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The Acetoacetyl-Coenzyme A Thiolases of Rat Brain and their Relative Activities during Postnatal Development

By B. MIDDLETON*
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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1. The apparent 3-oxoacyl-CoA thiolase activity of rat brain extracts is due to two different acetoacetyl-CoA thiolases, one cytoplasmic and the other mitochondrial. By the methods developed in the preceding paper (Middleton, 1973), the changes in activities of these two enzymes were determined during postnatal development. 2. Although the total brain acetoacetyl-CoA thiolase activity changes not more than 2-fold from birth to adulthood this masks large changes in the relative proportions of the two types of thiolase present. 3. Cytoplasmic acetoacetyl-CoA thiolase activity declines slowly from 4 units/g fresh wt. at birth to an adult value of 1.3 units/g fresh wt. 4. The mitochondrial acetoacetyl-CoA thiolase (activated by K^+) rises rapidly in activity from 1 unit/g fresh wt. at birth to a peak value of 5 units/g fresh wt. at 20 days. After weaning the activity declines to 2.3 units/g fresh wt. in the adult. 5. These different developmental patterns are discussed in terms of the probable metabolic roles of the two brain acetoacetyl-CoA thiolases.

In their investigation of the activities of enzymes involved in ketone-body utilization during the postnatal development of rat brain Page *et al.* (1971) found an extremely good correlation between the activities of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and 3-oxo acid CoA-transferase (EC 2.8.3.5). The activities of both of these enzymes showed a dramatic rise after birth, followed, after weaning, by a slow fall to the adult values. Surprisingly, the apparent brain content of acetoacetyl-CoA thiolase was not found to change. In the preceding paper (Middleton, 1973) I reported that three types of oxoacyl-CoA thiolase exist in many tissues and that the apparent acetoacetyl-CoA thiolase activity of brain was due to two separate enzymes. The present paper investigates the changes in relative activities of these two acetoacetyl-CoA thiolases during the postnatal development of rat brain. A preliminary report of this work has appeared (Middleton, 1971).

Materials and Methods

Animals

As a routine, adult rats were fed on a normal commercial diet containing approx. 15% protein, 3% fat and 80% carbohydrate (Oxoid Ltd., London S.E.1, U.K.). Those on a high-fat diet were fed on beef suet for 3 days before use, their average weight gain being 10g/rat per day. Infant rats were kept with their mother until weaned at 28 days.

* Present address: The Chemical Laboratory, University of Cambridge, Cambridge CB2 1EW, U.K.

Preparation of brain extracts

Brains were homogenized for 2min in 5–10vol. of 0.05M-sodium phosphate buffer, pH7.2, containing 0.3% (w/v) sodium deoxycholate. This was carried out in a ground-glass homogenizer with a motor-driven glass pestle. The homogenate was then centrifuged at 100000*g* for 30min and the clear supernatant fraction retained for determination of enzyme activities. Contrary to the findings of Dierks-Ventling & Cone (1971*a*), I found the use of detergent essential for complete release of thiolase activity from both frozen-thawed and fresh brain. Deoxycholate at 0.3% (w/v) or Triton X-100 at 1% (w/v) were used. Higher concentrations of either gave no further increase in soluble thiolase activity. Neither 0.6% (w/v) deoxycholate nor 1.5% (w/v) Triton X-100 had any effect on the activity of purified thiolases. Although Triton X-100 at 0.5% (w/v) gave complete release of thiolase activity from other rat tissues (Middleton, 1973) and from brains of newborn and adult rats, the higher concentration (1%, w/v) was necessary for complete release of enzyme activity from brains of suckling rats.

