Acetoacetate Metabolism in Rat Brain

DEVELOPMENT OF ACETOACETYL-COENZYME A DEACYLASE AND 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A SYNTHASE

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1. Data are provided that indicate that the rat brain acetoacetyl-CoA deacylase is almost exclusively mitochondrial. Developmental studies show that this enzyme more than doubles its activity during suckling (0–21 days) and then maintains this activity in adults (approx. 1.1 units/g wet wt.). 2. Kinetic studies (on the acetoacetyl-CoA deacylase) in a purified brain mitochondrial preparation give a $V_{\max}$ of 47 nmol/min per mg of protein, and a K_m for acetoacetyl-CoA of $5.2 \mu\text{M}$ and are compatible with substrate inhibition by acetoacetyl-CoA above concentrations of $47 \mu\text{M}$. 3. The total brain 3-hydroxy-3-methylglutaryl-CoA synthase remains constant in the developing and adult rat brain (approx. 1.2 units/g wet wt.). This enzyme is located in both the mitochondrial and cytosolic fractions. During suckling (0–21 days) the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase represents approx. one-third of the total, but this increases markedly to about 60% of the total in the adult. The cytosolic enzyme correspondingly falls to approx. 40% of the total. 4. The role of the acetoacetyl-CoA deacylase in providing cytosolic acetoacetate for biosynthetic activities in the developing brain is discussed.

Ketone bodies are important substrates for the neonatal mammalian brain (Hawkins *et al.*, 1971; Cremer & Heath, 1974), and this is reflected in the relatively high activities of the enzymes of ketone-body metabolism in the brains of young suckling mammals (Page *et al.*, 1971; Middleton, 1973; Buckley & Williamson, 1973). It has been shown that the ketone bodies are important, not only as energy fuels (Itoh & Quastel, 1970; Page *et al.*, 1971; Hawkins *et al.*, 1971), but also as precursors for lipids and possibly transmitters (acetylcholine) (Edmond, 1974; Patel & Owen, 1976, 1977). The major proportion of the steady-state concentrations of the ketone bodies exist as 3-hydroxybutyrate (Hawkins *et al.*, 1971), but the route of incorporation of carbon into lipids and transmitters from 3-hydroxybutyrate is via acetoacetate and acetyl-CoA. Since the 3-hydroxybutyrate dehydrogenase, which is responsible for converting 3-hydroxybutyrate into acetoacetate, is exclusively mitochondrial, this has led to considerable interest in the mechanisms by which intracellular acetoacetate concentrations are regulated, and in particular how the mitochondrial/cytosolic compartments interact to provide carbon from 3-hydroxybutyrate for cytosolic biosynthetic activities, e.g. lipid synthesis. However, Buckley & Williamson (1973) have reported the existence of a cytosolic acetoacetyl-CoA synthetase (EC 6.2.1.2; butyryl-CoA synthetase), which together with the cytosolic acetoacetyl-CoA thiolase may provide

cytosolic acetyl-CoA from acetoacetate without mitochondrial involvement.

Two main pathways for cytosolic acetoacetate production have been described in liver: (a) the formation of 3-hydroxy-3-methylglutaryl-CoA by 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) followed by its breakdown to acetoacetate and acetyl-CoA by the 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) (Lynen *et al.*, 1958), and (b) the deacylation of acetoacetyl-CoA by the acetoacetyl-CoA deacylase (Stern & Miller, 1959; Sauer & Erfle, 1966; Williamson *et al.*, 1968). The first of these two pathways is not significant in brain, owing to the absence of the 3-hydroxy-3-methylglutaryl-CoA lyase (Bachhawat *et al.*, 1955), and the presence of the second (until the present report) has not been verified.

This present paper reports on the developmental pattern and subcellular distribution of the acetoacetyl-CoA deacylase and 3-hydroxy-3-methylglutaryl-CoA synthase in rat brain as part of a study on the availability of acetoacetate in the cytosol for synthetic activities in the developing brain.

