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Changes in the Activities of the Enzymes of Hepatic Fatty Acid Oxidation during Development of the Rat

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1. Changes in the activities of several enzymes involved in mitochondrial fatty acid oxidation were measured in the livers of developing rats between late foetal life and maturity. The enzymes studied are medium- and long-chain ATP-dependent acyl-CoA synthetases of the outer mitochondrial membrane and matrix, GTP-dependent acyl-CoA synthetase, carnitine acyltransferase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, general 3-oxoacyl-CoA thiolase and acetoacetyl-CoA thiolase. Developmental changes in the activity of cytoplasmic acetoacetyl-CoA thiolase were also investigated. 2. The activities of all the mitochondrial enzymes studied increase markedly at birth and, except for enoyl-CoA hydratase, decrease at weaning. However, all the developmental patterns other than those of enoyl-CoA hydratase and acetoacetyl-CoA thiolase exhibit a fall followed by a rise in enzyme activity during the mid-suckling period. 3. The cytoplasmic acetoacetyl-CoA thiolase activity is low throughout suckling, but high before birth and after weaning. 4. The results are discussed in relation to changes in fatty acid supply to the liver and hormonal changes occurring during development.

Changes in the metabolism of fatty acids by the developing rat liver appear to follow changes in the dietary regime of the animal such that the capacity of the liver to degrade fatty acids is higher during the suckling period when the high-fat-content milk diet is being consumed, than at other stages of life (foetal and postweaning) when a predominantly carbohydrate diet is ingested (Bailey & Lockwood, 1973). During the suckling period, hepatic lipogenesis is suppressed (Ballard & Hanson, 1967; Taylor *et al.*, 1967), the rat obtains most of its energy from fatty acid oxidation (Drahota *et al.*, 1964), a physiological state of ketosis exists (Lockwood & Bailey, 1971; Page *et al.*, 1971) and ketone bodies become major metabolic fuels of respiration of extrahepatic tissues such as the brain (Hawkins *et al.*, 1971). Clearly the developing rat provides a particularly suitable system for studying the influence of nutritional and endocrinological factors on hepatic fatty acid oxidation, since the spontaneous and quite marked dietary and hormonal changes occurring during neonatal life afford one the opportunity of studying control with minimum deviation from the physiological state. However, developmental changes in the oxidation of [^{14}C]-palmitate by rat liver slices and mitochondria (Augenfeld & Fritz, 1970; Lockwood & Bailey, 1970; Taylor *et al.*, 1967) and in the activities of carnitine palmitoyltransferase (Augenfeld & Fritz, 1970; Lockwood & Bailey, 1970; Warshaw, 1972) and acetoacetyl-CoA thiolase (Lockwood & Bailey, 1971; Dierks-Ventling & Cone, 1971) only have so far been reported. Moreover, such studies were not carried out in sufficient detail to preclude the possibility that significant changes may take place in hepatic fatty acid oxidation between birth and weaning, a period of development when not only is a high-fat-content milk diet consumed, but also large alterations in some hormonal secretions are taking place (Bartova, 1968; Samel, 1968). In the present paper we report detailed developmental changes in the activities of enzymes involved in the oxidation of medium- and long-chain fatty acids by rat liver mitochondria.

Experimental

Materials

All reagents were of analytical grade or the purest available. GTP, ATP, NADH, NAD$^+$, GSH, *N*-acetyl-*S*-acetoacetylcysteamine and coenzyme A were obtained from C. F. Boehringer Corp. (London) Ltd., London W.5, U.K. Dithiothreitol, (−)-carnitine and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Palmitic acid and decanoic acid were obtained from Fluorochem Ltd., Glossop, Derbyshire, U.K. Crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. and made fat-free by the method of Chen (1967). Palmitoyl-coenzyme A and decanoyl-coenzyme A were obtained from International Enzymes Ltd., Windsor, Berks., U.K.

