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Regional enzyme development in rat brain

Enzymes associated with glucose utilization

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The development of key enzyme activities concerned with glucose metabolism was studied in six regions of the rat brain in animals from just before birth (−2 days) through the neonatal and suckling period until adulthood (60 days old). The brain regions studied were the cerebellum, medulla oblongata and pons, hypothalamus, striatum, mid-brain and cortex. The enzymes whose developmental patterns were investigated were hexokinase (EC 2.7.1.1), aldolase (EC 4.1.2.13), lactate dehydrogenase (EC 1.1.1.27) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Hexokinase, aldolase and lactate dehydrogenase activities develop as a single cluster in all the regions studied, although the timing of this development varies from region to region. Glucose-6-phosphate dehydrogenase activity, however, declines relative to glycolytic enzyme activity as the brain matures. When the different brain regions are compared, it is clear that the medulla develops its glycolytic potential, as indicated by its potential enzyme activity, considerably earlier than the other regions (hypothalamus, striatum and mid-brain), with the cortex and cerebellar activities developing even later. This enzyme developmental sequence correlates well with the neurophylogenetic development of the brain and adds support to the hypothesis that the development of the potential for glycolysis in the brain is a necessary prerequisite for the development of neurological competence.

It has long been recognized that one of the major problems related to the study of brain development is its heterogeneity in terms of both cell type and function [see Himwich (1970) for review]. Furthermore, the biological activity of the brain varies not only with respect to age but also with respect to the region (Himwich & Fazekas, 1941). One of the most important events of brain development in the rat and human is the switch-over in energy fuels from a mixture of ketone bodies and glucose during the suckling period to an obligatory dependence on glucose in the adult (Page *et al.*, 1971; Persson *et al.*, 1972; Cremer & Heath, 1974; Kraus *et al.*, 1974), except in cases of long-term starvation (Owen *et al.*, 1967). In previous studies from our laboratory on both the rat (Land *et al.*, 1977) and the guinea pig (Booth *et al.*, 1980) it has been suggested that the switch-over may be related to the development of neurological competence, and that the acquisition of the latter is dependent on the brain's capability for the complete oxidation of glucose. Enzyme studies on whole brains have suggested that in particular the development of the activity of pyruvate dehydrogenase may be a critical part of this process.

Although there have been previous regional brain studies (see Himwich, 1970), there is little information on regional energy requirements, and particularly on the development of enzymes associated with these requirements. Furthermore, there have been reports that have suggested that regions of the brain may differ in their capabilities for using ketone bodies for energy metabolism in the fed and starved states (Hawkins & Biebuyck, 1979). This prompted us to study the activities of several key enzymes of glucose metabolism in six different regions of rat brain as a function of development. The results indicate that different regions of the brain possess different enzyme

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profiles, which develop at different rates after birth. The data may be interpreted as providing further support for the hypothesis that the development of the capacity for full aerobic glycolysis may be correlated with the development of neurological maturity in brain, since the order in which full glycolytic capacity is developed follows approximately the phylogenetic age of the brain region.

Methods

Chemicals

Dithiothreitol, ATP, fructose 1,6-bisphosphate, glucose 6-phosphate and α-glycerophosphate dehydrogenase/triose phosphate isomerase mixture were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. $NADP^+$, NADH and glucose-6-phosphate dehydrogenase were from Boehringer Corp. (London), Lewes, East Sussex, U.K. All other reagents were of the highest purity available commercially, except pyruvic acid, which was twice distilled under reduced pressure before use. All solutions were made up in double-glass-distilled water.

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. The birth dates of all litters were carefully recorded after daily inspection and the litters were culled to eight to ten pups. Animals of either sex were used up to weaning (21 days), but, after this age and when adult, only male animals were used. Where foetuses were used, the age of the foetus was calculated from the recorded date of mating or determined by examination of vaginal lavages for spermatozoa.

