Regional enzyme development in rat brain

Enzymes of energy metabolism

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(Received 26 July 1983/Accepted 17 October 1983)

The development of several key enzymes of pyruvate and 3-hydroxybutyrate metabolism and of the tricarboxylic acid cycle was studied in six regions (cerebellum, medulla oblongata and pons, hypothalamus, striatum, mid-brain and cortex) of the neonatal, suckling and adult rat brain (2 days before birth to 60 days after birth). The enzymes whose developmental patterns were studied were: pyruvate dehydrogenase (EC 1.2.4.1), 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), citrate synthase (EC 4.1.3.7), NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) and fumarase (EC 4.2.1.2). Citrate synthase, isocitrate dehydrogenase and pyruvate dehydrogenase develop as a cluster in each region, although the pyruvate dehydrogenase appears to lag slightly behind the others. As with the glycolytic-enzyme cluster [Leong & Clark (1984) *Biochem. J.* 218, 131–138] the timing of the development of the activity of this group of enzymes varies from region to region; 50% of the adult activity developed first in the medulla oblongata, followed by the hypothalamus, striatum and mid-brain, and then in the cortex and cerebellum respectively. The 3-hydroxybutyrate dehydrogenase activity also develops earlier in the medulla oblongata than in the other regions. The results are discussed with respect to the neurophylogenetic development of the brain regions studied and the importance of the development of the enzymes of aerobic glycolysis in relationship to the development of neurological maturation.

The brain is a complex heterogeneous organ whose regional morphological and anatomical development proceeds at different rates. Associated with and related to this morphological diversity is the functional maturation of the various regions of the brain. One of the major changes that occurs during the normal development of the human and rat brain is the switch-over in respiratory fuels from a situation in the young developing brain where both ketone bodies and glucose are utilized, to the adult situation in which the brain is obligatorily dependent on glucose (Page *et al.*, 1971; Cremer & Heath, 1974; Kraus *et al.*, 1974). Previous studies from our laboratory (Land *et al.*, 1977; Booth *et al.*, 1980) have suggested that the development of fully active aerobic glycolytic flux may be correlated with the development of neurological maturity. Such a postulate has been formulated on the basis of studying enzyme development in species either born neurologically mature at

birth (precocial: guinea pig) or immature at birth (non-precocial: rat). As part of a study to secure further support, or otherwise, for this postulate we have studied the development of a series of key enzymes in various regions of the brain of a single species, the rat, working on the basis that, since the various regions of the brain develop and mature at different rates, so indeed might the enzyme activities associated with energy metabolism. In the preceding paper (Leong & Clark, 1984), the regional development of several enzymes associated with glucose utilization is reported. In the present paper the developmental profiles of key enzymes involved in the aerobic breakdown of the main product of glycolysis, i.e. pyruvate, and other energy fuels are described for various regions of the rat brain.

Methods

Chemicals

Dithiothreitol, CoA and thiamin pyrophosphate were supplied by Sigma (London) Chemical Co.,

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Poole, Dorset, U.K. Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)] was from British Drug Houses, Poole, Dorset, U.K., and *p*-(*p*-aminophenylazo)benzenesulphonic acid (sodium salt) from Fluka A.G., Büchs, Switzerland.

Animals

Albino rats of the Wistar strain were used throughout and fed *ad libitum* on laboratory diet no. 1 (Spratts, Reading, Berks., U.K.); drinking water was always available. Neonatal rats were used as described previously (Leong & Clark, 1984).

Preparation of brain tissue

Brain regions were dissected out essentially as described by Glowinski & Iversen (1966) and 1:10 (w/v) homogenates were made of pooled samples in 0.32M-sucrose/0.5mM-potassium EDTA/10mM-Tris/HCl, pH 7.4, as described previously (Leong & Clark, 1984).