Subcellular fractionation of brain

Rat brains were homogenized manually in a Dounce homogenizer in 10vol. of 0.3M-sucrose. After a preliminary low-speed spin (1000*g* for 10min) the crude mitochondrial fraction was sedimented at 10000*g* for 10min. Cytoplasm was prepared from the supernatant of the crude mitochondrial pellet by centrifugation at 100000*g* for 1h. The method of

Gray & Whittaker (1962), employing centrifugation through discontinuous sucrose-density gradients, was used to fractionate the crude mitochondrial fraction (obtained as described above) into a myelin-rich fraction, a synaptosomal fraction and purified mitochondria. Pure brain mitochondria were also prepared by the method of Clark & Nicklas (1970) by using a gradient of Ficoll (Pharmacia, Uppsala, Sweden).

Subcellular organelles and membranous structures were disrupted for enzyme assay by treatment with 0.5% (w/v) Triton X-100. Protein concentrations were determined by using the Folin–Ciocalteu reagent (Lowry *et al.*, 1951) with crystalline bovine serum albumin as the standard.

Chromatographic separation of oxoacyl-CoA thiolases

This was performed exactly as described in the preceding paper (Middleton, 1973). Particle-free extracts from brain were obtained after homogenizing with Triton X-100 at a final concentration of 1% (w/v). This gave as good extraction of thiolase activity as the deoxycholate used as a routine and, being non-ionic, avoided any chromatographic complications.

Determination of enzyme activities

Acetoacetyl-CoA thiolase was determined as previously described (Middleton, 1973) in media of carefully controlled cation composition. Activation by K^+ was determined in extracts that had been carefully freed from any K^+ by passage through Sephadex G-25 equilibrated with 10mM-sodium phosphate, pH7.8. The activation was expressed as the ratio of the rate of acetoacetyl-CoA thiolysis measured in a K^+-containing medium to that measured in a Na^+-containing medium. Citrate synthase activity (EC 4.1.3.7) was measured at 30°C by the assay of Srere *et al.* (1963). Occluded (bound) lactate dehydrogenase activity (EC 1.1.1.27) was measured as described by Johnson & Whittaker (1963).

Calculation of the content of individual acetoacetyl-CoA thiolases in brain extracts

The total thiolase activity of brain extracts when measured with acetoacetyl-CoA is entirely due to the sum of the activities of the mitochondrial and cytoplasmic acetoacetyl-CoA thiolases, there being virtually no general 3-oxoacyl-CoA thiolase detectable (Middleton, 1973). This preceding paper described a general method for calculating the tissue content of the mitochondrial acetoacetyl-CoA thiolase. The cytoplasmic enzyme content therefore can be obtained by subtracting the calculated mitochondrial acetoacetyl-CoA thiolase activity from the total acetoacetyl-CoA thiolase activity of brain.

Units of enzyme activity

A unit is defined as the amount of enzyme that transforms 1 μmol of substrate/min under the conditions of the assay. Activities in brain are expressed as units/g fresh wt. of tissue; specific activities are given as units/mg of soluble protein. Kinetic results expressed graphically have the velocities adjusted to correspond to the use of 10munits of enzyme per cuvette.

Results

Intracellular localization of brain acetoacetyl-CoA thiolases

The apparent 3-oxoacyl-CoA thiolase activities of rat brain subcellular fractions are shown in Table 1. The cytoplasmic fraction contains an acetoacetyl-CoA-specific thiolase unaffected by K^+. Particles sedimenting at 10000*g* and designated 'crude mitochondria' show acetoacetyl-CoA thiolase activity activated 3.2-fold by K^+. After purification by centrifugation on a Ficoll density gradient both the K^+ effect on the acetoacetyl-CoA thiolase and the enzyme's specific activity are enhanced. This suggests

Table 1. *Apparent 3-oxoacyl-CoA activities in adult rat brain subcellular fractions*

Fractions were prepared and assayed as described in the Materials and Methods section. Mitochondria were purified by the technique of Clark & Nicklas (1970). Abbreviations: C_4, acetoacetyl-CoA; C_6, 3-oxohexanoyl-CoA.