Preparation of brain tissue

After decapitation of the rat, the brain was removed on to an ice-cooled glass plate, and dissected essentially as described by Glowinski & Iversen (1966), except that the hippocampus was left associated with the cerebral cortex. This gave six regions: (1) cerebellum, (2) medulla oblongata and pons, (3) hypothalamus, (4) striatum, (5) midbrain and (6) cortex. Pooled preparations of these regions were made from ten animals for 1–5-day-old rats, six animals for 10–15-day-old rats and four aminals for 20–60-day-old rats. These pooled preparations were minced finely and washed in isolation medium (0.32M-sucrose/0.5mM-potassium EDTA/10mM-Tris/HCl, pH7.4) to remove blood. A 1:10 (w/v) homogenate was then made in the isolation medium by hand in a glass homogenizer (0.19mm clearance) and used for enzyme assays.

Enzyme assays

These were done at 25°C with a Pye–Unicam SP. 1800 or SP. 8–100 recording spectrophotometer. All assays were performed in the presence of a concentration of Triton X-100 which released maximal activity but did not inhibit enzyme activity (each individual enzyme assay was titrated for this concentration). In all experiments, duplicate determinations were performed at two different protein concentrations for each homogenate fraction. Hexokinase (EC 2.7.1.1) was assayed as described by Land *et al.* (1977). In a volume of 1ml, these final concentrations were present: 50mM-Tris/HCl, pH7.5, 6mM-glucose, 1mM-dithiothreitol, 10mM-$MgCl_2$, 0.5mM-$NADP^+$, 1.4 units of glucose-6-phosphate dehydrogenase, 0.1% (v/v) Triton X-100, 8mM-ATP and tissue sample. Aldolase (EC 4.1.2.13) was assayed by a modification of the method of Lebherz & Rutter (1975). The reaction mixture (1ml) contained (final concns.): 100mM-glycylglycine/KOH, pH7.5; 0.2mM-NADH; α-glycerophosphate dehydrogenase/triose phosphate isomerase mixture (1 unit: 6.7 units); 0.1% Triton X-100; and sample. The reaction was started by the addition of 5mM-fructose 1,6-bisphosphate.

Lactate dehydrogenase (EC 1.1.1.27) was measured as described by Clark & Nicklas (1970). The reaction mixture (1ml) contained (final concns.): 100mM-potassium phosphate, pH7.4; 0.2mM-NADH; 0.5% (v/v) Triton X-100; 1mM-pyruvate; and tissue homogenate. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was measured by a modification of the method of Langdon (1966), in which the reaction mixture (1ml) contained (final concns.): 100mM-Tris/HCl, pH7.5; 10mM-$MgCl_2$; 0.5mM-$NADP^+$; 0.1% (v/v) Triton X-100; and homogenate. The reaction was started with 1mM-glucose 6-phosphate. Protein was determined in the presence of 2% sodium deoxycholate by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard.

Expression of results

Tissue enzyme activities are expressed as units, where 1 unit corresponds to 1 μmol of substrate transformed/min per g wet wt. at 25°C. Graphics were produced on an ICL 2980 computer (Queen Mary College, London) by using graphic packages GINO-F and GINO GRAF (Computer Aided Design Centre, Cambridge, U.K.). In all cases, the raw data (means ± S.D.) were used for plotting and curves were smoothed through the data points by using a parametric cubic fit by GINO routines. In certain cases (e.g. hexokinase) a theoretical curve was fitted by using the SPSS (Statistical Package for Social Sciences; Vogelbach Computing Center, North Western University, Chicago,

IL, U.S.A.) on a CDC computer at the University of London Computer Centre. From these theoretical profiles, derivative curves have been plotted.

Results

The increases in the wet weight (Fig. 1) and protein content (mg/g wet wt.) (Fig. 2) of each of the brain regions studied in this investigation are indicated. Presentation of these data is important for two reasons; firstly, it allows the further calculation of total and specific enzyme activities, and secondly, it allows an assessment of the reproducibility of the dissection technique. All regions studied showed rapid increases in both wet weight and protein content during the first 15 days after birth, and those of the cortex and medulla continued to increase until 20 days *post partum* and of the cerebellum until 30 days after birth. It is also important to recognize the relative differences in sizes between the regions and the influence that the development profiles have on these differences. The protein contents of the brain regions in general showed a similar profile to the wet-weight increases, with an increase of approx. 50% occurring from about 5 days *post partum* to adulthood (Fig. 2). Interestingly, the cortex and medulla showed a decrease in protein as related to wet weight during the period from just before birth to 5 days *post partum*. This may be related to the very profound decreases in extracellular space and hence water content that occur during this period [Kuthan (1964), cited by Mourek (1970)].

