Biochem. J. (1971) **124**, 249–254
Printed in Great Britain

The Course of Ketosis and the Activity of Key Enzymes of Ketogenesis and Ketone-Body Utilization During Development of the Postnatal Rat

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(*Received* 29 *March* 1971)

1. The highest blood concentrations of ketone bodies were found at 5 days of age, after which time the concentration fell to reach the adult value by 30 days of age. 2. Both mitochondrial and cytoplasmic hydroxymethylglutaryl-CoA synthase activities were detected, with highest activities being found in the mitochondria at all stages of development. Activity of the mitochondrial enzyme increases rapidly immediately after birth, showing a maximum at 15 days of age, thereafter falling to adult values. The cytoplasmic enzyme, on the other hand, increased steadily in activity after birth to reach a maximum at 40 days of age, after which time activity fell to adult values. 3. Both mitochondrial and cytoplasmic acetoacetyl-CoA thiolase activities were detected, with the mitochondrial enzyme having considerably higher activities at all stages of development. The developmental patterns for both enzymes were very similar to those for the corresponding hydroxymethylglutaryl-CoA synthases. 4. The activity of heart acetoacetyl-CoA transferase remains constant from late foetal life until the end of the suckling period, after which time there is a gradual threefold increase in activity to reach the adult values. The activity of brain 3-oxo acid CoA-transferase increases steadily after birth, reaching a maximum at 30 days of age, thereafter decreasing to adult values, which are similar to foetal activities. Although at all stages of development the specific activity of the heart enzyme is higher than that of brain, the total enzymic capacity of the brain is higher than that of the heart during the suckling period.

Nutritionally the early life of the laboratory rat is characterized by two changes: the first is physiologically defined when the high-carbohydrate-content transplacental food is replaced by the high-fat-content milk diet after birth; the second change is less strictly defined and occurs at weaning when the milk diet of suckling is replaced by the laboratory diet, which usually has a high-carbohydrate/low-fat content. It is now clear that the hepatic metabolism of the rat, particularly lipogenesis and gluconeogenesis, adapts to the nutritional changes that occur during development (Taylor, Bailey & Bartley, 1967; Ballard & Hanson, 1967; Yeung, Stanley & Oliver, 1967; Vernon & Walker, 1968; Lockwood, Bailey & Taylor, 1970). Less is known about changes in lipid breakdown during development of the rat despite the fact that during the suckling period the neonatal rat derives most of its energy from the fat content of the milk (Drahota, Hahn, Kleinzeller & Kostolánská, 1964). Lockwood & Bailey (1970) and Augenfeld & Fritz (1970) studied the breakdown of palmitate to carbon dioxide and ketone bodies by liver homogenates and found that during the suckling period there was an increased production of ketone bodies relative to other periods of development. Drahota *et al.* (1964) had found elevated concentrations of blood acetoacetate during the suckling period and also that the production of acetoacetate by liver slices was greatest during suckling. Clearly during suckling a physiological state of ketosis exists and use of the developing rat allows the study of the onset of ketosis at birth and the reversion to the non-ketotic state at weaning.

The present paper is concerned with developmental changes in blood acetoacetate and β-hydroxybutyrate concentrations, in the activities of enzymes concerned with hepatic ketone-body formation, and in the activity of a key enzyme in ketone-body utilization by heart and brain. The enzymes involved in hepatic ketogenesis that have

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been studied are hydroxymethylglutaryl-CoA synthase, which is probably the rate-limiting enzyme in the pathway (Williamson, Bates & Krebs, 1968) and acetoacetyl-CoA thiolase. The enzyme involved in ketone-body utilization that has been investigated is 3-oxo acid CoA-transferase. Since, in the adult rat liver, both mitochondrial and cytoplasmic pathways of ketogenesis appear to exist (Sauer & Erfle, 1966; Williamson *et al.* 1968) we have investigated developmental changes in the intracellular distribution of enzymes of hepatic ketogenesis.

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, acetyl phosphate and coenzyme A. Sodium acetoacetate was prepared from freshly distilled ethyl acetoacetate as described by Krebs & Eggleston (1945). Acetoacetyl-CoA was prepared by the interaction of diketene with CoA (Wieland & Rueff, 1953) and was kindly provided by Dr D. H. Williamson (Metabolic Research Laboratory, Nuffield Department of Medicine, Radcliffe Infirmary, Oxford, U.K.). It was found to be stable for at least 3 weeks when stored at −20°C. Acetoacetyl-CoA was determined quantitatively by measuring NADH oxidation in the presence of 3-hydroxyacyl-CoA dehydrogenase (Decker, 1963).

