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Changes in Activity of some Enzymes Involved in Glucose Utilization and Formation in Developing Rat Liver

By R. G. VERNON and D. G. WALKER
Department of Biochemistry, University of Birmingham

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1. The activities of some enzymes involved in both the utilization of glucose (pyruvate kinase, ATP citrate lyase, NADP-specific malate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-specific isocitrate dehydrogenase, all present in the supernatant fraction of liver homogenates) and the formation of glucose by gluconeogenesis (glucose 6-phosphatase in the whole homogenate and fructose 1,6-diphosphatase, phosphopyruvate carboxylase, NAD-specific malate dehydrogenase and fumarase in the supernatant fraction) have been determined in rat liver around birth and in the postnatal period until the end of weaning. 2. The activities of those enzymes involved in the conversion of glucose into lipid are low during the neonatal period and increase with weaning. NADP-specific malate dehydrogenase first appears and develops at the beginning of the weaning period. 3. The marked increase in cytoplasmic phosphopyruvate carboxylase activity at birth is probably the major factor initiating gluconeogenesis at that time. 4. The results are discussed against the known changes in dietary supplies and the known metabolic patterns during the period of development.

The last decade has seen many advances in our knowledge of the homoeostatic mechanisms whereby several types of mammalian cells, such as those of the kidney and of adipose tissue, but particularly hepatic parenchymal cells, can adapt to various environmental stimuli such as changes in the diet, changes in hormonal status and the introduction of foreign compounds. These mechanisms, which have been studied mainly in the adult animal, involve the control of enzyme activity by several types of activating and inhibitory processes and control of the absolute amount of enzyme protein by regulation of both enzyme synthesis and enzyme degradation.

Mammalian development, from the intrauterine foetal stage through birth and the immediate postnatal, suckling and weaning phases, presents many changes in the nutritional status and the hormonal environment and includes many other genetically controlled developmental changes, only some of which are well understood. Many changes in hepatic enzyme activities have been recorded (some of which are noted below) during the development of several species and of the rat in particular, and it is already apparent that these changes include both modifications in the amounts of some enzymes and the initial appearance of measurable activities of others (Herrmann & Tootle, 1964; Moog, 1966). The present studies follow the changes in the activities of a number of key enzymes that are known to be rate-controlling steps in the utilization and formation of glucose in the adult rat liver. The aim has been not simply to verify many isolated facts reported in the literature about changes that occur particularly around birth, but to obtain an overall picture of the hepatic enzyme profile in the neonatal period in particular, and to follow the changes during weaning, a period that has often been neglected in earlier studies.

The present paper presents an account of the normal changes observed and discusses them in relation to known dietary modifications. The succeeding paper (Vernon & Walker, 1968) examines the regulation of the normal changes effected by modification of the diet during the weaning period, and discusses the significance of enzyme adaptation and new enzyme synthesis in the developmental period. A preliminary report has been given (Walker & Vernon, 1967).

MATERIALS AND METHODS

Materials. ATP, AMP, potassium phosphoenolpyruvate, NADH, $NADP^+$, CoA, oxaloacetic acid, fructose 1,6-diphosphate (sodium salt), 6-phosphogluconate (sodium salt), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and muscle lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer Corp. (London) Ltd. (London, W. 5) and ADP, ITP, glucose 6-phosphate and L-malic acid from

Sigma (London) Chemical Co. Ltd. (London, S.W. 6). All other reagents were AnalaR or next best grade from British Drug Houses Ltd. (Poole, Dorset). Solutions of CoA, oxaloacetate and NADH were prepared fresh daily. 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was prepared by the method of Glock & McLean (1953).

Animals. The rats were a Wistar albino strain fed *ad lib.* on a pelleted diet (Thompson Rat Cubes; Heygate and Sons, Bugbrooke Mills, Northants.), which contains (manufacturers' figures) 3·5% of oil, 21·5% of protein and 5% of fibre. The normal practice in the rat colony was to cull litters to a maximum of ten 2 days after birth and to remove the weanling rats from their mothers when 30–32 days old. All the results in this paper refer to neonatal animals removed from such litters at random. When comparing results in the present paper with others reported in the literature it should be noted that it is the practice in some Laboratories to wean rats at an earlier age. Foetal age was assessed on the basis of foetal crown–rump length and weight (Stotsenberg, 1915). All adult animals were males weighing 200–250g.

Animals were always killed for assays between 10 and 11 a.m.