Fig. 3 shows the development in the brain regions studied of hexokinase, a key regulatory enzyme of glycolysis. These results indicated that the development of hexokinase to adult activities occurred considerably earlier in the medulla oblongata than in the other regions. The data also showed that in the adult brain the activity of the hexokinase was highest in the cortex, followed by hypothalamus > midbrain > cerebellum = striatum, with that in the medulla oblongata being significantly lower than in any of these other regions. Fig. 4 indicates the development

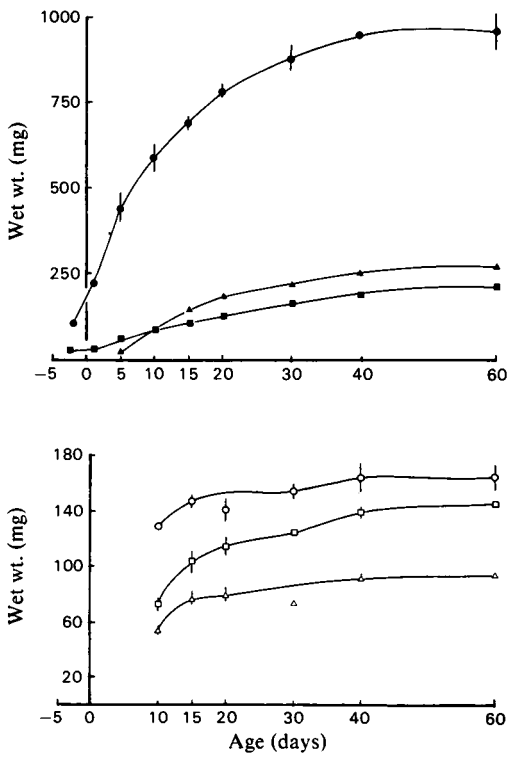

Fig. 1. *Rat brain regional wet-weight development*
The number of animals used in each experiment was at least four (see the Methods section for details). Each time point is expressed as the mean ± S.D. (vertical bar) for three or more distinct experiments. If no bars are shown, the S.D. was within the size of the symbol. Brain regions (▲, cerebellum; ■, medulla oblongata; ●, cortex; △, hypothalamus; □, striatum; ○, midbrain) were dissected out as reported by Glowinski & Iversen (1966). Curves are smoothed by the computer method outlined in the Methods section.

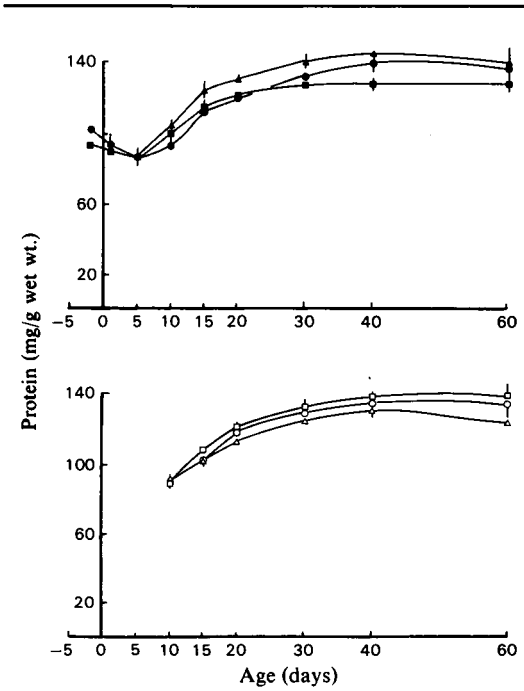

Fig. 2. *Rat brain regional protein development*
All conditions and symbols were as in Fig. 1. Protein is expressed as mg/g wet wt. and was measured by the method of Lowry *et al.* (1951).