Enzyme assays

These were done at 25°C with a Pye-Unicam SP.1800 or SP.8-100 recording spectrophotometer, with sufficient Triton X-100 present to release maximal activity [except with 3-hydroxybutyrate dehydrogenase; see Leong & Clark, (1984) and the individual assay below for concentration used]. All assays were performed at least in duplicate, each estimate being determined at two different protein concentrations. Pyruvate dehydrogenase (EC 1.2.4.1) was measured in the brain homogenates by coupling with the enzyme arylamine acetyltransferase (EC 2.3.1.5) such that the acetyl moiety of acetyl-CoA formed by the pyruvate dehydrogenase complex was transferred to form *N*-acetyl-*p*-(*p*-aminophenylazo)benzenesulphonate (Na^+ salt) (see Land & Clark, 1973). This latter reaction was monitored as a decrease in A_{460} (Jacobson, 1961). The reaction mixture contained (final concns.): 100mM-Tris/HCl, pH 8.0, 5mM-MgCl₂, 2.5mM-dithiothreitol, 10mM-NAD⁺, 0.2mM-CoA, 2mM-thiamine pyrophosphate, 0.3mM-*p*-(*p*-aminophenylazo)benzenesulphonate (Na^+ salt), arylamine acetyltransferase (80–100munits), 0.1% (v/v) Triton X-100, 10mM-pyruvate and tissue sample. The reaction was initiated by the addition of pyruvate and the mixture read against a blank containing all components except pyruvate. After a short lag period (3–4 min) the reaction was run for a further 5–10 min, during which time the rate was linear with respect to time and up to at least 1.0 mg of homogenate protein. Comparison on brain mitochondrial preparations of this method of pyruvate dehydrogenase measurement with other non-coupling methods, e.g. by NADH measurement (Reed & Willms, 1966), showed a complete agree-

ment in rates of enzyme activity (Leong, 1981). The coupling enzyme arylamine acetyltransferase was prepared from pigeon liver essentially by the method of Tabor *et al.* (1953) and assayed essentially as described by Jacobson (1961). Batches were stored at -20°C.

Citrate synthase (EC 4.1.3.7) was assayed essentially as described by Shepherd & Garland (1969), in which the CoA released in the reaction is coupled to Ellman's reagent. Production of the 5-thio-2-nitrobenzoate fragment was monitored at 412nm. The reaction mixture contained (final concns.): 100mM-Tris/HCl, pH 8.0, 0.1mM-acetyl-CoA, 0.2mM-5,5'-dithio-bis-(2-nitrobenzoic acid), 0.1% Triton X-100, 0.2mM-oxaloacetate and the tissue sample. The assay was initiated by the addition of oxaloacetate.

Isocitrate dehydrogenase (NAD⁺-linked; EC 1.1.1.41) was measured by a modification of the method of Plaut (1969). The reaction mixture contained (final concns.): 50mM-Tris/HCl, pH 7.2, 1mM-MnCl₂, 1mM-ADP, 1mM-NAD⁺, 0.1% Triton X-100, 50mM-DL-isocitrate and tissue sample. The rate of NADH formation was measured at 340nm.

Fumarase (EC 4.2.1.2) was determined by the method of Lai & Clark (1976) in which the reaction mixture was (final concns.): 100mM-potassium phosphate, pH 7.4, 0.1% Triton X-100, 50mM-L-malate and tissue sample. The reaction was initiated by the addition of malate and the increase in A_{240} was monitored.

D-3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was assayed by an adaptation of the method of Williamson & Corkey (1969). The reaction mixture contained (final concns.): 100mM-Tris/HCl, pH 8.5, 10mM-MgSO₄, 5mM-potassium EDTA, 400mM-hydrazine hydrate, 1μM-rotenone, 10mM-NAD⁺, 20mM-DL-3-hydroxybutyrate and tissue sample. The tissue homogenates were subjected to three cycles of alternate freezing and thawing to ensure maximum release of enzymes.

Protein was determined in the presence of 2% sodium deoxycholate by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard.

Results

The tissue enzyme activities are expressed as units, where 1 unit corresponds to 1μmol of substrate transformed/min per g wet wt. at 25°C. Data fitting and theoretical profiles have been generated by graphic packages outlined previously (Leong & Clark, 1984).

The key role that pyruvate dehydrogenase plays in the regulation of carbon flux from glucose into the citric acid cycle in both brain (Land *et al.*,

Fig. 1. Development of pyruvate dehydrogenase activity in regions of rat brain

The numbers of animals used in each experiment was at least four (see the Methods section for details). Each time point is expressed as the mean $\pm$ S.D. (vertical bar) for three or more distinct experiments. If no bars are shown, the S.D. was within the size of the symbol. Brain regions ($\blacktriangle$, cerebellum; $\blacksquare$, medulla oblongata; $\bullet$, cortex; $\triangle$, hypothalamus; $\square$, striatum; $\circ$, midbrain) were dissected out as reported by Glowinski & Iversen (1966) and curves are smoothed by the computer methods outlined previously (Leong & Clark, 1984). Pyruvate dehydrogenase complex activity was measured in homogenates of brain regions by the method of Land & Clark (1973) and is expressed in units ($\mu\text{mol}/\text{min}/\text{g}$ wet wt.