Assay procedures. Homogenates (10%, w/v) of liver tissue were prepared in 0·25M-sucrose and centrifuged at 100000*g* in an MSE Super Speed 40 centrifuge for 1 hr. to prepare the supernatant fractions. Glucose 6-phosphatase activity was estimated on the whole homogenate and all other enzyme assays were performed on the supernatant fraction. Protein was assayed in the supernatant fraction by a biuret method (Gornall, Bardawill & David, 1949) standardized with bovine serum albumin.

Pyruvate kinase (EC 2.7.1.40) was assayed by the method of Krebs & Eggleston (1965), except that bovine serum albumin (1mg./ml.) was included in the incubation mixture and the concentration of the potassium phosphate buffer, pH 7·4, was 16·7mM. ATP citrate lyase (EC 4.1.3.6) was assayed by a procedure based on the method of Srere (1959); the incubation mixture (1ml.) contained (final concentrations): 15mM-tris–HCl buffer, pH 7·8, 10mM-$MgCl_2$, 20mM-potassium citrate, 5mM-ATP, 0·3mM-NADH, 0·5mM-CoA, 10mM-mercaptoethanol, 0·1mM-EDTA, and an appropriate volume of enzyme. NADP-specific malate dehydrogenase (EC 1.1.1.40) was assayed by the method of Ochoa (1955*b*), but with an $NADP^+$ concentration of 0·23mM; glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed by the methods of Glock & McLean (1953); and NADP-specific isocitrate dehydrogenase (EC 1.1.1.42) was assayed by the method of Ochoa (1948), with 0·6mM-DL-isocitrate and 0·23mM-$NADP^+$.

Glucose 6-phosphatase (EC 3.1.3.9) activity was assayed by the method of Swanson (1950) except that the incubation time was 10min. A spectrophotometric method for the assay of fructose 1,6-diphosphatase activity (EC 3.1.3.11), based on that of Taketa & Pogell (1963), as described by Walker, Lea, Rossiter & Addison (1967), was used; this is very similar to that described by Underwood & Newsholme (1965). The effects of higher substrate concentrations and of AMP on the enzyme in supernatant preparations of livers from foetal, newborn and adult rats suggested that the characteristic properties of the enzyme (Underwood & Newsholme, 1965) were similar at all stages of development. Phosphopyruvate carboxylase (EC 4.1.1.32) was assayed by the method of Nordlie & Lardy (1963) except that K^+ ions were omitted from the incubation medium to minimize removal of the phosphoenolpyruvate formed by pyruvate kinase. P_i was determined by the method of Fiske & Subbarow (1925). Fumarase activity (EC 4.2.1.2) was determined by the method of Racker (1950) and NAD-specific malate dehydrogenase (EC 1.1.1.37) by the method of Ochoa (1955*a*), except that 100mM-potassium phosphate buffer, pH 7·5, and 0·375mM-oxaloacetate were employed.

All enzyme activities are expressed as μmoles of substrate used or product formed/min., either per g. wet wt. of tissue, per 100mg. of supernatant protein or per 100g.

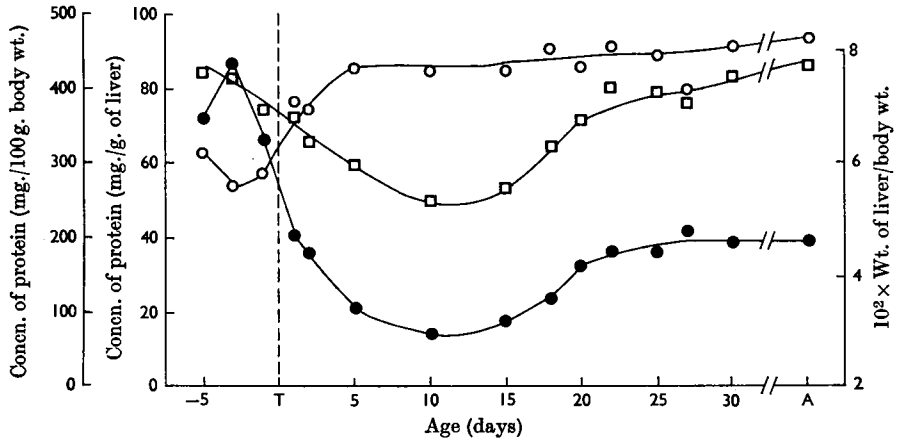

Fig. 1. Changes in liver wt./body wt. ratio and the liver supernatant protein concentration in the neonatal and weanling rat. Protein was determined in supernatant fractions by the biuret method, and is expressed as mg./g. of liver (○) or mg./100g. body wt. (□). ●, 100 × Liver wt./body wt. T, term; A, adult. Each point represents the mean of 5–26 determinations.

body wt. The temperature used for all assays was 30°, except that 37° was used for glucose 6-phosphatase.