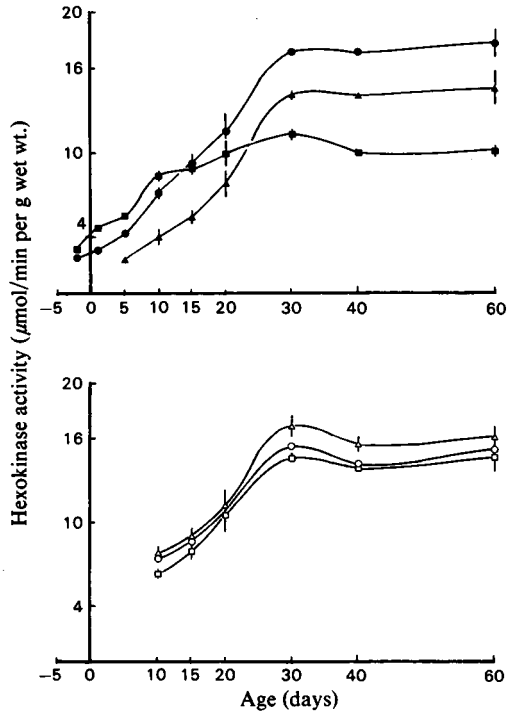

Fig. 3. *Development of hexokinase in regions of rat brain*
Conditions and symbols were as in Fig. 1. Hexokinase activity was measured by the method of Land *et al.* (1977) and is expressed in μmol/min per g wet wt.

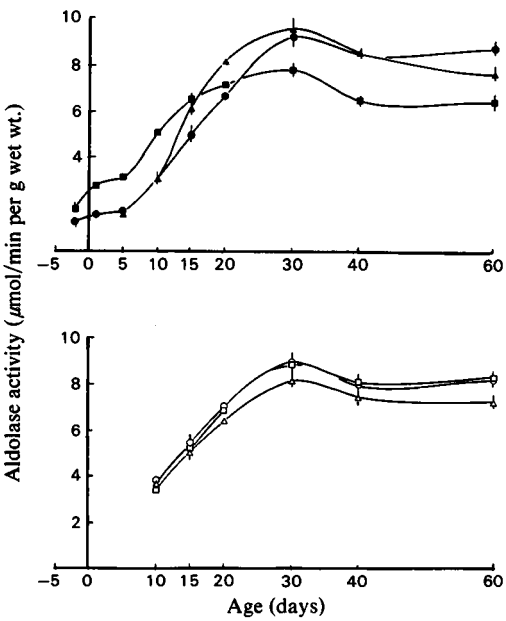

Fig. 4. *Development of aldolase in regions of rat brain*
Conditions and symbols were as in Fig. 1. Aldolase activity was measured by the method of Lebherz & Rutter (1975) and is expressed in μmol/min per g wet wt.

profiles for aldolase activity in the various brain regions. Aldolase activity appeared to develop earlier than hexokinase, and in all regions reached a peak at 30 days *post partum* and thereafter decreased slightly to adult values. In the adult the aldolase activities are in the order cortex > striatum = midbrain > cerebellum > hypothalamus > medulla oblongata. Fig. 5 describes the development profile of lactate dehydrogenase. In the adult brain the lactate dehydrogenase activities are in the order striatum > cortex > midbrain = hypothalamus > cerebellum > medulla oblongata. Overall, in the cortex, this represents an approx. 4-fold increase in lactate dehydrogenase activity from birth to adulthood, which correlates reasonably with the data of Kuhlman & Lowry (1956) from histochemical techniques and those obtained by Booth *et al.* (1980) by enzymic assay on whole brain. The developmental profiles of the above glycolytic enzymes contrast with that of the glucose-6-phosphate dehydrogenase, the first enzyme in the metabolism of glucose-6-phosphate by the pentose phosphate pathway (Fig. 6). This enzyme in all the regions studied shows relatively little change in activity from 10 days *post partum*, all activities in all regions being