Fig. 2. Development of citrate synthase activity in regions of rat brain

Conditions and symbols were as in Fig. 1. Citrate synthase activity was measured essentially by the method of Shepherd & Garland (1969), and results are expressed as the mean activity ($\pm$ S.D.) in units/g wet wt.

1977; Booth *et al.*, 1980) and other tissues (Denton & Halestrap, 1979) has been amply documented. Although several groups have studied the development of this enzyme complex in whole rat brain (Cremer & Teal, 1974; Wilbur & Patel, 1974; Land & Clark, 1975; Land *et al.*, 1977; Booth *et al.*, 1980), no data are available for its development in the various regions of the brain. Such information is detailed in Fig. 1, where the pyruvate dehydrogenase activity of the various regions of the brain is presented as a function of age. The increases in activity of the pyruvate dehydrogenase complex from the period *post partum* to adulthood ranged from factors of approx. 3 for the medulla oblongata, 5–6 for the hypothalamus, midbrain and striatum and 7–10 for the cortex and cerebellum. The activities reported here for the adult cortex are comparable with those previously reported (Booth

et al., 1980), but higher than those found by Cremer & Teal (1974) and Wilbur & Patel (1974). Taking the first derivative of Fig. 1 provided an estimate of the time of maximum rate of increase of pyruvate dehydrogenase complex activity in the different brain regions. In the medulla this was at 5 days, in the hypothalamus at 10 days, with the midbrain and striatum at 11 and 12 days respectively. Both the cortex and cerebellum had their maximum rate of increase at 15 days *post partum*.

The regional development of the citrate synthase activity is shown in Fig. 2. Previous studies in whole brain (Wilbur & Patel, 1974; Baquer *et al.*, 1975; Land *et al.*, 1977; Booth *et al.*, 1980) showed a rapid increase in activity from birth up to 20 days *post partum*, at which time the forebrain activity attains the adult value. Fig. 2 indicates that the cortex, hypothalamus, striatum and midbrain (which may collectively be regarded as the forebrain) activities reach 80% or more of their adult values at weaning (21 days), but that the citrate synthase activity developed earliest in the medulla oblongata and latest in the cerebellum. The activities in the adult were in the order cortex = striatum >

cerebellum, hypothalamus and midbrain > medulla oblongata. The maximum rate of enzyme increase was earliest in the medulla, followed by the striatum, midbrain, hypothalamus and cortex, and lastly the cerebellum.

The development of the NAD⁺-linked isocitrate dehydrogenase is shown in Fig. 3. The isocitrate dehydrogenase in all regions appeared to develop its peak activity at about 40 days *post partum*, after which there was a slight decrease to the adult (60-day) value. In the adult the cortex possesses the highest activity and the medulla the lowest. The development of fumarase activity is shown in Fig. 4. In common with the previously studied enzymes (Figs. 1–3), the medulla oblongata shows a higher activity as a percentage of the adult value at 1 day *post partum* (40%) than other regions, e.g. cortex (25%). Adult activities were highest in the cerebellum, followed by midbrain > medulla oblongata = cortex > striatum = hypothalamus.

Fig. 5 shows the regional development of D-3-hydroxybutyrate dehydrogenase, a key enzyme in ketone-body utilization. Previous studies on whole rat brain for both this enzyme and other enzymes of ketone-body utilization (3-keto acid CoA-trans-

Fig. 4. Development of fumarase activity in regions of rat brain

Conditions and symbols were as in Fig. 1. Fumarase activity was measured by the method of Lai & Clark (1976), and the results are expressed as the mean activity ($\pm$ S.D.) in units/g wet wt.

Fig. 3. Development of isocitrate dehydrogenase activity in regions of rat brain

Conditions and symbols were as in Fig. 1. Isocitrate dehydrogenase (NAD⁺-linked) activity was measured by a modification of the method of Plaut (1969) (see the Methods section), and the results are expressed as the mean activity ($\pm$ S.D.) in units/g wet wt.