RESULTS

The rate of growth of the liver in the neonatal rat lags behind that of the whole body, so that the liver weight/body weight ratio, after being high at the end of gestation, is at a minimum at about 10 days after birth (Fig. 1). These marked changes, which have been noted before (Geschwind & Li, 1949; Widdowson & McCance, 1960; Oliver, Ballard, Shield & Bentley, 1962), mean that, although the absolute content of protein in the supernatant fraction of the liver homogenate only increases slightly from 5 days after birth, there is also a marked trough in the plot of total supernatant protein/100g. body wt. (Fig. 1). This must be taken into account when the physiological effectiveness of the reported specific enzyme activities (given as units/100mg. of protein) to the whole animal is being considered.

Enzymes of glucose utilization. Krebs & Eggleston (1965) drew attention to the regulatory importance of the control of phosphoenolpyruvate–pyruvate interconversion in glycolysis and gluconeogenesis, and to the adaptive behaviour of hepatic pyruvate kinase. The specific activity of hepatic pyruvate kinase decreases from late gestation to a minimum at 15–20 days after birth and then increases to adult values at 30 days (Fig. 2). Similarly, ATP citrate lyase, which is considered to be of rate-controlling significance in the formation of lipid from carbohydrates via citrate (Srere, 1959; Kornacker & Lowenstein, 1965; Leveille & Hanson, 1966), also shows a trough in specific activity in the neonatal period. The decrease around birth is more dramatic than that for pyruvate kinase; those found between 10 and 20 days after birth are less than 5% of the maximum activities recorded.

NADP-specific dehydrogenases. A gradual decrease of about 50% in the specific activities of the two dehydrogenases of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, from parturition to 15 days after birth was reported by Burch *et al.* (1963), and has been confirmed by us. These activities then gradually increase from 20 days (Fig. 3) to adult values; the activity of glucose 6-phosphate dehydrogenase is always less than that of the succeeding enzyme. The specific activity of soluble isocitrate dehydrogenase (NADP) shows no significant change throughout the whole of the neonatal and weaning period (Fig. 4), but significant activities of NADP-specific malate dehydrogenase are not found until about 20 days

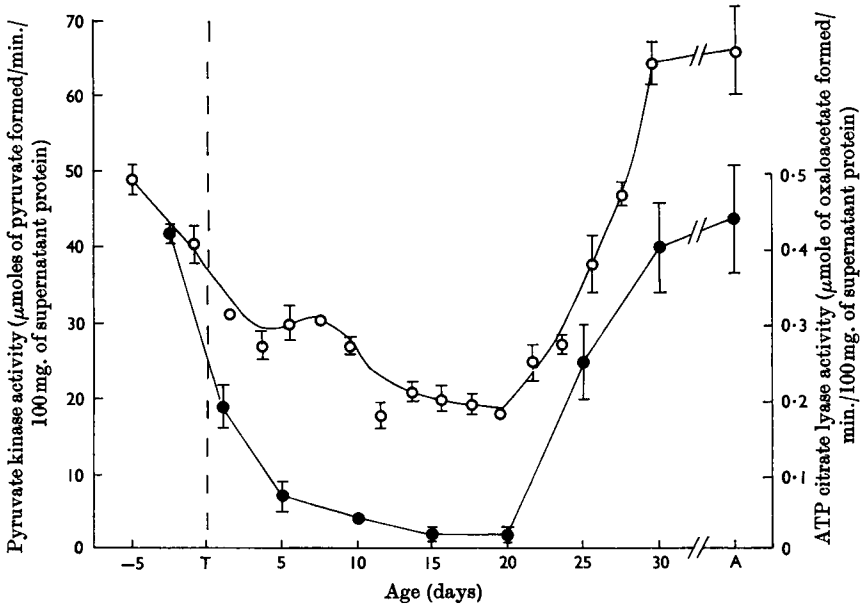

Fig. 2. Activities of pyruvate kinase (○) and ATP citrate lyase (●) in the liver supernatant as a function of age. Each point represents the mean of 4–11 determinations and the vertical bar shows ± S.E.M., when large enough to record. For methods, see the text. Activities are expressed as μmoles/min./100mg. of supernatant protein. T, term; A, adult.

after birth and then increase steadily over the next 10 days (Fig. 4).