Fraction	Specific activity with different 3-oxoacyl-CoA substrates (nmol/min per mg)		Activation by K^+
	C_6	C_4	
Cytoplasm	<0.7	10.3	1.0
Extract of 'crude mitochondria'	3.0	44.3	3.2
Extract of purified mitochondria	8.0	130	4.1

Table 2. *Acetoacetyl-CoA thiolase localization in membranous fractions from adult rat brain*

Crude mitochondrial fraction (6ml of 20mg of protein/ml) in 0.32M-sucrose was separated by the density-gradient method of Gray & Whittaker (1962) into three particulate fractions enriched respectively in myelin, synaptosomes and mitochondria. These fractions were assayed for enzyme activity and protein as described in the Materials and Methods section. Enzyme distribution is expressed in terms of the relative specific activity:

$$\left(\frac{\%\text{ of total activity recovered in each fraction}}{\%\text{ of total protein recovered in each fraction}}\right)$$

The observed K^+ activation of acetoacetyl-CoA thiolase activity is given in parentheses.

	Relative specific activity in each fraction		
Enzyme activity	Myelin	Synaptosomes	Mitochondria
Occluded (bound) lactate dehydrogenase	0.23	2.3	0.72
Citrate synthase	0.06	0.72	3.0
Acetoacetyl-CoA thiolase	<0.02	0.55 (3.0)	3.3 (3.8)

that the K^+-activated acetoacetyl-CoA thiolase of brain is truly mitochondrial. To check that this is indeed so the 'crude mitochondria' were separated into a myelin-rich fraction, a synaptosomal fraction and mitochondria by the technique of Gray & Whittaker (1962). Citrate synthase was used as a mitochondrial marker and occluded (bound) lactate dehydrogenase (Johnson & Whittaker, 1963) as a synaptosomal marker. The relative specific activities (de Duve *et al.*, 1955) of these enzymes in the different fractions are shown in Table 2, values greater than unity expressing enrichment of the enzyme concerned in the fraction. It is clear that the K^+-activated acetoacetyl-CoA thiolase is mitochondrial and that contamination by entrapped cytoplasmic thiolase was responsible for the smaller effect of K^+ on the activity from 'crude mitochondria'.

It is noteworthy that thiolase activity with 3-oxohexanoyl-CoA as substrate was low in brain subcellular fractions, confirming previous findings (Middleton, 1973). The low thiolase activity with this longer-chain 3-oxoacyl-CoA substrate in brain mitochondria correlates well with the observed low rates of β-oxidation in these mitochondria (Clark & Nicklas, 1970).

Isolation of individual acetoacetyl-CoA thiolases from rat brain

Chromatography on DEAE-cellulose at pH8.2 separates the cytoplasmic thiolase from mitochondrial thiolases (Middleton, 1973) and a typical result for the chromatography of an extract of brain from newborn rats is shown in Fig. 1(*a*). Fig. 1(*b*) shows that the retarded peak of activity is due to the cytoplasmic thiolase. This technique gives quantitative recoveries of purified cytoplasmic acetoacetyl-CoA thiolase. The unretained enzyme activity is due to the mitochondrial acetoacetyl-CoA thiolase, as demonstrated

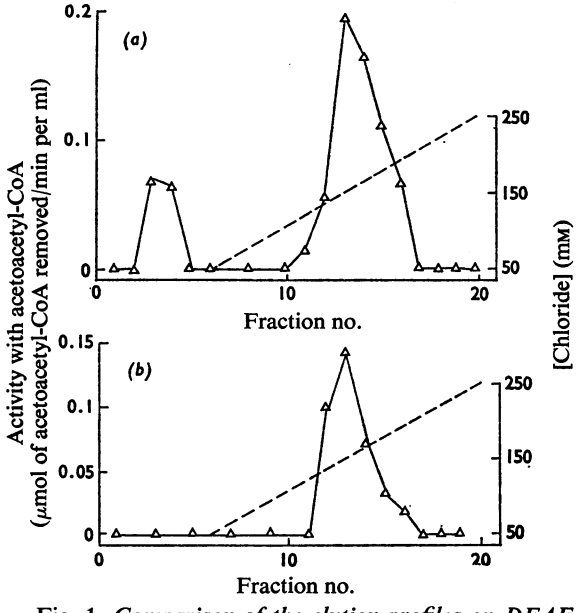

Fig. 1. *Comparison of the elution profiles on DEAE-cellulose of acetoacetyl-CoA thiolase activity from cytoplasm and whole-tissue extracts of brain*