[*methyl*-^{3}H](±)-Carnitine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Acetoacetyl-coenzyme A was prepared by the method of Wieland & Rueff (1953) and assayed by that of Decker (1963). Crotonyl-coenzyme A was prepared as described by Simon & Shemin (1953). All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals

The rats used at all ages were females of an albino Wistar strain from the Sheffield University Animal House colony. The animals were kept in an environment of constant temperature and humidity on a 12h lighting schedule. They had free access to water and food, the latter being Oxoid Diet 86 obtained from Herbert C. Styles (Bewdley) Ltd., Bewdley, Worcs., U.K. Rats were removed from the mother on day 21 after birth and allowed to eat the same diet. Foetal ages were determined by dated matings. Animals were only taken from litters containing seven to ten rats. Separate series of rats were used for the acyl-CoA synthetases, the carnitine acyl transferases, the enoyl-CoA hydratase and cytoplasmic thiolase, the mitochondrial thiolases and 3-hydroxyacyl-CoA dehydrogenase. Assays of enzyme activity were carried out in random order, two or more age groups being included in any batch of assays.

Animals were killed by a blow to the head. The livers were rapidly excised and cooled on ice.

General preparation of mitochondria

Mitochondria were normally prepared by homogenizing livers in 5vol. of a solution containing 220mM-mannitol, 60mM-sucrose and 10mM-Tris/HCl buffer, pH7.4, in a Potter–Elvehjem homogenizer with a loose-fitting Teflon pestle. The homogenate was centrifuged at $700g_{av.}$ for 5min and the supernatant at $10000g_{av.}$ for 20min. The pellet was carefully resuspended in the same medium, again centrifuged at $10000g_{av.}$ for 20min and finally resuspended in the homogenizing medium.

Measurement of enzyme activities

Enzyme activities were determined immediately after preparation of the appropriate subcellular fraction. No appreciable decrease in activity occurred during the time required to carry out a batch of assays.

The various assay temperatures used are given in the legends to the Figures. The data in the Figures have not been corrected for the recovery values given below; however, the absolute values may be calculated from the data given.

Acyl-CoA synthetase activities of the outer mitochondrial membrane and matrix [acid–CoA ligase (AMP) (EC 6.2.1.3) and acid–CoA ligase (GDP) (EC 6.2.1.10)] were measured essentially as described by Aas (1971) but the assay medium was modified so that the final concentration of palmitate or decanoate was 0.5mM; KCl, 0.1M (for outer membrane enzymes) and absent for matrix enzymes; GTP, 2.0mM for the GTP-dependent enzyme; and Triton X-100 was not added to any assay. The mitochondria were prepared as in the general method except that 0.5mM-ATP was added to the homogenizing medium and the pH was 7.2. Whole mitochondria were used for the assay of outer-membrane activities. Matrix activities were measured in the supernatant after centrifuging (at $150000g_{av.}$ for 60min) a five-times frozen–thawed mitochondrial suspension.

The 'outer' carnitine acyltransferase activities were measured spectrophotometrically at 412nm by following the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by reduced coenzyme A released from acyl-CoA, by a modification of the method of Bieber *et al.* (1972). A molar extinction coefficient of 1.36×10^4litre·mol^{-1}·cm^{-1}, was used for the reduction product 5-thio-2-nitrobenzoate (Bieber *et al.*, 1972). The assay mixture contained 60mM-sucrose, 220mM-mannitol, 10mM-Tris/HCl buffer, pH7.4, 1mM-EDTA, 0.12mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.4mg of fat-free bovine plasma albumin/ml, 0.08–0.16mg of mitochondrial protein/ml, and 1mM-(−)-carnitine. The reaction was started by the addition of palmitoyl-CoA or decanoyl-CoA (16μM final concentration) after 3min preincubation. The activity was corrected for acyl-CoA hydrolase activity by using an assay mixture as above, but without the carnitine. The reaction was linear with time for 2–3min.

Enoyl-CoA hydratase [L-3-hydroxyacyl-CoA hydro-lyase (EC 4.2.1.17)] was measured by a modification of the method of Stern (1955). Livers were homogenized in the mannitol/sucrose/Tris buffer as above, with the addition of 1mM-mercaptoethanol. The homogenate was frozen–thawed five times in rapid succession (in liquid N_2) and samples of this were used in the assay, which contained 100mM-Tris/HCl, pH7.5, 5mM-EDTA and 2mg of bovine plasma albumin/ml, the reaction being started by the addition of 0.16mM-crotonyl-CoA (final concn.). Experiments showed that more than 90% of the total cell activity was in the mitochondrial fraction at all ages (P. C. Foster, unpublished work).