Enzymes of gluconeogenesis. The marked changes in activity of glucose 6-phosphatase (see Dawkins, 1966) and fructose 1,6-diphosphatase (Ballard & Oliver, 1962, 1963) that occur around birth are well documented, but Fig. 5 shows that it is some time before the specific activities fall to adult values. Although glucose 6-phosphatase activity decreases steadily from the peak activities found a few days after birth, the activity of fructose 1,6-diphosphatase remains significantly higher than in the adult at the end of weaning period. Expression of these activities per 100g. body wt. (Fig. 6) reveals a significant trough in the developmental progress curve for glucose 6-phosphatase between peaks at 5 and 20 days after birth.

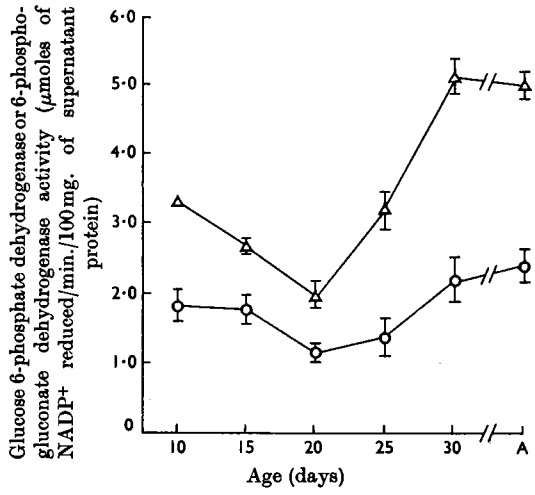

Fig. 3. Activities of glucose 6-phosphate dehydrogenase (○) and 6-phosphogluconate dehydrogenase (△) in the liver supernatant as a function of age. Each point and vertical bar represents the mean ± S.E.M. of 6–10 determinations. Assay methods are described in the text. Activities are expressed as μmoles/min./100mg. of supernatant protein. T, term, A, adult.

The results for the activity of phosphopyruvate carboxylase (transphosphorylating) in the soluble fraction of the liver homogenate, recorded in Figs. 5 and 6, indicate a rapid increase from very low values the day before birth to a value approximately double that of adult liver only 2 days after birth. Widely varying activities over the whole range of this dramatic increase were recorded in animals 6–36hr. old. The decrease in phosphopyruvate carboxylase activity after weaning begins is also more pronounced than the changes in the other gluconeogenic enzymes, and in particular is faster than the decrease in fructose 1,6-diphosphatase activity.

Recently described hypotheses (Lardy, Paetkau & Walter, 1965; Krebs, Gascoyne & Notton, 1967) about gluconeogenesis in rat liver introduce a role for cytoplasmic fumarase and NAD-specific malate dehydrogenase in the process. The fumarase activity in the supernatant fraction of foetal liver homogenates is low (Fig. 7) and shows a gradual increase from birth up to the end of weaning. The cytoplasmic NAD-specific malate dehydrogenase,

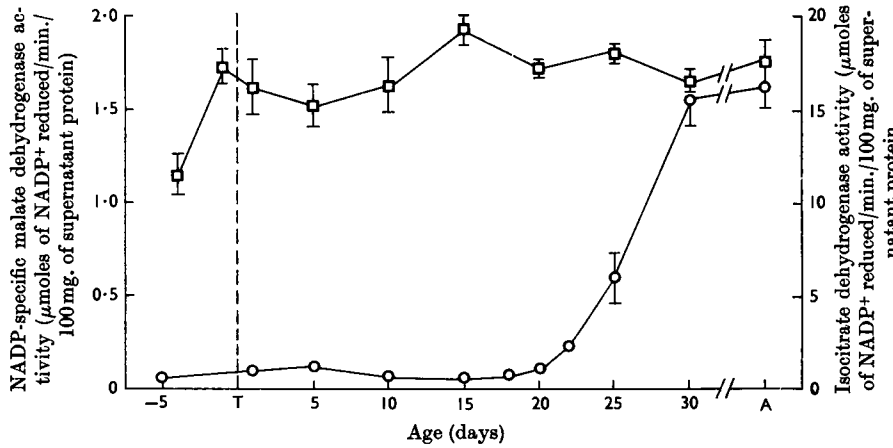

Fig. 4. Activities of hepatic malate dehydrogenase (decarboxylating) (NADP) (○) and isocitrate dehydrogenase (NADP) (□) as a function of age. Activities were determined, as described in the text, on the liver supernatant fraction and are expressed as μmoles/min./100mg. of protein. Each point represents the mean ± S.E.M. of 4–8 determinations. T, term, A, adult.