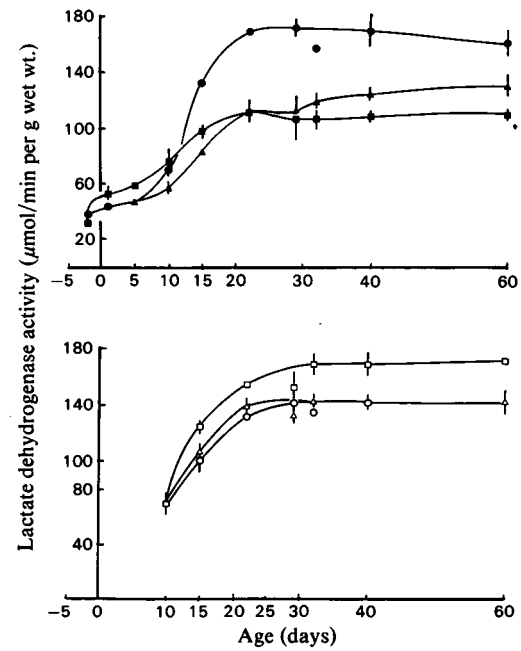

Fig. 5. *Development of lactate dehydrogenase in regions of rat brain*
Conditions and symbols were as in Fig. 1. Lactate dehydrogenase activity was measured as described by Clark & Nicklas (1970) and is expressed as μmol/min per g wet wt.

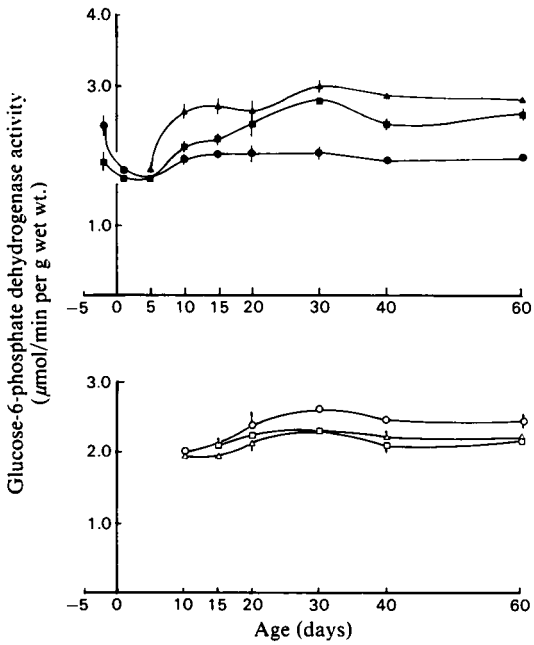

Fig. 6. *Development of glucose-6-phosphate dehydrogenase in regions of rat brain*
Conditions and symbols were as in Fig. 1. Glucose-6-phosphate dehydrogenase activity was measured by the method of Langdon (1966) and is expressed in μmol/min per g wet wt.

in the range of 2 (cortex) to about 3 (cerebellum) μmol/min per g wet wt. This agrees with previous histochemical (Kuhlman & Lowry, 1956) and enzymic (Baquer *et al.*, 1975) analyses on whole brain. However, during the period just before birth, up to 5 days *post partum* a decrease in activity of glucose-6-phosphate dehydrogenase has been observed in cortex and medulla which then increases to adult values by 10 days *post partum*.

Discussion

The data presented here (Figs. 1 and 2) on regional brain weight development are more extensive than have been previously published (see Benjamin & McKhann, 1976). The protein content of these areas in general follows the developmental profile of the wet weight (Fig. 2), and the particularly rapid increase in protein content observed for all the regions studied during the period 5–15 days *post partum* correlates with the cell proliferation and growth occurring during that period (Himwich, 1962; Benjamin & McKhann, 1976). However, one anomalous feature of the protein content is the apparent loss of protein (per g wet wt.) during the period just after birth until 5 days *post partum*. This was also observed on the whole brain by Himwich (1962).