Materials and Methods

Reagents

Substrates (except for acetyl-CoA and acetoacetyl-CoA), nucleotides and enzymes were purchased

either from Boehringer Corp., Lewes, Sussex BN7 1LG, U.K., or Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Diketen, for the synthesis of acetoacetyl-CoA, was obtained from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K., and twice distilled before use, 5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was obtained from BDH Chemicals, Poole, Dorset, U.K. All other reagents were of the highest purity available. Acetyl-CoA was prepared by the method of Ochoa (1955), and acetoacetyl-CoA by the method of Wieland & Rueff (1953). Ficoll was obtained from Pharmacia (Uppsala, Sweden) and purified by dialysis against glass-distilled water. Pyruvic acid was redistilled and stored at -20°C .

Animals

(a) *Adult*. Male rats of the Wistar strain (150–180 g body wt.) were used. They were fed *ad libitum* on laboratory diet no. 1 (Spratts, Reading, Berks., U.K.) and drinking water was always available.

(b) *Young animals*. The birth dates of all litters were carefully recorded after daily inspection and litters were culled to eight to ten pups. Animals of either sex were used up to weaning (21 days). After this age only male rats were used.

Subcellular fractionation

This was carried out essentially as described by Lai *et al.* (1977). Adult animals were killed by decapitation and the forebrains removed, minced and homogenized in the isolation medium [0.32M-sucrose, 1mM-EDTA (potassium salt), 10mM-Tris/HCl, pH 7.4] to yield a 10% (w/v) homogenate (A). This was then centrifuged in an MSE HS 18 centrifuge at 4°C at 2000g for 3min to yield a nuclear pellet (B), and a supernatant that on further centrifugation (15000g_{av.} for 10min) yielded a crude mitochondrial fraction (C) and a postmitochondrial fraction. The latter fraction was further centrifuged in an MSE SS 50 instrument at 99000g_{av.} for 1h to yield a microsomal pellet (D) and a cytosolic fraction (E).

The crude mitochondrial fraction (C) was further fractionated by centrifugation (MSE SS 50) on a 7.5%/10% Ficoll/sucrose gradient (Lai *et al.*, 1977) at 99000g for 30min. The myelin fraction was removed from the top of the gradient (isolation medium/7.5% Ficoll interphase), washed and re-centrifuged (25000g for 10min) to yield a myelin pellet (F). The synaptosomal fraction was removed from the 7.5% Ficoll/10% Ficoll interphase, washed and re-centrifuged (5500g for 10min) to yield a synaptosomal pellet (G). The 'free' mitochondrial pellet at the bottom of the tube was washed, resuspended and re-centrifuged (11500g for 10min) to yield a free mitochondrial pellet (H).

All the pellets of the various fractions were resuspended in the isolation medium to a concentration of about 15mg of protein/ml. Rat brain 'free' mitochondria, for studying the kinetics of acetoacetyl-CoA deacylase, were prepared by the method of Clark & Nicklas (1970).

Enzyme assays

All assays were carried out at 25°C . 3-Hydroxy-3-methylglutaryl-CoA synthase was measured essentially by the method of Clinkenbeard *et al.* (1975). The assay mixture contained the following, in a final volume of 1ml: glycylglycine (sodium salt), pH 8.8, 100mM; EDTA, 0.1mM; acetyl-CoA, 0.2mM; acetoacetyl-CoA, 50 μM ; Triton X-100, 0.2%, v/v; and up to 2mg of protein to be assayed. The disappearance of acetoacetyl-CoA was monitored by using an Aminco Chance spectrophotometer, on a dual-wavelength mode at 300nm, with 373nm as the reference wavelength. Under these conditions the molar absorption coefficient of acetoacetyl-CoA is equal to $7.8 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. 3-Hydroxy-3-methylglutaryl-CoA synthase activity is equal to half the rate of acetoacetyl-CoA consumption for preparations (e.g. mitochondrial or cytoplasmic extracts) in which acetoacetyl-CoA thiolase activity is greater than 3-hydroxy-3-methylglutaryl-CoA synthase activity (Clinkenbeard *et al.*, 1975). This correction has been made for the CoA-dependent acetoacetyl-CoA disappearance due to thiolase activity.