In both cases the material from 1-day-old rats was applied to a column (5cm×2.5cm) of DEAE-cellulose at pH8.2 and eluted with a linear gradient of Tris–HCl. The fraction size was 8ml. The broken line (– –) represents the calculated chloride concentration in the eluate. Acetoacetyl-CoA thiolase activity (△) was measured under standard assay conditions. (*a*) Acetoacetyl-CoA thiolase elution profile from an extract of whole brain; 78mg of soluble protein applied; (*b*) acetoacetyl-CoA thiolase elution profile from rat brain cytoplasm; 64mg of protein applied. See the Materials and Methods section for further details.

by its 4-fold activation by K^+. This material can be further purified by chromatography on cellulose phosphate (Fig. 2). All the acetoacetyl-CoA thiolase activity is eluted as a single peak, which showed no detectable activity with longer-chain 3-oxoacyl-CoA substrates. Similar behaviour was observed on chromatography on DEAE-cellulose and cellulose phosphate of extracts of rat brains at all stages in development. The good recovery of acetoacetyl-CoA thiolase activity enables these chromatographic methods to be used for the direct determination of the activities of the two acetoacetyl-CoA thiolases in brain.

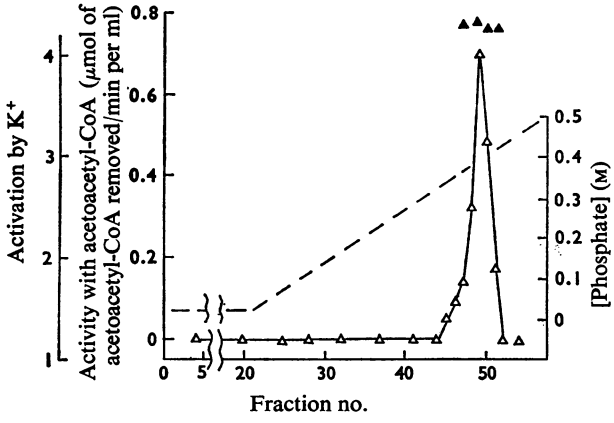

Fig. 2. *Chromatography of rat brain mitochondrial acetoacetyl-CoA thiolase on cellulose phosphate*

Acetoacetyl-CoA thiolase, extracted from brains of 23-day-old rats, was first chromatographed on DEAE-cellulose under the conditions of Fig. 1(*a*). The unretarded activity (260mg of protein) was then applied to a column (7cm×3cm) of cellulose phosphate at pH6.6 and eluted with a linear gradient of potassium phosphate. The fraction size was 8ml. The broken line (--) represents the calculated phosphate concentration in the eluate. Acetoacetyl-CoA thiolase activity (△) and the stimulation of this by K^+ (▲) were determined as described in the Materials and Methods section.