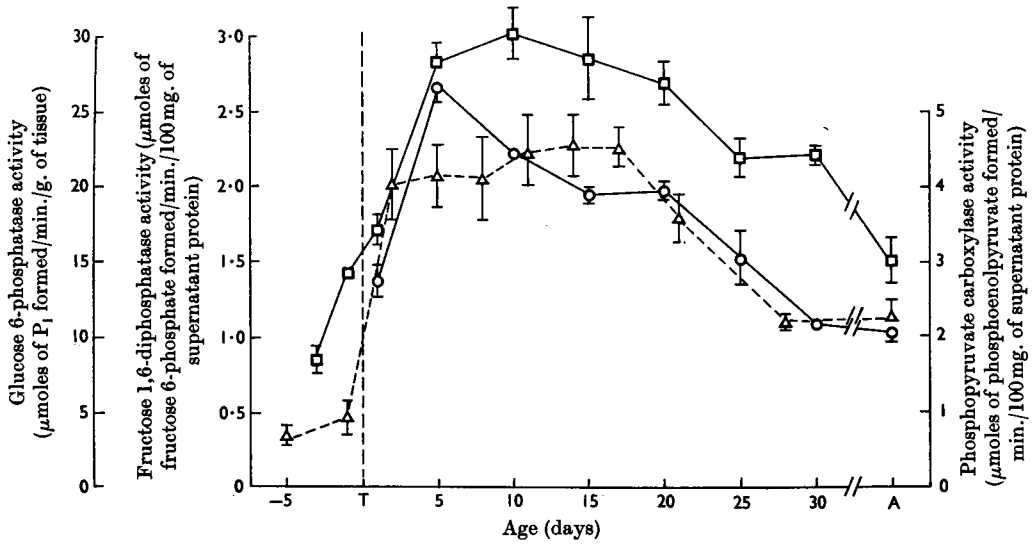

Fig. 5. Activities of the hepatic gluconeogenic enzymes glucose 6-phosphatase (○), fructose 1,6-diphosphatase (□) and phosphopyruvate carboxylase (transphosphorylating) (△) as a function of age. The activity of glucose 6-phosphatase was determined on the liver homogenate and is expressed as μmoles/min./g. of liver; activities of the other two enzymes were determined on the liver supernatant fraction and are expressed as μmoles/min./100 mg. of protein, as described in the text. Each point represents the mean ± S.E.M. for at least five determinations. T, term; A, adult.