Animals. Wistar rats from the Sheffield University Animal House Colony were used throughout, only male rats being used after weaning. The rats were killed by a blow on the head. Foetuses were removed from the mother near term, i.e. 19–20 days as estimated by foetal body weight (Lang, 1965). The young were weaned at 21 days on to a rat-cube diet [Oxoid diet 86 obtained from Herbert C. Styles (Bewdley) Ltd., Bewdley, Worcs., U.K.].

Determination of blood ketone bodies. The thoracic cavity was opened immediately after the animals were killed and blood was withdrawn from the heart by a syringe and needle (previously flushed with heparin to prevent the blood from clotting). The blood was transferred to a heparinized tube and 2 vol. of 6% (w/v) $HClO_4$ added. The precipitated protein was removed by centrifugation and the supernatant solution neutralized with 20% (w/v) KOH. Treatment of the blood sample was carried out at 0°C to prevent spontaneous breakdown of acetoacetate to acetone and CO_2. After the solution had stood for 1 h at 0°C the $KClO_4$ precipitate was sedimented by centrifugation and 0.5 ml of the supernatant solution taken for the determination of acetoacetate and β-hydroxybutyrate by the enzymic method of Williamson, Mellanby & Krebs (1962).

Preparation of tissue for enzyme assay. For hydroxymethylglutaryl-CoA synthase, acetoacetyl-CoA thiolase and 3-oxo acid CoA-transferase, tissue was homogenized in 4 vol. of ice-cold 0.25 M-sucrose containing 1 mM-mercaptoethanol and 10 mM-tris–HCl buffer, pH 7.4. To determine the activity of the whole homogenate, the homogenate was exposed to ultrasonic vibration for 2 min at maximum current in an M.S.E. ultrasonic disintegrator. The homogenate was then centrifuged at 30000 *g* for 30 min. Cytoplasmic enzyme activity was determined on the supernatant solution after a similar centrifugation of an homogenate that had not been sonicated. Mitochondrial enzyme activity was calculated by subtracting the cytoplasmic activity from that of the whole homogenate. All procedures were carried out at 0–4°C.

Determination of acetoacetyl-CoA thiolase activity. Acetoacetyl-CoA thiolase (acetyl-CoA–acetyl-CoA *C*-acyltransferase, EC 2.3.1.9) was assayed by the method of Williamson *et al.* (1968) by following the decrease in E_{313} resulting from the disappearance of the acetoacetyl-CoA–Mg^{2+} complex. The molar extinction coefficient of the complex was taken to be 11.8×10^3 $cm^2 \cdot mol^{-1}$ (Williamson *et al.* 1968). The assay mixture (final volume 2 ml) contained 50 mM-tris–HCl buffer, pH 8.5, 5 mM-$MgCl_2$, 0.2 mM-CoA and 0.05 mM-acetoacetyl-CoA. The reaction was started by adding enzyme to the reaction mixture, which had been preincubated for 5 min.

Determination of hydroxymethylglutaryl-CoA synthase activity. Hydroxymethylglutaryl-CoA synthase [3-hydroxy-3-methylglutaryl-CoA acetoacetyl-CoA-lyase (CoA-acetylating), EC 4.1.3.5.] was assayed by the method of Williamson *et al.* (1968). Activity was determined by incubation of the enzyme sample with an acetyl-CoA-generating system and subsequent spectrophotometric assay of acetoacetate (Williamson *et al.* 1962). The incubation mixture (final volume 1 ml) contained 50 mM-tris–HCl buffer, pH 8.5, 5 mM-$MgCl_2$, 0.85 mM-CoA, 10 mM-acetyl phosphate and phosphotransacetylase (10 units). The reaction was stopped by adding 1 ml of 3% (w/v) $HClO_4$ and the supernatant solution remaining after removal of precipitated protein by centrifugation was neutralized by addition of 10% (w/v) KOH. After the neutral solution had stood on ice for 1 h the $KClO_4$ formed was removed by centrifugation and acetoacetate determined in the supernatant solution. The reaction rate was linear with enzyme concentration within the range of enzyme concentrations used.