Some kinetic properties of the brain acetoacetyl-CoA thiolases

The substrate specificities and K^+-activation properties of the two brain thiolases have been reported and are similar to those of thiolases isolated from other rat tissues (Middleton, 1973). To extend the information available for comparison, the apparent K_m values for acetoacetyl-CoA and CoA and the apparent K_a for K^+ activation (of the mitochondrial enzyme) were determined under standard assay conditions (Table 3). The enzymes used for this study were purified as described above from extracts of brains of suckling rats, the age of the rat, however, had no effect on the kinetic parameters presented in Table 3. The effect of K^+ concentration on the activation of the mitochondrial acetoacetyl-CoA specific thiolase is shown in Fig. 3. The kinetic parameters given in Table 3 are close to those found for the rat liver enzymes (Middleton, 1973) and the similarity even extends to the high-substrate inhibition patterns (Figs. 4). Substrate inhibition by CoA occurs readily only with the cytoplasmic brain enzyme, whereas substrate inhibition by acetoacetyl-CoA readily occurs only with the mitochondrial acetoacetyl-CoA thiolase of brain. The existence of substrate inhibition by CoA for the cytoplasmic acetoacetyl-CoA thiolase and by acetoacetyl-CoA for the mitochondrial enzyme means that it is impossible to measure maximum tissue thiolase capacities by a simple assay. The concentrations of acetoacetyl-CoA and CoA used in the standard thiolase assay in this study are such that neither enzyme is grossly inhibited (Fig. 4) and, although maximum velocities are not attained, comparisons of relative tissue thiolase activities are perfectly valid.

Table 3. *Kinetic properties of acetoacetyl-CoA thiolases isolated from brains of suckling rats*

Acetoacetyl-CoA thiolases were isolated from brains of 23-day-old rats and purified as described in the Materials and Methods section. The apparent kinetic constants were determined under the standard assay conditions. For further details see the Materials and Methods section.

Enzyme	Apparent K_m values: Acetoacetyl-CoA (μM)	Apparent K_m values: CoA (μM)	Apparent K_a for activation by K^+ (mM)
Cytoplasmic acetoacetyl-CoA thiolase	23	5*	—
Mitochondrial acetoacetyl-CoA thiolase	11*	15	1.3

* Substrate inhibition occurs.

Changes in acetoacetyl-CoA thiolase activities during postnatal development

Fig. 5 shows the total brain acetoacetyl-CoA thiolase activity and its stimulation by K^+ as a function of the age of the rat. A significant rise in total activity soon after birth is accompanied by a doubling in the observed K^+ activation. Fig. 6 shows the resolution of this total activity into constituent mitochondrial and cytoplasmic thiolase activities. Results were obtained both by chromatographic isolation of the enzymes (open symbols) and by calculation (closed symbols) from the values in Fig. 5. The two acetoacetyl-CoA thiolases show quite different developmental patterns: the cytoplasmic enzyme activity is high at birth (4 units/g) and falls slowly to the adult value of 1.3 units/g; the mitochondrial thiolase rises rapidly after birth from an initial value of 1 unit/g to about 5 units/g at 20 days and then, after weaning, declines to the adult value of 2 units/g.

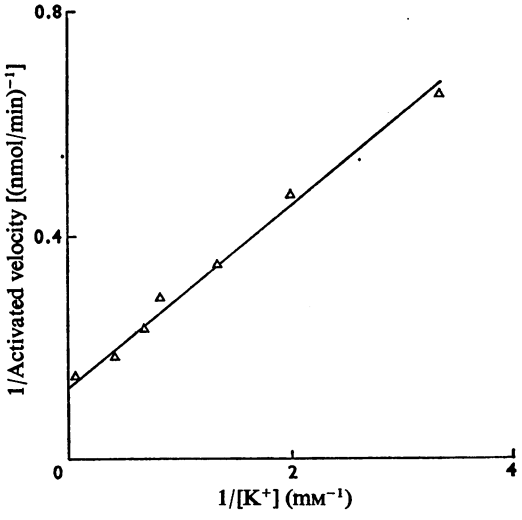

Fig. 3. *Effect of varying the concentration of K^+ on the activation of the suckling rat brain mitochondrial acetoacetyl-CoA thiolase*

The reciprocal of the activated velocity (velocity in the presence of K^+ minus the velocity in the absence of K^+) is expressed as a function of the reciprocal K^+ concentration. All other variables were kept constant at standard assay values (see the Materials and Methods section). The purified enzyme had a specific activity of 4.3 units/mg and was isolated from the brains of 23-day-old rats.