3-Hydroxyacyl-CoA dehydrogenase [L-3-hydroxyacyl-CoA–NAD$^+$ oxidoreductase (EC 1.1.1.35)] activity was measured spectrophotometrically by observing the decrease in E_{340} due to the oxidation of NADH by *N*-acetyl-*S*-acetoacetylcysteamine (Bunyan & Greenbaum, 1965). The assay mixture

contained 0.1 M-sodium pyrophosphate buffer, pH 7.3, 0.25 mM-NADH, and five-times frozen–thawed homogenate, which had been prepared in 66 mM-potassium phosphate buffer, pH 7.0. The reaction was started with 5 mM-*N*-acetyl-*S*-acetoacetylcysteamine (final concn.). Other experiments showed that more than 85% of the total cell activity was in the mitochondrial fraction at all ages (P. C. Foster, unpublished observations).

Cytoplasmic acetoacetyl-CoA thiolase (Middleton, 1973) activity was measured as described by Williamson *et al.* (1968). A liver homogenate was prepared as described for enoyl-CoA hydratase but without freeze–thawing, and centrifuged at 30000$g_{av.}$ for 30 min. The supernatant was used for the assay. The mitochondrial general oxoacyl-CoA thiolase and acetoacetyl-CoA-specific thiolase activities were measured as described by Middleton (1973). The mitochondria were prepared as in the general method but with the addition to the medium of 1 mM-mercaptoethanol.

Determination of glutamate dehydrogenase activity (Schmidt, 1974) in the various cell fractions at the different age groups used shows that mitochondrial contamination of the cytoplasmic fraction was always less than 1.5% and therefore would have no significant effect on the activity of the cytoplasmic acetoacetyl-CoA thiolase. At all ages, 55–65% of the cellular glutamate dehydrogenase activity was found in the mitochondrial fraction, most of the remainder being found in the 'nuclear' fraction. Thus the results obtained at various stages of development are similar to those previously observed for adults (Beaufay *et al.*, 1959).

Protein determinations were by the method of Lowry *et al.* (1951), crystallized bovine plasma albumin being used as the standard.

All spectrophotometric assays were carried out in a Gilford 222 spectrophotometer with a modified Unicam SP.500 monochromator.

Radioactivity was measured in a Nuclear–Chicago Isocap 300 liquid-scintillation counter and corrected by using an external-standard ratio. The scintillator used was that described by Aas (1971).

Results and Discussion

Long-chain (greater than C_{14}) and medium-chain (C_6–C_{14}) fatty acids are converted into the CoA derivatives by the acyl-CoA synthetases on the outer-mitochondrial and endoplasmic-reticulum membranes, whereas short-chain and medium-chain fatty acids are converted into the CoA derivatives in the mitochondrial matrix (Aas, 1971). Fig. 1 shows that the activity of the outer-membrane palmitoyl-CoA synthetase is very low in late foetal life, but rises rapidly at birth, reaching a maximum at 3–7 days, followed by a fall during the second week after birth.

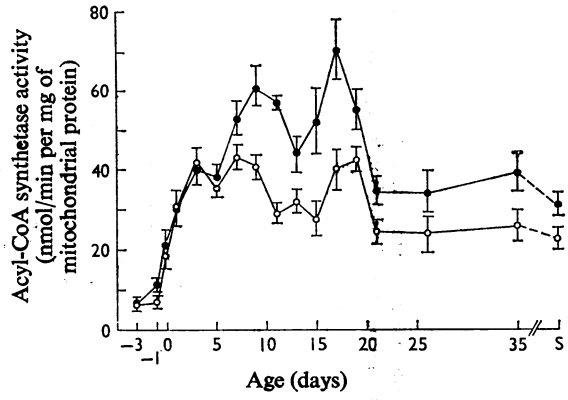

Fig. 1. *Developmental changes in the activities of rat liver outer-mitochondrial-membrane acyl-CoA synthetases*

●, Decanoyl-CoA synthetase activity; ○, palmitoyl-CoA synthetase activity. Enzyme activities were measured at 37°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means ± S.E.M. of six determinations, animals from at least three litters being used.