Fig. 5. Development of D-3-hydroxybutyrate dehydrogenase activity in regions of rat brain

Conditions and symbols were as in Fig. 1. D-3-Hydroxybutyrate dehydrogenase activity was measured by a modification of the method of Williamson & Corkey (1969), and the results are expressed as the mean activity ($\pm$ S.D.) in units/g wet wt.

ferase, acetoacetyl-CoA thiolase) have shown a characteristic enzyme-activity profile, with a peak activity at about weaning (20 days) followed by a decline to about 30% of this value in the adult (Klee & Sokoloff, 1967; Page *et al.*, 1971; Sherman & Wilson, 1978; Booth *et al.*, 1980). Although the regional development shows the same general profile (Fig. 5), it is clear that each region shows a different rate of development and attains its maximum activity at a slightly different time. In the adult brain, the cortical region maintained the highest specific activity of D-3-hydroxybutyrate dehydrogenase, whereas the lowest activity was observed in the medulla oblongata. In whole rat brain both Page *et al.* (1971) and Booth *et al.* (1980) observed a 4-fold increase in activity of D-3-hydroxybutyrate dehydrogenase from 5 to 25 days *post partum*. The results presented for the cortex in the present study are comparable with those previous results, although for the medulla there is only a 3-fold increase in activity over the same period. The maximum rate of increase of D-3-hydroxybutyrate dehydrogenase activity was in the medulla at 5 days, which is much earlier than in other regions; cf. the midbrain and striatum (8 and 9 days respectively) and the cerebellum, hypothalamus and cortex (10 days).

Discussion

As with the glycolytic enzymes (Leong & Clark, 1984), the activities of pyruvate dehydrogenase,

citrate synthase, NAD⁺-isocitrate dehydrogenase and fumarase develop differently in each region of the brain studied, and the development of each enzyme differs to some extent from that of other enzymes in the same region. However, the relative activities of each of the above enzymes remain fairly consistent between the brain regions. In the cortical region (Fig. 6), there is a general pattern of development of enzyme activities common to pyruvate dehydrogenase, citrate synthase and isocitrate dehydrogenase, with an upsurge in activity occurring a few days *post partum* and adult activities being established in the region of 30–40 days *post partum*. Hence, these enzymes do appear to develop as a 'cluster', although the timing does not necessarily fall into 'neonatal' or 'late suckling' clusters, as originally defined by Greengard (1971). In addition, analysis of Figs. 1–5 suggests that the timing of the development of this cluster of enzymes may be specific to each region, with, for example, the medulla showing the upsurge in enzyme activities earliest, and the cerebellum the latest, of the regions studied. Close inspection of Fig. 6, however, does reveal, despite the overall similarity, slight discrepancies between the actual timing of the development of individual enzymes in the cortex, with pyruvate dehydrogenase activity development lagging slightly behind that of isocitrate dehydrogenase, citrate synthase and fumarase until about 15 days *post partum*. This lag in pyruvate dehydrogenase activity has been observed previously in rat forebrain (Land *et al.*, 1977; Booth *et al.*, 1980). The retarded development of pyruvate dehydrogenase activity in the rat brain regions, with respect to other enzymes, may have an important influence on the maturation of brain regional activity, since this enzyme has a key regulatory role in controlling the flux of glycolytic products via pyruvate into the citric acid cycle for energy metabolism. Table 1 contains data derived from Figs. 1–5 in which the developmental time at which a particular enzyme has attained 50% of the adult activity for a specific brain region has been noted. Also included is the mean developmental time at which a group of seven enzymes associated with energy metabolism have achieved 50% of the adult activity. The enzymes in this group include not only those listed in Table 1 (pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and fumarase), but also some key enzymes associated with glycolysis (hexokinase, aldolase, lactate dehydrogenase), the details of which are listed in the preceding paper (Leong & Clark, 1984). It is very clear from Table 1 that, however the data are analysed, the medulla oblongata stands out as developing its glycolytic/citric-acid-cycle capability (as measured by potential enzyme activity) much earlier (approx. 7 days) than other regions of

Fig. 6. Comparative development of pyruvate dehydrogenase, citrate synthase and isocitrate dehydrogenase (NAD⁺-linked) in cortex of rat brain

The data are taken from Figs. 1–3 and replotted as percentages of the adult value (60 days). These were: pyruvate dehydrogenase, 2.15 ± 0.03 ($n=8$) units/g wet wt. (▲); citrate synthase, 36.0 ± 2.7 ($n=6$) units/g wet wt. (●); isocitrate dehydrogenase, 5.84 ± 0.78 ($n=6$) units/g wet wt. (△). (—) represents profile for one enzyme; (---) represents profile common to all three enzymes.

