malic' enzyme in rat liver.

The diet experiments demonstrate that the change in diet is an important controlling factor of the changes in hepatic enzyme activity at weaning. It seemed possible that the change from a high-fat to a low-fat-high-carbohydrate diet would lead to changes in hepatic metabolite concentrations. Thus metabolite concentrations were measured during development to see if the rapid changes in enzyme activity at weaning were preceded by and possibly result from an accumulation of substrates of the enzymes. The results, however, show that there is little direct correlation between substrate concentration and enzyme activity. Certainly, concentrations of phosphoenolpyruvate do not appear relevant to the changes in activity of pyruvate kinase. Malate concentrations increase during the suckling period when 'malic' enzyme activity is very low. The increase in concentration of pyruvate after 20 days may reflect an increase in the glycolytic rate. Alterations in malate and pyruvate concentrations possibly indicate changes in the redox state of NAD and NADP in the liver (Veech, Eggleston & Krebs, 1969) during development. The increase in concentration of glucose 6-phosphate before weaning may be associated with the increase in glucose 6-phosphate dehydrogenase activity just after weaning. As discussed above, the fall in the concentration of citrate at 30 days corresponds to the time of increasing activity of ATP citrate lyase and decreasing activities of extramitochondrial aconitate hydratase and NADP-dependent isocitrate dehydrogenase.

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