To establish the validity of using the factor of one-half for the calculation of the 3-hydroxy-3-methylglutaryl-CoA synthase activity, this enzyme was assayed by an alternative method in both homogenates and mitochondria derived from rat brain. The assay was based on the measurement of 3-hydroxy-3-methylglutaryl-CoA produced (see reaction 1) at timed intervals rather than measurement of acetoacetyl-CoA disappearance, which is the basis of the Clinkenbeard *et al.* (1975) assay. This is possible in rat brain preparations, since 3-hydroxy-3-methylglutaryl-CoA lyase is absent (Bachhawat *et al.*, 1955; T. B. Patel & J. B. Clark, unpublished work). The 3-hydroxy-3-methylglutaryl-CoA thus generated is then measured by a linked enzyme assay as follows (reactions 2, 3 and 4):

Acetoacetyl-CoA + acetyl-CoA

3-Hydroxy-3-methylglutaryl-CoA synthase

3-Hydroxy-3-methylglutaryl-CoA

3-Hydroxy-3-methylglutaryl-CoA lyase

Portions (1 ml) of the assay mixture for the 3-hydroxy-3-methylglutaryl-CoA synthase (see above) were removed at timed intervals and transferred to tubes containing 50 μl of 6M-HCl, and vortex-mixed to stop the reaction. The samples were neutralized with 3M-K₂CO₃ in 3M-triethanolamine, and 500 μl was then mixed in a cuvette with the following (final concns. in 1 ml); 200mM-Tris/HCl, pH 8.1; 5mM-glutathione (reduced); 5mM-MgCl₂; 0.3mM-NAD⁺; 125 μg (150 units) of malate dehydrogenase (EC 1.1.1.37); 50 μg (5.5 units) of citrate synthase (EC 4.1.3.7). The reaction mixture was incubated for 25 min until all the endogenous acetyl-CoA in the extract had been converted into citrate and a steady baseline was recorded on a dual-wavelength spectrophotometer at 340nm versus 373nm. The 3-hydroxy-3-methylglutaryl-CoA was then measured by the addition of a portion (20 μl ; approx. 1 unit) of 3-hydroxy-3-methylglutaryl-CoA lyase (see below). One unit of activity, both here and elsewhere, is the amount of enzyme required to convert 1 μmol of substrate/min. This reaction caused a stoicheiometric release of acetyl-CoA from the 3-hydroxy-3-methylglutaryl-CoA, which was recorded as a stoicheiometric increase in NADH by the linked enzyme assay recorded at 340nm versus 373nm.

Preparation of 3-hydroxy-3-methylglutaryl-CoA lyase

The enzyme was prepared from rat liver by the method of Stegink & Coon (1968) up to the (NH₄)₂SO₄ precipitation stage. This precipitate was dissolved in 0.1M-potassium phosphate, pH 8.1, containing 1mM-2-mercaptoethanol and kept deep-frozen.

Fig. 1 shows the results obtained by using a rat brain mitochondrial preparation in which the activity of the 3-hydroxy-3-methylglutaryl-CoA synthase has been measured by acetoacetyl-CoA disappearance measured by the method of Clinkenbeard *et al.* (1975) involving the factor of one-half (open symbols), and secondly by measuring the appearance of 3-hydroxy-3-methylglutaryl-CoA by the linked enzyme assay reported here (closed symbols). It is clear that there is a close correlation between the two methods that justifies the use of the factor of one-half in the Clinkenbeard *et al.* (1975) method. Similar correlations were also obtained with rat brain homogenates. By using the 3-hydroxy-3-methylglutaryl-CoA lyase method to measure the 3-hydroxy-3-methylglutaryl-CoA synthase the following specific activities (means

Fig. 1. Measurement of 3-hydroxy-3-methylglutaryl-CoA synthase

Rat brain mitochondria were prepared by the method of Clark & Nicklas (1970). The 3-hydroxy-3-methylglutaryl-CoA synthase activity was measured at two protein concentrations (○, ●, 0.307mg of mitochondrial protein/ml; ▲, △, 0.615mg of mitochondrial protein/ml) under the conditions outlined in the Materials and Methods section (Clinkenbeard *et al.*, 1975). The enzyme activity was assayed either by acetoacetyl-CoA removal by measuring continuously on a dual-wavelength spectrophotometer at 300nm versus 373nm and dividing by 2 (○, △; cf. Clinkenbeard *et al.*, 1975) or by stopping the reaction and measuring the 3-hydroxy-3-methylglutaryl-CoA by adding 3-hydroxy-3-methylglutaryl-CoA lyase in conjunction with the linked enzyme system (see the Materials and Methods section) and measuring the increased NADH production at 340nm versus 373nm (●, ▲).