Table 1. *Estimation of developmental time when enzyme activity is 50% of adult activity*

Time values are estimated in days after birth (or, if before birth, as negative numbers). The estimation of the time when the enzyme activity is half that in adult (60-day-old) rats was derived from Figs. 1–5. When the estimated time values are before 10 days *post partum* for hypothalamus, striatum and midbrain they are denoted by the value <10 days. The last column represents a time average ($\pm$ S.D.) when a number (*n*) of enzymes concerned with aerobic glycolysis have achieved 50% of adult activity in the various regions of the brain. The enzymes taken for consideration are pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and fumarase (this paper) and hexokinase, aldolase and lactate dehydrogenase (Leong & Clark, 1984).

Enzyme Brain region	Time (days) when enzyme activity reaches 50% of adult value					Enzyme group
	Pyruvate dehydrogenase	Citrate synthase	Isocitrate dehydrogenase	Fumarase	D-3-Hydroxybutyrate dehydrogenase	
Cerebellum	17	17	13	16	6	15.0 $\pm$ 3.0 (7)
Medulla oblongata	4	–1	3	6	–1	3.0 $\pm$ 2.4 (7)
Hypothalamus	11	10	11	12	<10	10.8 $\pm$ 0.8 (6)
Striatum	12	11	11	12	<10	11.7 $\pm$ 0.8 (7)
Midbrain	10	11	13	13	<10	11.4 $\pm$ 1.3 (7)
Cortex	13	12	13	13	6	13.0 $\pm$ 1.5 (7)

the brain. The hypothalamus, striatum and mid-brain all develop 50% of their adult enzyme activity around 11 days *post partum*, with the cortex (13 days) and the cerebellum (15 days) following soon after. This order recapitulates the embryological and neurophylogenetic development of the brain regions, which in its simplest concept proceeds from the medulla to the end brain (telencephalon). It therefore appears that the development of morphological and neurological maturity in the various regions of the brain may be correlated with the development of the potential for aerobic glycolysis, and in particular with pyruvate dehydrogenase activity. These observations are consistent with previous observations that the pyruvate dehydrogenase activity of the brain of species born neurologically mature (precocial, e.g. guinea pig) is fully developed at birth, whereas that of a species born neurologically immature (non-precocial, e.g. rat) is low and that the subsequent development of neurological activity is dependent on the development of pyruvate dehydrogenase activity (Booth *et al.*, 1980).

The data may also be discussed from the viewpoint of brain regional substrate utilization and the switch-over in energy fuels; i.e. from a mixture of ketone bodies and glucose to one of glucose alone, that occurs during the normal development of the rat and human brain (Cremer & Heath, 1974; Kraus *et al.*, 1974). The rate at which this switch-over occurs will be critically dependent on the development of the pyruvate dehydrogenase activity, which is necessary for the full utilization of glucose. Other factors of importance will also be the circulatory ketone-body concentration, which is relatively high in the newborn rat (Page *et al.*, 1971) and the activity of the ketone-body-transport system across the blood/brain barrier (Cremer *et al.*, 1976; Moore *et al.*, 1976), which is 6 times more

active in the suckling-rat brain than in the adult-rat brain. It appears from the data presented here that the various regions of the brain vary in their potential enzymic capabilities to utilize both glucose and ketone bodies. Considerable evidence is available from the work of Sokoloff and his colleagues (Sokoloff, 1975; Reivich *et al.*, 1975; Sokoloff *et al.*, 1977) that *in vitro* different regions of the adult brain utilize glucose at different rates. Hawkins & Biebuyck (1979) have suggested that ketone bodies may be used as a supplementary respiratory fuel for adult brain and that this may have regional variations, depending on the blood/brain-barrier characteristics of the region. However, little is known of the regional brain variations in glucose or ketone-body utilization as a function of development. Despite this, one important conclusion that may be drawn from the present work is that, because of the variation in the timing of the development of the brain regional capability for aerobic glycolysis and ketone-body utilization, the chemical nature and timing of any insult to the brain during development may be expected to have markedly different effects on the various brain regions and their associated functions.

S. F. L. is grateful to the British Council for financial support. We thank Mr. P. Browne for helping in the analysis of the data by computer graphics.

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