Determination of 3-oxo acid CoA-transferase activity. 3-Oxo acid CoA-transferase (succinyl-CoA–3-oxo acid CoA-transferase, EC 2.8.3.5) was assayed by the method of Williamson, Bates, Page & Krebs (1971). The assay follows the decrease in E_{313} resulting from the disappearance of the acetoacetyl-CoA–Mg^{2+} complex in the presence of iodoacetamide, which inhibits acetoacetyl-CoA thiolase activity, which would otherwise interfere. The assay mixture (final volume 2 ml) contained 50 mM-tris–HCl buffer, pH 8.5, 10 mM-$MgCl_2$, 0.05 mM-acetoacetyl-CoA, 50 mM-iodoacetamide and 50 mM-sodium succinate. The rates of spontaneous decomposition of acetoacetyl-CoA and its disappearance resulting from acetoacetyl-CoA lyase were always very low and were subtracted from the final rate. The reaction was started by adding succinate to the reaction mixture. The reaction rate was linear with enzyme concentration within the range of enzyme concentrations used.

RESULTS

Blood ketone bodies. Changes in concentrations of the ketone bodies acetoacetate and β-hydroxybutyrate in the blood during neonatal development are shown in Fig. 1. The concentration of acetoacetate

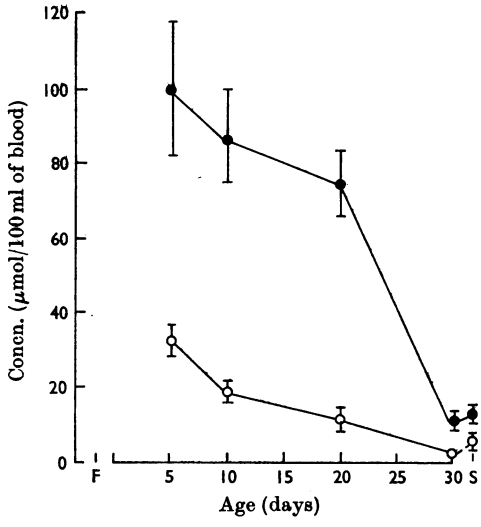

Fig. 1. Changes in blood concentrations of ketone bodies during development of the rat. Acetoacetate (○) and β-hydroxybutyrate (●) were determined enzymically in deproteinized blood as described in the Experimental section. On the abscissa F denotes foetal rats and S denotes adult stock rats. The results are the means ± S.E.M. of five determinations.

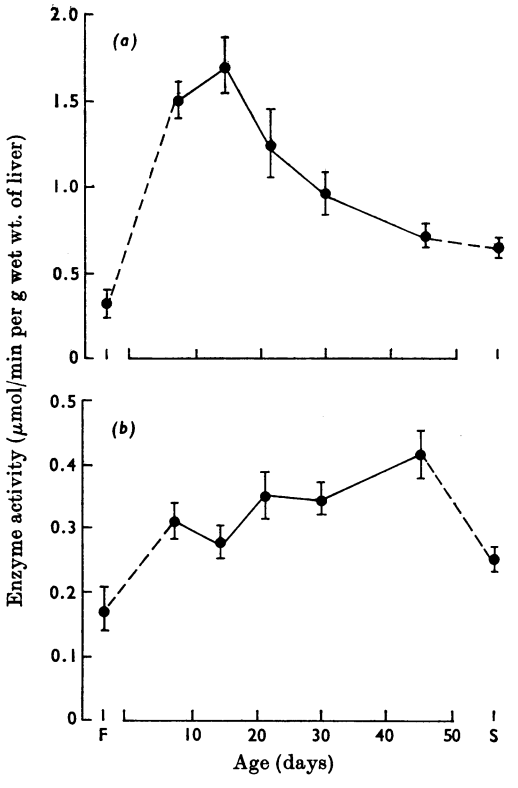

Fig. 2. Changes in the activity of hepatic hydroxymethylglutaryl-CoA synthase during development of the rat. Mitochondrial (*a*) and extramitochondrial (*b*) enzyme activities were determined as described in the Experimental section. The results are the means ± S.E.M. of five determinations. On the abscissa F denotes foetal rats and S denotes adult stock rats.

is highest at 5 days of age (33 μmol/100ml) thereafter gradually decreasing to the much lower adult concentrations (5 μmol/100ml). The elevated blood acetoacetate concentration observed during the suckling period confirms the results of Drahota *et al.* (1964). Blood concentrations of β-hydroxybutyrate vary in a similar manner during development to those of acetoacetate. There is a gradual decrease in concentration from 5 to 20 days of age, after which time the concentration rapidly falls to adult values by 30 days of age. Similar results have been reported by Page, Krebs & Williamson (1971), although we find higher β-hydroxybutyrate/acetoacetate ratios during part of the suckling period.