±s.d., $n=3$) were obtained: rat brain homogenate (adult) 4.8 ± 0.3 nmol/min per mg of protein and 1.17 ± 0.07 $\mu\text{mol/min}$ per g wet wt. These values correlate well with those obtained by using the Clinkenbeard *et al.* (1975) method involving the factor of $\frac{1}{2}$ (see Tables 1 and 2). This would therefore justify the claim by Clinkenbeard *et al.* (1975) that for crude preparations of the 3-hydroxy-3-methylglutaryl-CoA synthase containing acetoacetyl-CoA thiolase, provided that the activity of the latter enzyme is in excess of the former, then the activity of the 3-hydroxy-3-methylglutaryl-CoA synthase is equal to $\frac{1}{2}$ of the rate of acetoacetyl-CoA disappearance.

Acetoacetyl-CoA deacylase was assayed by the method of Williamson *et al.* (1968). The rate of disappearance of acetoacetyl-CoA in the presence of iodoacetamide was monitored at 303nm, by using 373nm as the reference wavelength on a dual-wavelength mode as above. Under these conditions, acetoacetyl-CoA has a molar absorption coefficient of 20.5×10^{-3} litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹.

Table 1. *Subcellular distribution of acetoacetyl-CoA deacylase and 3-hydroxy-3-methylglutaryl-CoA synthase*

The fractions were isolated and enzymes were assayed as described in the Materials and Methods section. Results are expressed as means $\pm$ s.d. for three separate fractionation studies, in each of which fractions were derived from six to eight rats and each enzyme determination was carried out at least in duplicate. Relative specific activities were calculated by determining the ratio of the specific activity of the fraction to that of the homogenate. Recoveries for all enzymes for the basic fractionation (e.g. fractions B-E inclusive) were in excess of 80%.