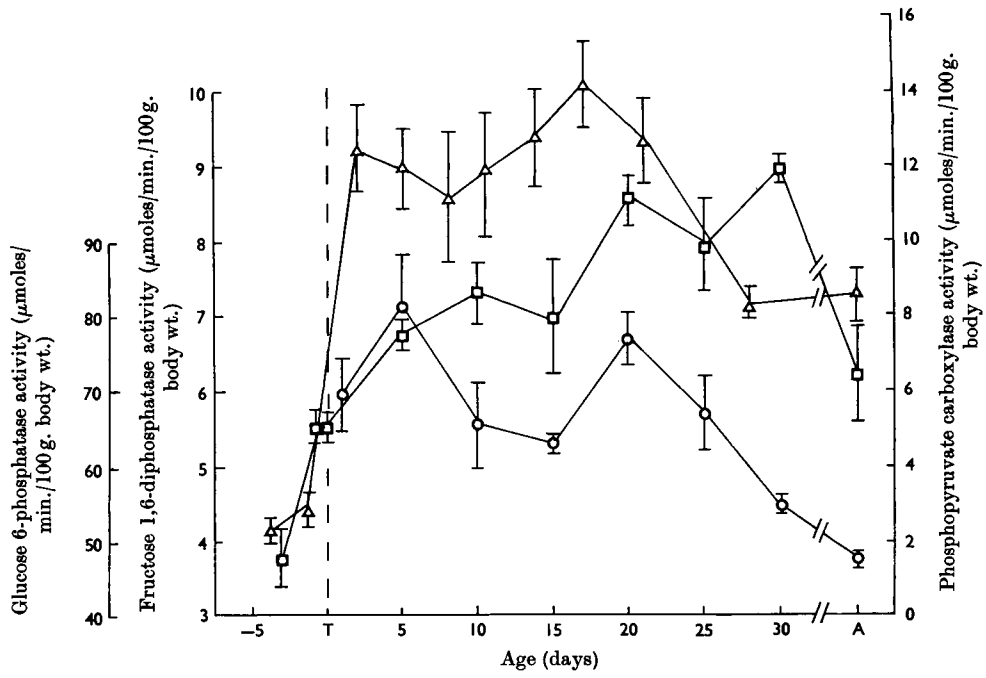

Fig. 6. Activities of the hepatic gluconeogenic enzymes as a function of age, expressed as μmoles/min./100 g. body wt. The activities were determined as in Fig. 5 for glucose 6-phosphatase (○), fructose 1,6-diphosphatase (□) and phosphopyruvate carboxylase (transphosphorylating) (△). Each point represents the mean ± S.E.M. for at least five determinations. T, term; A, adult.

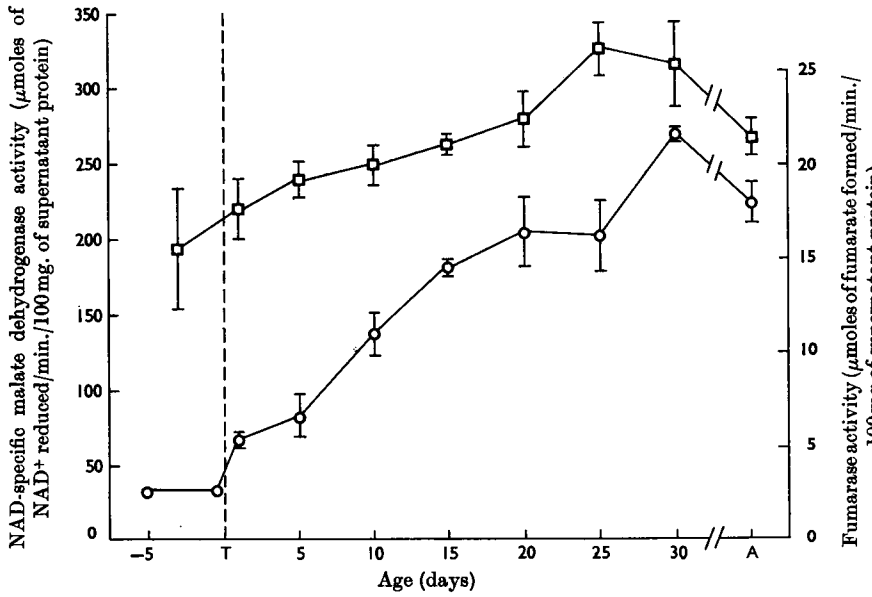

Fig. 7. Activities of fumarase (○) and malate dehydrogenase (NAD) (□) in the supernatant fraction of liver homogenates as a function of age. The activities were determined as described in the text and are expressed as μmoles/min./100 mg. of protein. Each point represents the mean ± S.E.M. of 4–6 determinations. T, term; A, adult.

Table 1. *Absence of sex differences in the activities of ATP citrate lyase, malate dehydrogenase (decarboxylating) (NADP), glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the livers of rats at 30 days of age*

Activities were determined on the supernatant fractions of homogenates, as described in the text. The results are means ± S.E.M. for five animals in each group taken at random from two litters that had remained with the mothers.

	Males		Females	
Body wt. (g.)	76·3 ± 2·8		71·8 ± 3·3	
Liver wt. (g.)	4·04 ± 0·33		3·79 ± 0·25	
100 × Liver wt./body wt.	5·25 ± 0·25		5·26 ± 0·16	
Supernatant protein (mg./g. wet wt. of liver)	77·6 ± 2·08		78·5 ± 3·07	
	Activities (μmoles of product formed/min.)			
	(per g. wet wt.)	(per 100 mg. of protein)	(per. g. wet wt.)	(per 100 mg. of protein)
ATP citrate lyase	0·55 ± 0·06	0·71 ± 0·09	0·47 ± 0·08	0·62 ± 0·13
Malate dehydrogenase (decarboxylating) (NADP)	1·15 ± 0·07	1·48 ± 0·09	1·20 ± 0·11	1·56 ± 0·20
Glucose 6-phosphate dehydrogenase	2·32 ± 0·25	3·02 ± 0·38	2·65 ± 0·33	3·46 ± 0·55
6-Phosphogluconate dehydrogenase	3·99 ± 0·39	5·13 ± 0·25	3·84 ± 0·41	4·90 ± 0·46

however, shows no marked change in activity throughout the neonatal period.