Changes in the activity of hepatic hydroxymethylglutaryl-CoA synthase. Fig. 2 shows the changes in activity of both mitochondrial (Fig. 2*a*) and cytoplasmic (Fig. 2*b*) hepatic hydroxymethylglutaryl-CoA synthase during development. A low activity (0.3 μmol/min per g of tissue) of the mitochondrial enzyme is found in foetal liver. However, the activity of the enzyme increases rapidly after birth to reach a maximum (1.7 μmol/min per g of tissue) at 15 days of age, thereafter decreasing gradually to the adult value of 0.7 μmol/min per g of tissue. The activity of the extramitochondrial enzyme increases gradually after birth, reaching a maximum at 45 days of age, after which time the activity declines to the adult value. The activity of the extramitochondrial enzymes is at all ages considerably less than that of the mitochondrial enzyme, although during later development the activity of the extramitochondrial enzyme accounts for 30–35% of the total hepatic enzyme activity. It is possible that some of the cytoplasmic hydroxymethylglutaryl-CoA synthase arises by leakage from the mitochondria during preparation of cell fractions, since the relative amount of enzyme activity in the cytoplasm is higher than previously reported (Williamson *et al.* 1968). However, since the developmental patterns for the two enzymes are completely different it seems unlikely that mitochondrial leakage accounts for much of the cytoplasmic enzyme activity.

Changes in activity of hepatic acetoacetyl-CoA thiolase. The activities of the hepatic mitochondrial and cytoplasmic acetoacetyl-CoA thiolases throughout development are shown in Fig. 3 (*a*) and

3 (*b*) respectively. Both activities are always far in excess of the respective hydroxymethylglutaryl-CoA synthase activities. The activity of the mitochondrial enzyme increases rapidly after birth to a maximum (160 μmol/min per g of tissue) at 14 days of age. It then decreases equally rapidly until 30 days of age to reach the adult value, which is very similar to that of foetal liver (30 μmol/min per g of tissue). Extramitochondrial enzyme activity is much lower than that of the mitochondrial enzyme. It increases after birth to reach maximum activity at about 20 days of age. This activity is maintained until 50 days of age, after which time it decreases to a slightly lower adult value. In the adult the extramitochondrial activity contributes approx. 17% of the total hepatic enzyme activity.

Changes in activity of 3-oxo acid CoA-transferase in heart and brain. This enzyme, which is entirely mitochondrial, catalyses the first step in the utilization of acetoacetate by extrahepatic tissues. Changes in activity of the enzyme in the heart and brain during development are shown in Fig. 4. The activity of the enzyme in heart is similar in foetal and neonatal tissue from birth to 20 days of age (approx. 5 μmol/min per g of tissue). There is a two- to three-fold increase in activity from 20 to 30 days of age and adult activities are similar to those at 30 days of age. Specific enzyme activity is lower in brain than in heart at all ages. Foetal and 5-day-old rat brain activities are similar (approx. 1–2 μmol/min per g of tissue) and there is then a gradual increase in activity up to 20 days of age. After 30 days of age activity falls to adult values, which are similar to those of foetal and 5-day-old rats. Although the specific activity of the enzyme is higher in the heart than in the brain at all stages of development, the total enzymic capacity of the brain (see Table 1) is higher at 10 and 20 days of age, similar at 5 and 30 days of age and only lower than that of heart in adults, i.e. during much of the suckling period the brain has a greater capacity for ketone-body utilization than the heart. Qualitatively similar results have been reported by Page *et al.* (1971).