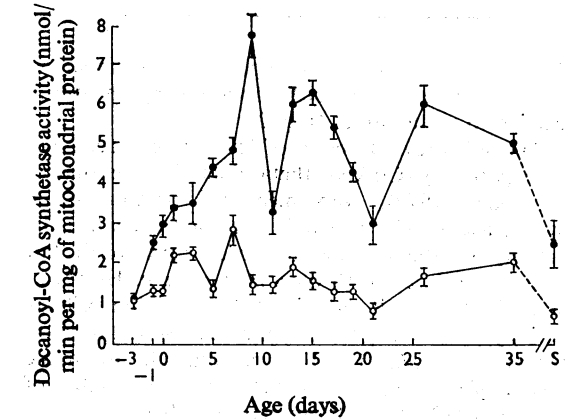

Fig. 2. *Developmental changes in the activities of rat liver mitochondrial-matrix decanoyl-CoA synthetases*

●, ATP-dependent enzyme; ○, GTP-dependent enzyme. Enzyme activities were measured at 37°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means ± S.E.M. of six determinations, animals from at least three litters being used.

A second peak of enzyme activity is observed at 17–19 days, after which the activity falls to adult values. The rate of formation of decanoyl-CoA follows a similar pattern. The matrix ATP-dependent decanoyl-CoA synthetase shows a developmental pattern similar to that of the enzyme of the outer membrane, except for a third rise after weaning at

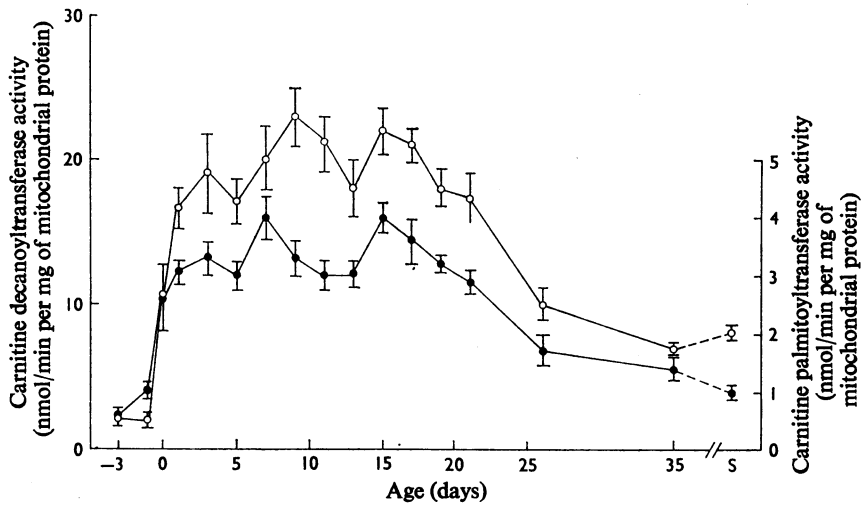

Fig. 3. *Developmental changes in the activities of rat liver mitochondrial acyl-CoA–carnitine acyltransferase*

●, Carnitine palmitoyltransferase activity; ○, carnitine decanoyltransferase activity. Enzyme activities were measured at 30°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means ± S.E.M. of six determinations, animals from at least three litters being used.

21 days, whereas the GTP-dependent enzyme shows a small slow rise after birth, followed by an overall decline to adult values (Fig. 2).

The only previous report of developmental changes of hepatic acyl-CoA synthetase activity was that of Warshaw (1972), who measured palmitoyl-CoA synthetase activity in whole liver homogenates; the activity of the enzyme increased after birth but changed little after 10 days *post partum*. However, since in the adult rat about 65% of the cellular activity of this enzyme is in the microsomal fraction (Farstad *et al.*, 1967) it is difficult to make meaningful comparisons between the results of Warshaw (1972) and those presented here.

Workers who have previously measured carnitine acyltransferase activity have assayed a combination of the activities of both the inner and outer transferases, uncorrected for acyl-CoA hydrolase activity (Augenfeld & Fritz, 1970; Lockwood & Bailey, 1971). We measured the outer transferase activity only, by using a method of assay that is independent of hydrolase activity (Bieber *et al.* 1972). The transferase activity measured with palmitoyl-CoA or decanoyl-CoA exhibits similar changes (Fig. 3). The activities increase rapidly after birth, remain high during the suckling period (with peaks at about 8 and 15 days), and fall to the adult values at weaning. These results essentially agree with those of Augenfeld & Fritz (1970) and Lockwood & Bailey (1971) who showed a large rise in activity at birth to values higher than in the adult. Norum (1965) has shown that the combined inner and outer transferase activity is increased in starvation and fat-feeding in the adult, situations in which the supply of fatty acids to the liver is increased.