Absence of sex differences at the end of the weaning period. The processes of sexual maturation commence when the rat is about a month old. Table 1 shows that, for the strain of animals and type of diet used in this study, there are no significant differences in several growth parameters and in the activities of ATP citrate lyase, malate dehydrogenase (NADP), glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase at 30 days of age. Examination of all the other results reported above

supports the general conclusion that there are no sex differences that influence the developmental patterns.

DISCUSSION

The foetus receives glucose and amino acids from the maternal blood via the placenta (Hagerman & Villee, 1960), but lipids do not traverse the placenta and are made by the foetus (Popják, 1954; Carroll, 1964). At birth the maternal source of nutrients is altered abruptly and the stores of glycogen (Shelley, 1961) and lipid (Carroll, 1964) built up in the liver and other tissues just before birth are rapidly depleted; milk becomes the dietary supply. Rat milk (Dymsza, Czajka & Miller, 1964) can be regarded as a high-fat high-protein low-carbohydrate diet. The lactose concentration is only about 3% (w/v), so that carbohydrate can supply less than 10% of the total calories required by the neonatal animal (Hahn, Koldovský, Melichar & Novák, 1961). As the percentage utilization of amino acids formed by digestion of milk proteins for synthesis of new protein is very high (Hahn, Koldovský, Křeček, Martínek & Vacek, 1961), the oxidation of lipid must be the main supply of energy until weaning, when carbohydrate again becomes the major dietary source.

Two other features of the diet must also be considered. First, although the rat pup eats a comparatively large quantity of food, there is a sense in which it may be undernourished. Thus Widdowson & McCance (1960) have shown that individuals in small litters of rats grow faster than those in large litters. The trough in the liver wt./body wt. ratio (Fig. 1) is not so pronounced in small litters (Widdowson & McCance, 1960). Secondly, observed enzyme activities depend not only on the quantity and nature of the food eaten but also on the eating behaviour (Tepperman & Tepperman, 1958; Hollifield & Parson, 1962; Leveille, 1966). The neonatal and early weanling rat is a 'nibbler' in that it eats very frequently during the whole 24hr. period whereas the fully weaned and mature rat eats mainly during the period of darkness. How far such a change in eating habits affects the measured enzyme activity during weaning on to the solid rat diet is unknown, but it is against such facts that the changes in the hepatic enzyme profile must be assessed.

The post-natal decrease in pyruvate kinase activity and the increase as the supply of carbohydrate increases at weaning (see also Weber, Lea, Fisher & Stamm, 1966) follow the changes of activity that occur for this enzyme in adult rat liver (Krebs & Eggleston, 1965; Bartley, Dean, Taylor & Bailey, 1967) in response to various diets. It is not known whether the changes involve one or more isoenzyme forms of pyruvate kinase, which have been reported to occur in rat liver (Tanaka, Harano, Morimura & Mori, 1965). The trough in the activity curve (Fig. 2) for ATP citrate lyase is more pronounced than that for pyruvate kinase. This decrease has also been noted by Ballard & Hanson (1967) and Bailey, Taylor & Bartley (1967); it reflects the small amount of carbohydrate available for conversion into lipid and the low rate of incorporation of [^{14}C]glucose into lipid by liver slices (Ballard & Hanson, 1967) during the neonatal period.

The absence of soluble NADP-specific malate dehydrogenase during the period of conversion of glucose into lipid by the foetal liver raises several points. Kinetic considerations and studies on its behaviour under various dietary and hormonal conditions (Shrago, Lardy, Nordlie & Foster, 1963; Lardy, Foster, Shrago & Ray, 1964; Seubert & Huth, 1965) indicated that this enzyme acts in the direction of malate decarboxylation and that formation of NADPH is one of its prime functions (Wise & Ball, 1964; Young, Shrago & Lardy, 1964) in rat liver. The quantitative significance of this role of NADP-specific malate dehydrogenase in adult rat liver is not clear; hydrogen-balance studies in adipose tissue (Flatt & Ball, 1964; Katz, Landau & Bartsch, 1966) indicate that in that tissue the formation of NADPH by the oxidation of glucose 6-phosphate and 6-phosphogluconate may not be sufficient to meet the demands for NADPH in lipid synthesis under some conditions. The activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the foetal liver are higher than in adult liver for both the rat (Burch *et al.* 1963) and the guinea pig (Lea & Walker, 1964). The absence of NADP-specific malate dehydrogenase from rat liver before weaning may signify that the pentose phosphate-pathway dehydrogenase can produce all the NADPH required for the lipid synthesis that occurs during later gestation (Villee & Hagerman, 1958; Carroll, 1964; Ballard & Hanson, 1967). It means also that the oxaloacetate formed by ATP citrate lyase cannot be converted into pyruvate in the cytoplasm via the NAD- and NADP-specific malate dehydrogenases (Young *et al.* 1964), although the NAD-specific enzyme is active (Fig. 7). The oxaloacetate is not used for gluconeogenesis, for this process does not occur in foetal rat liver (Ballard & Oliver, 1963, 1965); its fate is therefore uncertain. It is generally believed (Chappell, 1961) that oxaloacetate does not readily traverse the mitochondrial membrane, but it may be reduced to malate and pass back into the mitochondria in that form.