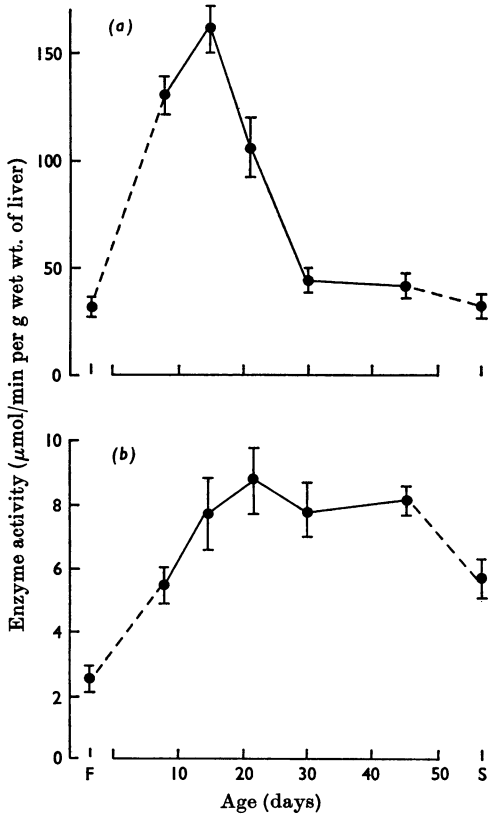

Fig. 3. Changes in the activity of hepatic acetoacetyl-CoA thiolase during development of the rat. Mitochondrial (*a*) and extramitochondrial (*b*) enzyme activities were determined as described in the Experimental section. The results are the means ± S.E.M. of five determinations. On the abscissa F denotes foetal rats and S denotes adult stock rats.

DISCUSSION

Our results on blood ketone-body concentrations during development confirm those of Page *et al.* (1971) and extend the findings of Drahota *et al.*

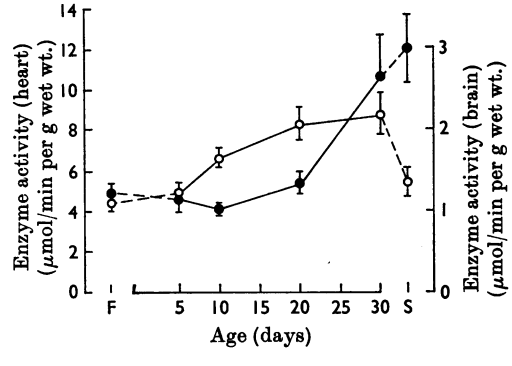

Fig. 4. Changes in the activity of 3-oxo acid CoA-transferase in the heart and brain of the developing rat. Enzyme activity in heart (●) and brain (○) was determined as described in the Experimental section. The results are the means ± S.E.M. of five or six determinations, except for foetal activities, which are the means of three determinations each carried out on pooled tissue from seven to ten animals. On the abscissa F denotes foetal rats and S denotes adult stock rats.

Table 1. *Changes in total activity of 3-oxo acid CoA-transferase on heart and brain during development*

Organ and body weights are the means of the number of observations shown in parentheses. Total enzyme activities are calculated from the mean organ weights and the mean specific activities shown in Fig. 4.

Age (days)	Body wt. (g)	Heart wt. (g)	Total heart enzyme activity (μmol/min per heart)	Brain wt. (g)	Total brain enzyme activity (μmol/min per brain)
5	9.9 (10)	0.062 (10)	0.3	0.28 (10)	0.28
10	20.1 (15)	0.11 (22)	0.5	0.63 (22)	0.95
20	33.2 (15)	0.14 (16)	0.7	0.85 (16)	1.7
30	74.8 (3)	0.27 (3)	2.7	1.3 (3)	2.6
Stock adult	205.4 (3)	0.73 (3)	7.5	1.3 (3)	1.3

(1964). Clearly these and previous results (Drahota *et al.* 1964; Lockwood & Bailey, 1970; Augenfeld & Fritz, 1970) indicate that an increased hepatic synthesis of ketone bodies takes place during suckling when a high-fat-content diet is being consumed. The source of ketone bodies *in vivo* is the plasma free fatty acids and it is noteworthy that Page *et al.* (1971) have found that the concentration of plasma free fatty acids is high during suckling and falls on weaning. Although the liver is the main site of ketone-body formation in the non-ruminant (for review of ketone-body metabolism see Williamson & Hems, 1970), it is unable to utilize ketone bodies, since it lacks the enzyme 3-oxo acid CoA-transferase.