Within the inner mitochondrial membrane, acyl-CoA is oxidized by acyl-CoA dehydrogenases. Unfortunately we were unable to obtain a satisfactory assay for this enzyme to use in developmental studies.

Fig. 4 shows that the measured activity of enoyl-CoA hydratase is considerably greater than the activity of the other fatty acid oxidation enzymes studied at all stages of development. It also exhibits a very different developmental pattern, since the enzyme activity rises rapidly immediately before and after birth, and then remains relatively constant from 5 days to weaning at 21 days, after which it rises approximately 50% to adult values. Waterson & Hill (1972) suggested that this enzyme could have some regulatory function in fatty acid oxidation; however, this seems unlikely, at least during the neonatal period, because of the differences in the developmental changes in activity of this enzyme compared with the others involved.

3-Hydroxyacyl-CoA dehydrogenase activity is low in the 16-day foetal liver but rises rapidly during the last few days of foetal life and after birth to give a maximum at 7–11 days *post partum* (Fig. 5). The activity declines to about one-half the maximum value by 13 days and then rises rapidly to a second peak of activity at 17 days, after which time the activity falls gradually to the adult value.

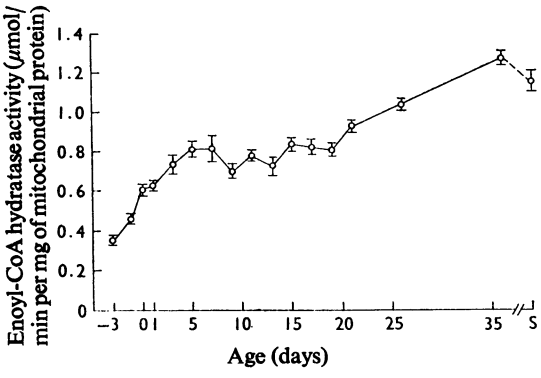

Fig. 4. *Developmental changes in the activity of rat liver mitochondrial enoyl-CoA hydratase*

Enzyme activities were determined at 25°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means±S.E.M. of eight determinations, animals from at least three litters being used.

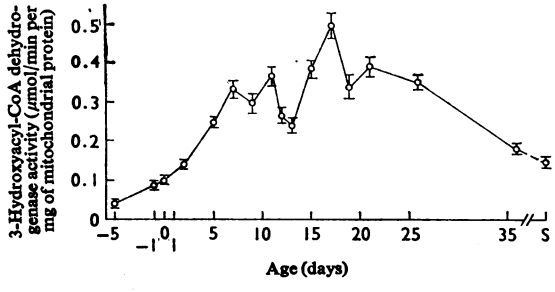

Fig. 5. *Developmental changes in the activity of rat liver mitochondrial 3-hydroxyacyl-CoA dehydrogenase*

Enzyme activities were determined at 25°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means±S.E.M. of eight determinations, animals from at least three litters being used.

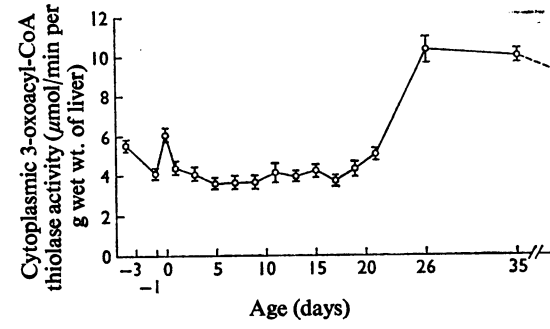

Fig. 6. *Developmental changes in the activity of rat liver cytoplasmic 3-oxoacyl-CoA thiolase*

Enzyme activities were determined at 25°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means±S.E.M. of eight determinations, animals from at least three litters being used.