Enzyme	Fraction ...	Homogenate (A)	Nuclear (B)	Crude mitochondria (C)	Microsomal (D)	Cytosol (E)	Myelin (F)	Synaptosomes (G)	Free mitochondria (H)
3-Hydroxy-3-methylglutaryl-CoA synthase	Total protein (mg)	1183 $\pm$ 36	553 $\pm$ 22	275 $\pm$ 19	76 $\pm$ 8	240 $\pm$ 11	33 $\pm$ 5	96 $\pm$ 5	9.5 $\pm$ 0.9
	% of total activity in homogenate	100	35.4 $\pm$ 3.4	39.8 $\pm$ 3	3.3 $\pm$ 0.2	13.8 $\pm$ 1.2	0.6 $\pm$ 0.04	11.0 $\pm$ 0.8	2.6 $\pm$ 0.2
	Specific activity (nmol/min per mg of protein)	5.3 $\pm$ 0.4	4.0 $\pm$ 0.3	9.1 $\pm$ 0.2	2.7 $\pm$ 0.07	3.6 $\pm$ 0.2	1.2 $\pm$ 0.1	7.2 $\pm$ 0.1	17.7 $\pm$ 1.1
	Relative specific activity	1	0.76	1.7	0.5	0.67	0.2	1.35	3.3
Acetoacetyl-CoA deacylase	% of total activity in homogenate	100	32.9 $\pm$ 1.7	43.0 $\pm$ 3.6	1.9 $\pm$ 0.1	2.5 $\pm$ 0.1	0.25 $\pm$ 0.02	17.3 $\pm$ 1.3	5.7 $\pm$ 0.4
	Specific activity (nmol/min per mg of protein)	5.3 $\pm$ 0.4	4.2 $\pm$ 0.2	9.8 $\pm$ 0.4	1.6 $\pm$ 0.06	0.7 $\pm$ 0.02	0.5 $\pm$ 0.02	11.3 $\pm$ 1	38.5 $\pm$ 1.6
	Relative specific activity	1	0.8	1.84	0.3	0.12	0.09	2.1	7.3
	% of total activity in homogenate	100	34.1 $\pm$ 3.6	44.1 $\pm$ 0.5	3.2 $\pm$ 0.2	1.1 $\pm$ 0.1	0.27 $\pm$ 0.06	17.8 $\pm$ 1.1	5.6 $\pm$ 0.4
Citrate synthase	Specific activity (nmol/min per mg of protein)	145 $\pm$ 4	106 $\pm$ 5	275 $\pm$ 16	71 $\pm$ 8	7.3 $\pm$ 0.8	15.1 $\pm$ 0.3	319 $\pm$ 10	1017 $\pm$ 32
	Relative specific activity	1	0.73	1.9	0.5	0.05	0.1	2.2	7.0
	% of total activity in homogenate	100	25.4 $\pm$ 0.7	14.6 $\pm$ 1.9	4.0 $\pm$ 0.4	39.7 $\pm$ 3.2	0.85 $\pm$ 0.2	8.1 $\pm$ 0.6	0.6 $\pm$ 0.03
	Specific activity (nmol/min per mg of protein)	348 $\pm$ 3	189 $\pm$ 7	217 $\pm$ 4	213 $\pm$ 11	702 $\pm$ 20	104 $\pm$ 2.8	323 $\pm$ 7	271 $\pm$ 20
Lactate dehydrogenase	Relative specific activity	1	0.54	0.62	0.61	2.0	0.3	0.93	0.78
	% of total activity in homogenate	100	38.0 $\pm$ 1.2	30.2 $\pm$ 2.8	23.6 $\pm$ 1.7	3.8 $\pm$ 0.3	1.4 $\pm$ 0.1	9.4 $\pm$ 0.7	1.5 $\pm$ 0.3
	Specific activity (nmol/min per mg of protein)	3.5 $\pm$ 0.3	2.8 $\pm$ 0.2	4.5 $\pm$ 0.1	13.3 $\pm$ 1.1	0.61 $\pm$ 0.04	1.9 $\pm$ 0.1	4.1 $\pm$ 0.4	6.5 $\pm$ 0.3
	Relative specific activity	1	0.8	1.3	3.7	0.17	0.53	1.2	1.83
NADPH-cytochrome c reductase (rotenone-insensitive)	% of total activity in homogenate	100	33.1 $\pm$ 0.2	34.2 $\pm$ 1.8	18.7 $\pm$ 0.3	4.2 $\pm$ 0.2	1.7 $\pm$ 0.2	12.4 $\pm$ 1.1	3.2 $\pm$ 0.3
	Specific activity (nmol/min per mg of protein)	52.2 $\pm$ 0.7	37.1 $\pm$ 0.7	76.8 $\pm$ 1.4	149 $\pm$ 4.2	11.1 $\pm$ 0.3	32.2 $\pm$ 1.6	77.5 $\pm$ 5.7	21.6 $\pm$ 0.2
	Relative specific activity	1	0.7	1.5	2.85	0.2	0.62	1.48	0.41
	% of total activity in homogenate	100	33.1 $\pm$ 0.2	34.2 $\pm$ 1.8	18.7 $\pm$ 0.3	4.2 $\pm$ 0.2	1.7 $\pm$ 0.2	12.4 $\pm$ 1.1	3.2 $\pm$ 0.3
Acetylcholinesterase	Specific activity (nmol/min per mg of protein)	52.2 $\pm$ 0.7	37.1 $\pm$ 0.7	76.8 $\pm$ 1.4	149 $\pm$ 4.2	11.1 $\pm$ 0.3	32.2 $\pm$ 1.6	77.5 $\pm$ 5.7	21.6 $\pm$ 0.2
	Relative specific activity	1	0.7	1.5	2.85	0.2	0.62	1.48	0.41
	% of total activity in homogenate	100	33.1 $\pm$ 0.2	34.2 $\pm$ 1.8	18.7 $\pm$ 0.3	4.2 $\pm$ 0.2	1.7 $\pm$ 0.2	12.4 $\pm$ 1.1	3.2 $\pm$ 0.3
	Specific activity (nmol/min per mg of protein)	52.2 $\pm$ 0.7	37.1 $\pm$ 0.7	76.8 $\pm$ 1.4	149 $\pm$ 4.2	11.1 $\pm$ 0.3	32.2 $\pm$ 1.6	77.5 $\pm$ 5.7	21.6 $\pm$ 0.2