Effect of fat feeding on adult rat brain acetoacetyl-CoA thiolases

Although Dierks-Ventling & Cone (1971*b*) had shown no change in brain acetoacetyl-CoA thiolase activities while the rats were being fed on fat, their assay method could not discriminate between the two types of thiolase present. The effect of feeding fat was therefore reinvestigated using the K^+-activation of the total acetoacetyl-CoA thiolase activity to calculate the amounts of the two thiolases. After being starved for 24h adult male rats were fed for 3 days on a high-fat diet or on the normal balanced diet. The results (Table 4) show no significant difference between the groups in the total acetoacetyl-CoA thiolase or in the apparent activation by K^+. Therefore neither the absolute amounts nor the relative proportions of the two brain acetoacetyl-CoA thiolases are changed by feeding fat to the adult rat.

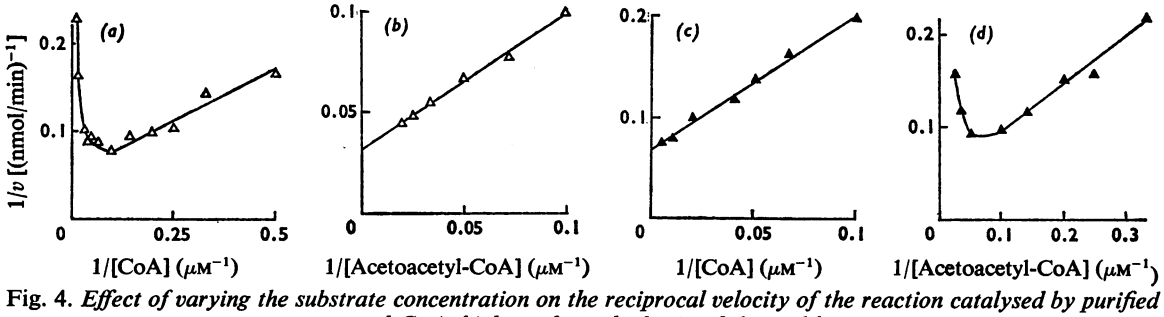

Fig. 4. *Effect of varying the substrate concentration on the reciprocal velocity of the reaction catalysed by purified acetoacetyl-CoA thiolases from the brain of the suckling rat*

The cytoplasmic acetoacetyl-CoA thiolase (△) had a specific activity of 1.1 units/mg, and mitochondrial acetoacetyl-CoA thiolase (▲) had a specific activity of 6.0 units/mg. The enzymes were purified from the brains of 23-day-old rats. (*a*) and (*c*), Reciprocal velocity as a function of reciprocal CoA concentration; acetoacetyl-CoA at 10μM. (*b*) and (*d*), Reciprocal velocity as a function of reciprocal acetoacetyl-CoA concentration; CoA at 50μM. Conditions, except for the variable substrate, were those of the standard assay (see the Materials and Methods section).