There are two pathways of hepatic ketogenesis, both of which involve the conversion of acetyl-CoA into acetoacetyl-CoA by the enzyme acetoacetyl-CoA thiolase (Lynen, 1953). The acetoacetyl-CoA can be converted into acetoacetate by deacylation (Stern & Miller, 1959). However, this pathway probably accounts for only a small proportion of the acetoacetate formed by adult rat liver (Williamson *et al.* 1968) or for that matter by livers of rats of any age (E. A. Lockwood & E. Bailey, unpublished work). The major pathway of acetoacetate formation (the hydroxymethylglutaryl-CoA pathway) involves conversion of acetoacetyl-CoA and acetyl-CoA into hydroxymethylglutaryl-CoA (by hydroxymethylglutaryl-CoA synthase), which is then split to acetoacetate and acetyl-CoA by hydroxymethylglutaryl-CoA lyase (Lynen, Henning, Bublitz, Sorbo & Kröplin-Rueff, 1958). The hydroxymethylglutaryl-CoA synthase is thought to be the rate-limiting enzyme of this pathway (Williamson *et al.* 1968). Our results show that the mitochondrial activity of this enzyme varies during development, with highest activities being found during suckling when a high-fat diet is being consumed. The developmental pattern tends to support the suggestion that the synthase is the rate-limiting enzyme in ketogenesis. It is noteworthy, however, that the mitochondrial acetoacetyl-CoA thiolase activity shows a very similar developmental curve and the ratio of thiolase to synthase activity is maintained at about 60:1 throughout development. It appears that during any adaptive change in ketogenesis a constant large excess of thiolase activity over synthase activity is maintained.

Williamson *et al.* (1968) found increased hepatic activities of hydroxymethylglutaryl-CoA synthase and thiolase on feeding adult rats on a high-fat diet, a situation somewhat analogous to the suckling rat. The question arises as to whether or not an increased activity of the enzymes of hepatic ketogenesis is essential for the increased synthesis of ketone bodies. It is possible that a rise in plasma free fatty acid concentration is followed by increased activity of the enzymes of ketogenesis followed by increased ketone-body formation. However, Williamson *et al.* (1968) showed that the ketosis of starvation is not accompanied by an increased synthesis of enzyme. Further, from our results it is probable that the increase in concentration of blood ketone bodies precedes synthesis of the enzymes of ketogenesis, suggesting that enzyme concentrations do not control ketogenesis directly. It would appear that either the rise in ketogenesis induces the synthesis of ketone-body-forming enzymes or that the rise in enzyme concentration is an independent phenomenon, which, nevertheless, ensures that there is always an excess of enzyme present. There is also the unlikely possibility that the rise in synthesis of the enzymes is a developmental process unrelated to the feeding of fat-rich milk.

The hydroxymethylglutaryl-CoA pathway is thought to be predominantly mitochondrial, but significant activity of enzymes of the pathway has been found in the cytoplasm of adult guinea-pig liver (Sauer & Erfle, 1966) and adult rat liver (Williamson *et al.* 1968). Our results indicate the presence of all enzymes necessary to support an extramitochondrial pathway of ketogenesis throughout development. Williamson & Hems (1970) have suggested that this pathway is involved in the utilization of cytoplasmic acetyl-CoA in

situations in which cholesterol and fatty acid synthesis have ceased. The developmental patterns of the enzymes of the pathway (unlike the mitochondrial enzymes) bear no resemblance to changes in blood ketone-body concentrations, supporting the suggestion that the cytoplasm is a minor site of ketogenesis. It is noteworthy, however, that as with the mitochondrial enzymes, the acetoacetyl-CoA thiolase and hydroxymethylglutaryl-CoA synthase of the extramitochondrial pathway exhibit almost identical developmental patterns, with a thiolase/synthase activity ratio of about 20 being maintained throughout development. As with the mitochondrial enzymes, changes in the activity of these two enzymes appear to be closely linked. It is possible that the developmental changes in the activity of these enzymes are associated more with cholesterol biosynthesis than with ketogenesis. Cholesterol synthesis and acetoacetate formation share a common pathway up to the formation of hydroxymethylglutaryl-CoA, which, in cholesterol formation, is reduced to mevalonic acid by hydroxymethylglutaryl-CoA reductase.

There is no correlation between the activity of the 3-oxo acid CoA-transferase of rat brain and heart during development and the concentration of blood ketone bodies. In heart in particular, lowest activities of the enzymes are found during suckling, when hepatic ketone-body production is highest. Similar results were obtained by Page *et al.* (1971). These authors concluded that the rate of ketone-body utilization in the brain of suckling rats is determined both by the activity of 3-oxo acid CoA-transferase and by the high concentration of ketone bodies in the blood.

We thank the Medical Research Council for a grant to E.A.L.

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