Citrate synthase (EC 4.1.3.7) was measured by the method of Coore *et al.* (1971). Lactate dehydrogenase (EC 1.1.1.27) was assayed as described by Clark & Nicklas (1970). Acetylcholinesterase (EC 3.1.1.7) was measured in the various fractions under the conditions described by Ellman *et al.* (1961).

NADPH-cytochrome *c* reductase (rotenone-insensitive) (EC 1.6.99.3) activity was determined essentially by the method of Duncan & Mackler (1966).

3-Hydroxy-3-methylglutaryl-CoA lyase was assayed by the method of Stegink & Coon (1968).

Proteins were determined by the method of Gornall *et al.* (1949).

Results

Table 1 shows the results of a study of the subcellular distribution of the acetoacetyl-CoA deacylase and 3-hydroxy-3-methylglutaryl-CoA synthase in rat brain. Also included in Table 1 are results of the distribution of citrate synthase (a mitochondrial marker), lactate dehydrogenase (a cytosolic marker), the rotenone-insensitive NADPH-cytochrome *c* reductase (a microsomal marker) and acetylcholinesterase (a synaptic-membrane marker). Results are means $\pm$ s.d. of three separate distribution studies in which the enzyme recoveries ($\Sigma = B + C + D + E$) were in excess of 80%. The data are expressed in three different ways to aid analysis: (a) as a percentage of total homogenate activity; (b) as a specific activity in nmol/min per mg of protein and (c) as a relative specific activity by taking the homogenate value as unity.

Preliminary analysis indicates in all cases substantial quantities of enzyme activity associated with the nuclear fraction. However, since this is an unwashed nuclear fraction this is most likely due to contamination from unbroken cells and other subcellular fractions.

Further analysis of the acetoacetyl-CoA deacylase data shows that this enzyme has an increased relative specific activity in the crude mitochondrial (C) and synaptosomal (G) fractions, and a particularly high relative specific activity in the free mitochondrial (H) fraction. This follows closely in both actual values and distribution the pattern of the citrate synthase relative specific activities, suggesting therefore a predominantly mitochondrial location for the acetoacetyl-CoA deacylase in rat brain.

The distribution data on the 3-hydroxy-3-methylglutaryl-CoA synthase suggest, however, a bimodal distribution. Although the highest relative specific activity (3.3) is present in the free mitochondrial fraction (H), suggesting a mitochondrial location, it is clear that a considerable proportion of the activity (14%) also resides in the cytosolic fraction (E),

indicating a dual localization of the 3-hydroxy-3-methylglutaryl-CoA synthase activity.

Fig. 2 shows the developmental pattern of the acetoacetyl-CoA deacylase in rat brain, which indicates an increase in activity of more than 2-fold during the period 5–21 days, which is maintained after weaning and into adulthood. This pattern of development is different from that of other enzymes involved in ketone-body metabolism, cf. 3-hydroxybutyrate dehydrogenase or 3-oxo acid CoA-transferase (Page *et al.*, 1971), where a marked fall in activity occurs after weaning. It is, however, a similar pattern of development to that described for the acetyl-CoA synthetase in rat brain cytosol by Buckley & Williamson (1973).

Fig. 3 represents the developmental pattern of the 3-hydroxy-3-methylglutaryl-CoA synthase. The total brain enzyme activity remains essentially constant from 5 days until adulthood at a value of about 1.2 μ mol/min per g wet wt. However, if the cytosolic activity itself is studied there is a marked decline in activity after weaning as the animal gets older, which

Fig. 2. Developmental pattern of total brain acetoacetyl-CoA deacylase

The total acetoacetyl-CoA deacylase activity was measured (see the Materials and Methods section) in homogenates of brains of different ages. The results are expressed as nmol/min per g wet wt., and are the means of at least three distinct experiments in each of which the homogenate was a pooled preparation from at least four rats and the enzyme activity was measured in duplicate at least. The s.d. in all cases were less than 6%.