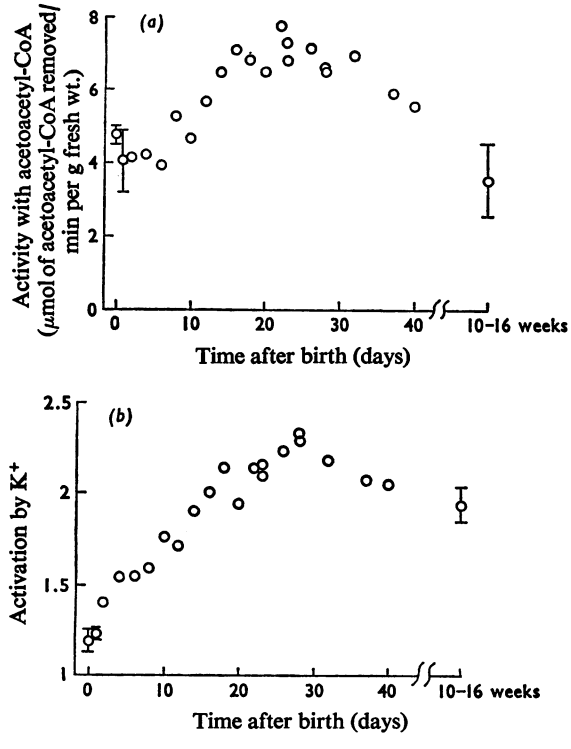

Fig. 5. *Effect of age on the total brain acetoacetyl-CoA thiolase activity and its activation by K^+*

(*a*) Total brain acetoacetyl-CoA thiolase activities; (*b*) apparent activation of the total acetoacetyl-CoA thiolase activity by K^+. Each point represents the mean value for two to ten rats from the same litter. The standard deviations of the mean values for 19 newborn rats (less than 12h after birth), nine 1-day-old rats and 30 adult rats are shown by the vertical bars. For further experimental details see the Materials and Methods section.

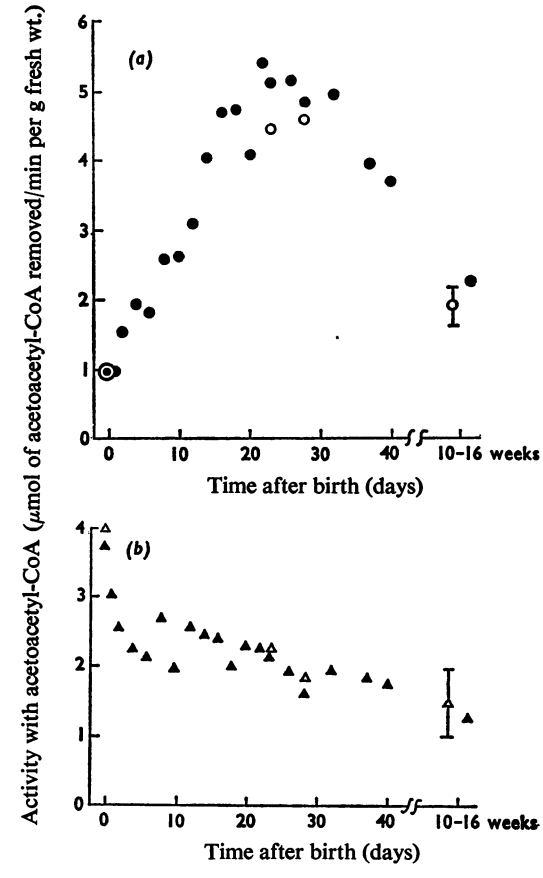

Fig. 6. *Changes in mitochondrial and cytoplasmic acetoacetyl-CoA thiolase activities in rat brain after birth*

(*a*) Mitochondrial acetoacetyl-CoA thiolase activity: ●, calculated from the results in Fig. 5; ○, experimentally determined by chromatographic separation. (*b*) Cytoplasmic acetoacetyl-CoA thiolase activity: ▲, calculated from the results in Fig. 5; △, experimentally determined by chromatographic separation. Experimentally determined points are the mean values from four to ten rats of the same litter. The standard deviations of the experimentally determined values from 11 adult rats are shown by the vertical bars. For further details see the Materials and Methods section.

Discussion

Although thiolase activity has been measured previously in brains of suckling rats (Lynen, 1957; Page *et al.*, 1971; Dierks-Ventling & Cone, 1971*a*) no evidence of any consistent changes has been reported. This failure is now seen to be due to the complications involved in attempting to measure acetoacetyl-CoA thiolase activity by simple assays. Thus, not only are there two acetoacetyl-CoA thiolases present in brain, but they are both substrate inhibited (by different substrates in each case) and further, one of the enzymes is greatly stimulated by K^+. The assays used by the above investigators always contained high enough concentrations of acetoacetyl-CoA to inhibit severely the mitochondrial acetoacetyl-CoA thiolase and thus completely mask the significant rise in total thiolase activity shown in Fig. 5. When the cytoplasmic and mitochondrial thiolase contributions to the total activity are separated, as in Fig. 6, the changes become even more significant.