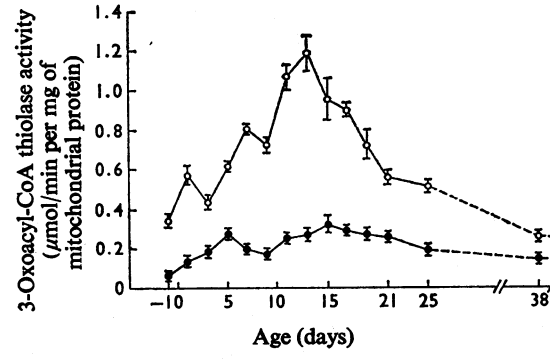

Fig. 7. *Developmental changes in the activities of rat liver mitochondrial 3-oxoacyl-CoA thiolase*

●, General 3-oxoacyl-CoA thiolase; ○, acetoacetyl-CoA-specific thiolase. Enzyme activities were determined at 30°C as described in the Experimental section. On the abscissa, S denotes adult stock rats. The results are the means±S.E.M. of eight determinations, animals from at least three litters being used.

At least three enzymes are present in adult rat liver that catalyse the thiolysis of acetoacetyl-CoA (Middleton, 1973; Huth *et al.*, 1974). Middleton (1973) suggested that the cytoplasmic acetoacetyl-CoA thiolase is involved in cholesterol biosynthesis, mitochondrial acetoacetyl-CoA-specific thiolase in ketogenesis, and a mitochondrial general oxoacyl-CoA thiolase in β-oxidation. Cytoplasmic acetoacetyl-CoA thiolase activity rises slightly at birth but then falls during the first day *post partum* and remains approximately constant until about 19 days, when it rises rapidly, reaching the adult value by 26 days (Fig. 6). A similar developmental pattern is obtained for the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (McNamara *et al.*, 1972) and for the biosynthesis of sterols from acetic acid and mevalonic acid (Wróbel *et al.*, 1973). In contrast, the mitochondrial acetoacetyl-CoA-specific thiolase activity increases slowly at birth reaching a maximum value at 13 days, and falls slowly thereafter to adult values (Fig. 7). This developmental pattern differs from those of the other enzymes of hepatic ketogenesis, 3-hydroxy-3-methylglutaryl-CoA synthetase and 3-hydroxy-3-methylglutaryl-CoA lyase

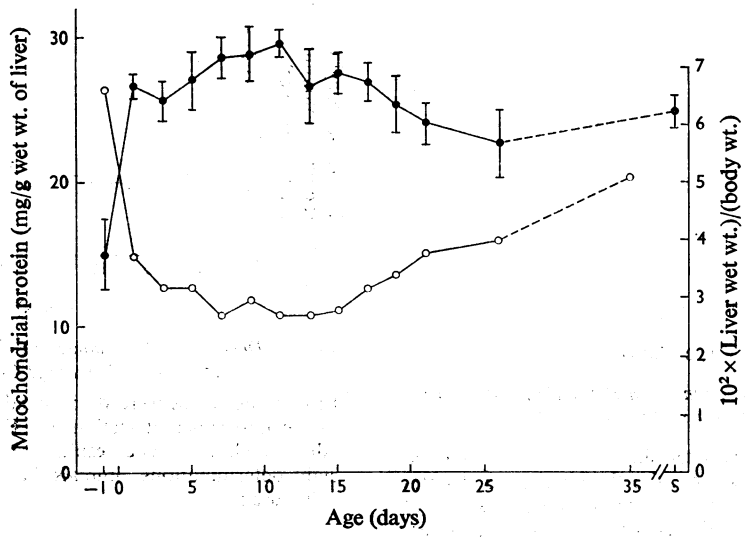

Fig. 8. *Developmental changes in the liver/body weight ratio and liver mitochondrial protein*

●, Liver mitochondrial protein. The results are the means±S.E.M. of eight determinations, animals from at least three litters being used. ○, (Liver wet wt.)/(body wt.) ratio. The results are the means of at least 35 determinations. The S.E.M. at each age is too small to be indicated.

(Hipolito-Reis *et al.*, 1974). Huth *et al.* (1973) have suggested that the acetoacetyl-CoA-specific thiolase is rate-limiting for ketone-body formation in the adult. However, the above results, taken in conjunction with those of Hipolito-Reis *et al.* (1974) suggest that the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase is of limiting activity, at least in the neonatal period, since there is a large and rapid increase in the synthase activity in the first 12h after birth, a time when there is a rapid rise in the rate of ketogenesis (Snell & Walker, 1973), whereas the thiolase activity increases by a relatively small amount in the same period.