Although several studies on hexokinase development have been reported for rat brain, these have dealt with the whole forebrain (Baquer *et al.*, 1973; Land *et al.*, 1977; Booth *et al.*, 1980), the cerebral cortex (Schwark *et al.*, 1972; Kellogg *et al.*, 1974; Takagaki, 1974), or the cerebellum (Lehrer *et al.*, 1970; Kellogg *et al.*, 1974). It is clear from the data reported here that not only does the adult specific activity of hexokinase vary from region to region, but the rate at which this is attained is also variable. This is also true for both aldolase and lactate dehydrogenase, the other glycolytic enzymes measured in this paper. If the activities of these enzymes are expressed as a percentage of the adult (60-day) value, a developmental time at which 50% of the adult activity is achieved may be calculated. If this is done with respect to the region studied, a comparison of the rates of enzyme development between regions can be made (see Table 1). The main conclusion to be drawn from Table 1 is that the medulla develops its potential glycolytic activity very much earlier (50% activity at an average 3 days *post partum*) than any of the other regions. The hypothalamus, striatum and midbrain develop an equivalent activity around 11 days *post partum*, the cortex at 13 days and the cerebellum at 15 days *post partum*. Despite these regional variations in the time of development of the glycolytic enzymes, if the development of this group of enzymes is considered within the context of a single region (see Fig. 7 for cortex) it is clear that they develop broadly as a group in certain regions of the brain and fit into the constant-proportion-group concept for enzyme development first proposed by Pette in muscle (Pette *et al.*, 1962*a*,*b*; Bass *et al.*, 1969; Staudte & Pette, 1972) and subsequently studied in whole brain by MacDonnell & Greengard (1974) and Baquer *et al.* (1975). The cortex (Fig. 7) shows these characteristics most clearly, and inspection of Table 1 suggests that the hypothalamus, striatum and midbrain will also exhibit a similar profile. It is, however, evident that both the cerebellum and medulla show more variability in this respect. In contrast with the glycolytic enzymes studied, glucose-6-phosphate dehydrogenase, a key enzyme of the pentose phosphate pathway of glucose metabolism, shows very little change in activity over the course of brain development in most of the regions studied. This might be considered anomalous, since data from several groups suggest that the carbon flux through the pentose phosphate pathway is higher in the neonatal rat brain than in the adult (Itoh & Quastel, 1970; Winick, 1970, 1974; Baquer *et al.*, 1975) and may be correlated with the high rate of

Table 1. *Development of glycolytic enzymes in regions of rat brain*
The adult activity is defined as that at 60 days. If the estimated time value for the midbrain, hypothalamus or striatum is before 10 days *post partum*, then this is denoted as < 10 days, since it was not possible to differentiate these regions before this time.

	Time for 50% of adult activity of enzyme to develop (days)		
Brain region	Hexokinase	Aldolase	Lactate dehydrogenase
Cerebellum	19	11	12
Medulla oblongata	3	5	1
Hypothalamus	11	< 10	10
Striatum	13	12	11
Midbrain	10	11	10
Cortex	16	13	11

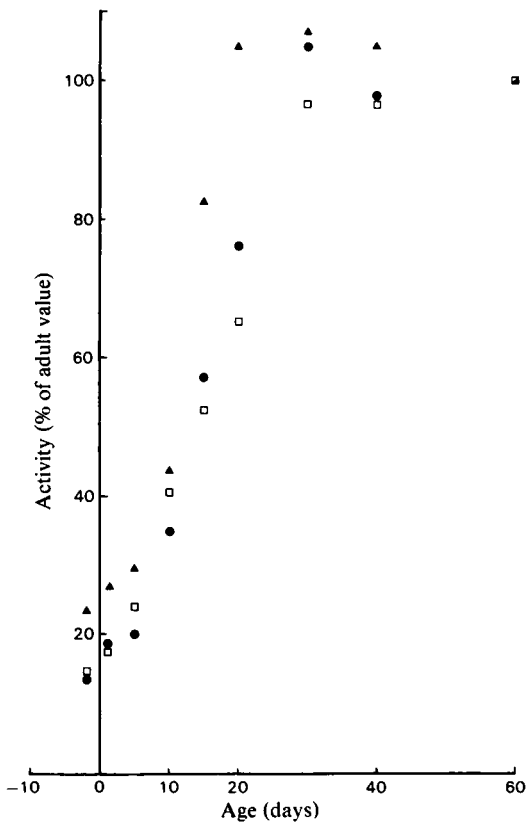

Fig. 7. *Comparative development of hexokinase, aldolase and lactate dehydrogenase in a single region of the rat brain cortex*
Enzyme activities are derived from Figs. 3, 4 and 5, and expressed as a percentage of the 60-day-old adult value. □, Hexokinase; ●, aldolase; ▲, lactate dehydrogenase.