Fig. 3. Developmental pattern of 3-hydroxy-3-methylglutaryl-CoA synthase

The total activity (●) of the 3-hydroxy-3-methylglutaryl-CoA synthase was determined in homogenates of brains of rats of different ages, as outlined in the Materials and Methods section. The cytosolic activity (▲) was measured in the cytosolic fraction (E) prepared as outlined in the Materials and Methods section. All activities are expressed as $\mu\text{mol}/\text{min}$ per g wet wt., and represent the means for at least two distinct experiments in each of which the homogenate/cytosol was derived from a pooled preparation from at least four rats and the enzyme activity was measured in duplicate at least. The standard deviations were in all cases less than 7%. The cytosolic enzyme activity was calculated on a whole-tissue basis by using the recovery of lactate dehydrogenase as a measure of the cytosolic recovery, the lactate dehydrogenase being measured alongside the 3-hydroxy-3-methylglutaryl-CoA synthase.

means that the mitochondrial enzyme (total minus cytosolic) increases from a value of approx. 33% of the total activity during suckling to 55–60% in the adult rat brain. The pattern of the decline in activity of the cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase during brain maturation follows closely that of the cytosolic acetoacetyl-CoA thiolase (Middleton, 1973) and also that of the rate of cholesterol synthesis in the brain (Davison, 1970). The total adult rat brain 3-hydroxy-3-methylglutaryl-CoA synthase activity reported here (approx. 1.2 units/g wet wt.) is approx. 15 times higher than that reported by McGarry & Foster (1969), which may reflect the indirect assay method used by the latter authors.

More detailed analysis of the kinetic parameters of the adult rat brain acetoacetyl-CoA deacylase (Fig. 4) gave an apparent K_m for acetoacetyl-CoA of

Fig. 4. Lineweaver-Burk plot for acetoacetyl-CoA deacylase

Rat brain mitochondria were prepared by the method of Clark & Nicklas (1970) from adult rats. The kinetics were studied at 25°C by the method of Williamson *et al.* (1968). The Michaelis parameters were computed by extrapolation, yielding a V_{max} of 47 nmol/min per mg of mitochondrial protein and a K_m for acetoacetyl-CoA of 5.2 μM .

5.2 μM with a V_{max} of 47 nmol/min per mg of mitochondrial protein. The kinetics were also compatible with substrate (acetoacetyl-CoA) inhibition of the deacylase activity at acetoacetyl-CoA concentrations in excess of 47 μM .

Discussion

The subcellular-distribution data provide good evidence for the localization of the rat brain acetoacetyl-CoA deacylase in the mitochondrial compartment (Table 1). This is in contrast with the liver, where Williamson *et al.* (1968) have reported that the enzyme is cytosolic. Although both the liver and brain enzymes have similar apparent K_m values for acetoacetyl-CoA (10 and 5 μM respectively), the adult brain has about 3 times the activity of the adult liver on a whole-tissue basis [1.12 ± 0.03 ($n=3$) units/g (Table 2): 0.4 unit/g (Williamson *et al.*, 1968)].

Brain, unlike liver, is a non-ketogenic tissue, as reflected by the absence of measurable 3-hydroxy-3-methylglutaryl-CoA lyase (Bachhawat *et al.*, 1955; T. B. Patel & J. B. Clark, unpublished work). However, the observations of Patel & Owen (1977) have suggested that ketone bodies in addition to glucose may be precursors for lipid synthesis, particularly in suckling animals, when lipid synthesis in the brain is maximal (6–8 days). What is at issue is the pathway by which the carbon from ketone bodies becomes incorporated into the lipid moieties, particularly in view of the low activity of the ATP citrate lyase in brain (D'Adamo & D'Adamo, 1968). Buckley &

Williamson (1973), however, have indicated that acetoacetate may be incorporated directly into cytosolic lipid synthesis in the brain by means of the acetoacetyl-CoA synthetase, which together with the cytosolic acetoacetyl-CoA thiolase (Middleton, 1973) may produce cytosolic acetyl-CoA for incorporation into lipid.