The NADP-specific malate dehydrogenase appears at about the same developmental stage as does glucokinase (Walker & Holland, 1965; Walker & Eaton, 1967). The functional significance of this

correlation in the appearance of two enzymes involved in carbohydrate–lipid interconversion is obvious, but whether there is any common feature in the mechanism for triggering the initiation of enzyme synthesis (if, indeed, this is the process involved, as seems likely) remains to be established.

The general developmental changes in glucose 6-phosphatase and fructose 1,6-diphosphatase were in agreement with the many earlier reports (reviewed by Dawkins, 1966; Walker, 1967). Not all of these studies followed the return of activity to normal values during weaning. It is clear from Fig. 5, however, that the activities of both enzymes only return to normal around the end of the weaning period and not before weaning begins (cf. Burch *et al.* 1963). The double peak for glucose 6-phosphatase activity when expressed per 100g. body wt. (Fig. 6) is noteworthy, for this enzyme will influence whether the end product of gluconeogenesis is glycogen or glucose. In contrast, the activity profile for fructose 1,6-diphosphatase shows a broad high plateau over the period; its functional activity is determined by the concentration of metabolites (Underwood & Newsholme, 1965).

The activity curves for phosphopyruvate carboxylase are particularly significant for the control of gluconeogenesis during development. Whereas the activities of both glucose 6-phosphatase and fructose 1,6-diphosphatase increase during the late foetal period and reach almost adult values at birth, the activity of phosphopyruvate carboxylase is very low in the foetal rat liver and increases rapidly within the first 2 days after birth (Fig. 5). This rapid increase could well be the factor that initiates gluconeogenesis in the neonatal period, when considered in terms of the processes believed to be involved in rat liver (Lardy *et al.* 1965). Expression of the activity per 100g. body wt. (Fig. 6) emphasizes this point. Fig. 6 also shows that, of the three rate-determining gluconeogenic enzymes, it is the activity of phosphopyruvate carboxylase that decreases most rapidly to the normal adult value during the weaning period. The regulatory role of phosphopyruvate carboxylase activity is emphasized by the results of experiments in which rats were weaned on to various diets (Vernon & Walker, 1968). The relative rates at which the measurable activities of the various enzymes change under a new set of conditions depend on many uncertain factors in our present stage of knowledge (cf. Schimke, 1966), but the rapid activity changes recorded are in themselves of significance to the overall rate of gluconeogenesis.

The higher activities of all the gluconeogenic rate-controlling enzymes in the liver during the neonatal period are certainly a reflection of the increased capacity for gluconeogenesis during this period (Ballard & Oliver, 1963; Yeung & Oliver, 1967; R. G. Vernon & D. G. Walker, unpublished work). This in turn reflects the needs, which are quantitatively uncertain, of many of the rapidly growing tissues for glucose. The gluconeogenic amino acids will be the major precursors (Yeung & Oliver, 1967). Neonatal gluconeogenesis is accompanied in the rat by increased ketone-body formation (Drahota, Hahn, Kleinzeller & Kostolánská, 1964; Drahota, Hahn & Honová, 1966). Ketone bodies arise as by-products of the increased fatty acid oxidation (Drahota *et al.* 1966) that occurs in the liver at the time when fatty acid synthesis and the activity of several associated enzymes are at a minimum. Krebs (1966) has pointed out a general association between gluconeogenesis and ketone-body formation. In the neonatal liver this association provides primarily a means of producing glucose, the dietary supply of which is very restricted during the neonatal period. The ketone bodies formed can be utilized, for example, by the brain of infant rats (Drahota, Hahn, Mourek & Trojanová, 1965) and in such a way may lower the requirement for glucose; they may also act as regulators of gluconeogenesis.

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