lipid and myelin synthesis during this period. However, this apparent disparity between the relatively constant regional activity of the glucose-6-phosphate dehydrogenase and the declining pentose phosphate pathway flux as a function of development can be more readily understood when the relative activities of the glucose-6-phosphate dehydrogenase and hexokinase of each region are compared. This is shown in Fig. 8 for several regions (cortex, medulla and cerebellum) and clearly reflects the relative decline in activity of the glucose-6-phosphate dehydrogenase activity as compared with hexokinase as the brain develops. It is noteworthy that in all the areas studied the final adult situation is one in which the hexokinase activity exceeds that of glucose-6-phosphate dehydrogenase activity by a factor ranging between 4 (medulla) and 10 (cortex) times.

The relationship between maximal enzyme activity and metabolic flux is complex, but several investigators have considered the measurement of enzyme activities as a useful approach to relating metabolic potential to tissue function, particularly when related to a variable physiological state, such as development or differentiation (Pette, 1966; Greengard, 1971; Baquer *et al.*, 1975, 1977). Investigations *in vitro* on brain region slices in humans and experimental animals (Siesjo, 1978) have suggested that the cortex and cerebellum utilize 3–5 times more oxygen than do other regions. Studies *in vivo* by Sokoloff and his colleagues (Sokoloff, 1975; Reivich *et al.*, 1975; Sokoloff *et al.*, 1977), using the 2-deoxyglucose technique, have also indicated regional differences in glucose uptake in the brain, which have been correlated with variations in cerebral blood flow and functional activity. Although the maximal enzyme activities of the glycolytic enzymes studied in the present paper do not reflect such large variations in the adult (except for the medulla, which has enzymic activities consistently 66–75% of those of the other regions), the outstanding observation relates to the time at which this 'adult' activity is attained. The medulla oblongata (Table

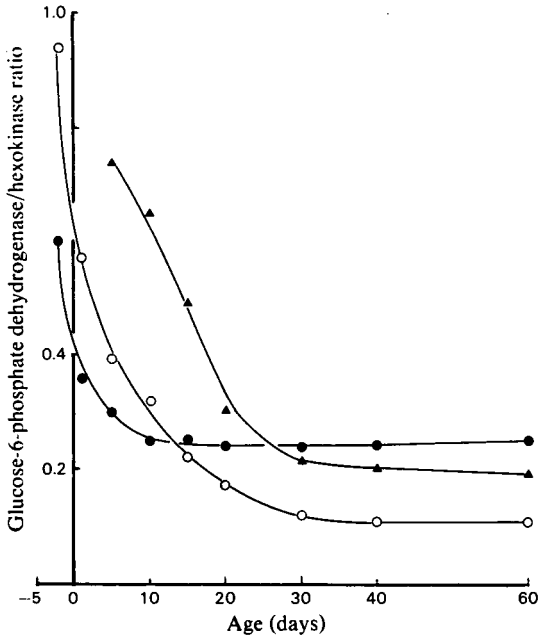

Fig. 8. *Relative activities of glucose-6-phosphate dehydrogenase and hexokinase in regions of the rat brain as a function of development*

The hexokinase and glucose-6-phosphate dehydrogenase activities are derived from Figs. 3 and 6. ●, Medulla oblongata; ▲, cerebellum; ○, cortex.

1) consistently attains its 'adult' enzyme complement almost a week before the other regions. This may reflect the early development of the caudal regions of the brain as compared with the rostral regions and may be related to the observations that the phylogenetically older regions of the brain, e.g. medulla, are more resistant to anoxic/hypoglycaemic insult than the regions that develop later (Himwich & Fazekas, 1941; Tyler & Van Harreveld, 1942).

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