The distinctive activity pattern of the mitochondrial thiolase closely resembles the patterns of postnatal development of enzymes of ketone-body utilization,

Table 4. *Brain acetoacetyl-CoA thiolase activities in normal and adult rats fed on fat*

The activities are expressed as μmol of acetoacetyl-CoA removed/min per g fresh wt. of brain and are means ±S.D. for four observations. Activation by K^+, determined as described in the Materials and Methods section, is expressed as the mean ±S.D. Individual cytoplasmic and mitochondrial acetoacetyl-CoA thiolase activities were calculated as described in the Materials and Methods section.

State of rats	Total acetoacetyl-CoA thiolase	Activation by K^+	Mitochondrial acetoacetyl-CoA thiolase	Cytoplasmic acetoacetyl-CoA thiolase
Normal	2.6±0.3	2.0±0.08	1.7	0.9
Fat-fed	2.3±0.4	2.0±0.1	1.5	0.8

3-hydroxybutyrate dehydrogenase (Klee & Sokoloff, 1967) and 3-oxo acid CoA-transferase (Page *et al.*, 1971; Tildon *et al.*, 1971). These enzymes are all located in the mitochondria of brain and the rapid increase in their activity during the 20 days after birth is associated with rapid increases in other mitochondrial enzymes such as cytochrome oxidase (EC 1.9.3.1; Klee & Sokoloff, 1967), glutamate dehydrogenase (EC 1.4.1.2; Page *et al.*, 1971) and citrate synthase (B. Middleton, unpublished work). However, these latter three enzymes retain the high activities reached after 20 days and no decline in tissue activity occurs on maturation. Therefore, although the initial activity rise may reflect a general increase in the brain mitochondrial protein per weight of tissue, the subsequent decrease in activities of 3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and mitochondrial acetoacetyl-CoA thiolase after weaning must represent a specific and co-ordinated control on the concentration of these enzymes within the mitochondrion. The results of the present investigation therefore strongly support the hypothesis (Middleton, 1973) that the mitochondrial K^+-activated acetoacetyl-CoA thiolase is concerned with the extrahepatic utilization of ketone bodies in animal tissues.

The co-ordinated decrease in the three enzymes of the ketone-body-utilization pathway after weaning may be triggered by the accompanying change in diet (Page *et al.*, 1971). Williamson *et al.* (1971) found no evidence for a reversal of this process when adult rats were starved, made alloxan-diabetic or fed on fat, the activities of 3-hydroxybutyrate dehydrogenase, 3-oxo acid CoA-transferase and total acetoacetyl-CoA thiolase remaining constant. As shown above, an analysis of the two constituent acetoacetyl-CoA thiolases of adult rat brain during fat-feeding shows that no change occurs in the relative proportions of mitochondrial and cytoplasmic enzymes or in their total. The ability of the activities of the enzymes of ketone-body utilization in the brain of suckling rats to alter according to the metabolic need does not appear to be carried through into the adult state.

In Fig. 6(*b*) is shown the change in the cytoplasmic acetoacetyl-CoA thiolase of brain after birth. Unlike the developmental patterns of the activities of the other enzymes described this shows a steady decrease throughout suckling to reach the low adult value. This behaviour is certainly consistent with the role, proposed in the preceding paper (Middleton, 1973), involving cytoplasmic acetoacetyl-CoA thiolase in the first step of the pathway for sterol synthesis *de novo*. It is known (Srere *et al.*, 1950) that the rate of this process is very high in the brain of the newborn rat and rapidly diminishes after birth.

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