As Fig. 7 shows, the general oxoacyl-CoA thiolase activity increases from the last day of foetal life to 5 days, after which it declines to 9 days and then rises to a new peak value at 15 days, before once more falling to the adult value. Previous studies (Lockwood & Bailey, 1971; Dierks-Ventling & Cone, 1971) have measured a combination of thiolase activities, but their results also showed higher thiolase activity during suckling than during foetal life or after weaning.

It can be seen from Figs. 1–7 that the activities of all the enzymes of hepatic mitochondrial fatty acid oxidation studied increase markedly at birth and, except for enoyl-CoA hydratase, decrease at weaning. Clearly these enzymes adapt to developmental changes in nutritional and hormonal status as has been shown for enzymes of other metabolic pathways (Greengard, 1971). Another feature common to all the enzyme developmental patterns (other than those of enoyl-CoA hydratase and acetoacetyl-CoA-specific thiolase) is a relatively low enzyme activity during the mid-suckling period. Such changes cannot be explained in terms of changing hepatic mitochondrial population or different body and liver growth rates. Since the amount of mitochondrial protein/g wet wt. of liver increases around birth (Fig. 8), then the observed increases in enzymic activities immediately after birth would be greater if expressed relative to liver weight. However, such an amplification of the postnatal rise would be negated if expressed per unit body weight since the ratio (liver wt.)/(body wt.) falls in the first postnatal day (Fig. 8). The results obtained after day 2 would not be significantly affected if expressed in either of the alternative ways mentioned above, since only minor changes occur in mitochondrial protein content/g wet wt. of liver and in the (liver wt.)/(body wt.) ratio (Fig. 8). The results shown in Fig. 8 are consistent with those obtained by Vernon & Walker (1968), Jakovcic *et al.* (1971) and Lang & Harbener (1972). Further, the method of selection of experimental animals described in the Experimental section ensures that external factors such as change of environment

or assay conditions do not contribute to the observed developmental changes.

It is noteworthy that similar developmental changes to those described above have been observed for the oxidation of palmitoylcarnitine, decanoylcarnitine and palmitate and the formation of ketone bodies from palmitate by rat liver mitochondria (P. C. Foster & E. Bailey, unpublished results), the activities of enzymes of hepatic ketogenesis (J. Shah & E. Bailey, unpublished results) and some of the enzymes of hepatic gluconeogenesis (Vernon & Walker, 1968), and that somewhat reciprocal changes occur in the activities of some enzymes of hepatic glycolysis and lipogenesis (Webb & Bailey, 1975). These results suggest that there may be a shift in relative importance of lipid and carbohydrate degradation in the liver during the mid-suckling period despite the continued intake of a high-fat-content diet. It is noteworthy that at this time there is also an increase in renal gluconeogenesis (Hauser & Bailey, 1975). With regard to acylcarnitine oxidation (P. C. Foster & E. Bailey, unpublished results), it should be noted that the rates obtained are at all ages considerably lower than are the activities of any of the enzymes reported here, e.g. the values for palmitoylcarnitine oxidation at −1, 5, 7, 17 and 35 days of age are 2, 10, 2.5, 5 and 3 nmol/min per mg of mitochondrial protein respectively. It thus appears that at all developmental stages the measured activities of the enzymes of fatty acid oxidation are in excess of the overall rates of β-oxidation. However, it is possible that in the mitochondrion *in vivo*, the activities of the enzymes are considerably lower than those measured *in vitro*. There is no evidence in the literature suggesting a decreased intake of lipid into the gut during the mid-suckling period. It is possible that at this age lipid is being directed to other tissues to provide both an energy source and structural components, e.g. the brain has an increased capacity to take up fatty acids and incorporate them into complex lipids (Dhopeshwarkar & Mead, 1973). However, endocrinological changes such as an increase in plasma thyroxine (Samel, 1968) and corticosterone (Bartova, 1968) are known to occur during the mid-suckling period and it is possible that such changes affect the activities of enzymes involved in hepatic fatty acid oxidation. Thyroxine is known to have a lipolytic effect on adipose tissue (Rudman, 1963) and may thus affect the supply of fatty acids to the liver, and the same hormone has also been shown to increase the activity of some mitochondrial enzymes in the adult rat (Tata *et al.*, 1963).

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