The importance of the mitochondrial acetoacetyl-CoA deacylase may be seen therefore in its role in providing additional cytosolic acetoacetate from mitochondrial 3-hydroxybutyrate. Table 2 outlines the relative activities of all the enzymes involved in acetoacetate metabolism in brain of suckling and adult rats. It is clear, particularly in the brain of suckling rats, that the enzymes involved in converting 3-hydroxybutyrate into acetyl-CoA in the mitochondria are present in activities that are much greater than those enzymes concerned with converting acetoacetyl-CoA either into 3-hydroxy-3-methylglutaryl-CoA or back into acetoacetate. However, should acetoacetyl-CoA become available over and above the requirements of the mitochondrial acetoacetyl-CoA thiolase, then this may be converted back into acetoacetate by the deacylase, the acetoacetate transported out of the mitochondria on the monocarboxylate transporter (Patel *et al.*, 1977) and become available for cytosolic synthetic activities via the cytosolic acetoacetyl-CoA synthetase (Buckley & Williamson, 1973). Recent evidence (Patel & Clark, 1977) supports this hypothesis in that brain mito-

chondria from suckling rats, utilizing 3-hydroxybutyrate as a substrate, transport acetoacetate outwards at a faster rate than citrate. Thus the presence of the mitochondrial acetoacetyl-CoA deacylase permits the proposal of an alternative/additional mechanism for the transport of carbon across the brain mitochondrial membrane to that involving citrate transport.

The developmental pattern of the cytosolic brain 3-hydroxy-3-methylglutaryl-CoA synthase follows closely that of the acetoacetyl-CoA thiolase (Middleton, 1973) in that the enzyme activity decreases after suckling. This may relate to the decreasing requirement for cholesterol synthesis as the brain matures (Davison, 1970).

However, the explanation for the presence of, and increase in activity with age of, the mitochondrially located 3-hydroxy-3-methylglutaryl-CoA synthase in rat brain is less clear. Preliminary reports indicate the presence of a 'particulate' 3-hydroxy-3-methylglutaryl-CoA reductase in rat brain (Sudjic & Booth, 1976), probably in the mitochondrial fraction (T. B. Patel & J. B. Clark, unpublished work). This suggests that the brain mitochondria are capable of producing mevalonic acid and possibly related products.

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Table 2. *Enzymes involved in the production and utilization of acetoacetate in rat brain*

The enzyme activities are expressed in units/g wet wt. and where indicated are means $\pm$ S.D. for at least four separate determinations.

Enzyme	Intracellular location	Maximal activity (units/g wet wt.)		Reference
		21-day-old	Adult	
3-Hydroxybutyrate dehydrogenase	Mitochondrial	1.7	0.58	Page <i>et al.</i> (1971)
		1.44 ± 0.06	0.5 ± 0.03	T. B. Patel & J. B. Clark (unpublished work)
3-Oxo acid CoA-transferase	Mitochondrial	6.0	2.5	Page <i>et al.</i> (1971)
		7.1 ± 0.2	3.0 ± 0.1	T. B. Patel & J. B. Clark (unpublished work)
Acetoacetyl-CoA thiolase	Mitochondrial	5.0	2.0	Middleton (1973)
		5.5 ± 0.3	2.1 ± 0.1	T. B. Patel & J. B. Clark (unpublished work)
	Cytosolic	2.2	1.3	Middleton (1973)
		2 ± 0.1	1.4 ± 0.08	T. B. Patel & J. B. Clark (unpublished work)
3-Hydroxy-3-methylglutaryl-CoA synthase	Mitochondrial	0.4 ± 0.03	0.7 ± 0.05	The present paper
	Cytosolic	0.86 ± 0.07	0.49 ± 0.03	The present paper
Acetoacetyl-CoA deacylase	Mitochondrial	1.08 ± 0.01	1.12 ± 0.03	The present paper
Acetoacetyl-CoA synthetase	Cytosolic	0.017	0.007	Buckley & Williamson (1973)

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