Development of Mitochondrial Energy Metabolism in Rat Brain

By JOHN M. LAND, ROBERT F. G. BOOTH, RUUD BERGER*
and JOHN B. CLARK†

Department of Biochemistry, St. Bartholomew's Hospital, Medical College, University of London,
Charterhouse Square, London EC1M 6BQ, U.K.

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1. The development of pyruvate dehydrogenase and citrate synthase activity in rat brain mitochondria was studied. Whereas the citrate synthase activity starts to increase at about 8 days after birth, that of pyruvate dehydrogenase starts to increase at about 15 days. Measurements of the active proportion of pyruvate dehydrogenase during development were also made. 2. The ability of rat brain mitochondria to oxidize pyruvate follows a similar developmental pattern to that of the pyruvate dehydrogenase. However, the ability to oxidize 3-hydroxybutyrate shows a different developmental pattern (maximal at 20 days and declining by half in the adult), which is compatible with the developmental pattern of the ketone-body-utilizing enzymes. 3. The developmental pattern of both the soluble and the mitochondrially bound hexokinase of rat brain was studied. The total brain hexokinase activity increases markedly at about 15 days, which is mainly due to an increase in activity of the mitochondrially bound form, and reaches the adult situation (approx. 70% being mitochondrial) at about 30 days after birth. 4. The release of the mitochondrially bound hexokinase under different conditions by glucose 6-phosphate was studied. There was insignificant release of the bound hexokinase in media containing high KCl concentrations by glucose 6-phosphate, but in sucrose media half-maximal release of hexokinase was achieved by 70 μ M-glucose 6-phosphate. 5. The production of glucose 6-phosphate by brain mitochondria in the presence of Mg^{2+} + glucose was demonstrated, together with the inhibition of this by atractyloside. 6. The results are discussed with respect to the possible biological significance of the similar developmental patterns of pyruvate dehydrogenase and the mitochondrially bound kinases, particularly hexokinase, in the brain. It is suggested that this association may be a mechanism for maintaining an efficient and active aerobic glycolysis which is necessary for full neural expression.

Although the capacity of adult-rat brain slices to oxidize ketone bodies has been known for over 40 years (Jowett & Quastel, 1935), it is only relatively recently that evidence has been obtained that ketone bodies may be used by the human brain as primary energy substrates under physiological conditions (starvation) (Owen *et al.*, 1967). Subsequently it was shown that the normal immature rat (Page *et al.*, 1971; Williamson & Buckley, 1973) and human brain (Kraus *et al.*, 1974) may also use ketone bodies as energy substrates, and a compartmental analysis of experiments by Cremer & Heath (1974) has suggested that in the 18-day-old rat the glucose and ketone bodies may make approximately equal contributions to carbon influx to the tricarboxylic acid cycle. These observations have in part been rationalized by the fact that the enzymes of ketone-

body utilization, namely 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), 3-oxo acid CoA-transferase (EC 2.8.3.5) and acetoacetyl-CoA thiolase (EC 2.3.1.9), are maximally active soon after birth (about 3 weeks in the rat), but then decline to approx. 40% of that value in the adult brain (Page *et al.*, 1971; Middleton, 1973).

In contrast, the enzymes of glycolysis and the tricarboxylic acid cycle appear in the rat to be relatively low in activity at birth and to develop and remain at adult values during the neonatal period (first 3–4 weeks) (Wilbur & Patel, 1974; MacDonnell & Greengard, 1974; Baquer *et al.*, 1975). There is, however, only limited information available on the development and control of pyruvate utilization in rat brain (Wilbur & Patel, 1974; Cremer & Teal, 1974), particularly with respect to the control of the pyruvate dehydrogenase complex.

Most previous studies on the development of enzyme systems in rat brain have used homogenates or crude particulate fractions as enzyme sources,

* Permanent address: Laboratory of Developmental Biochemistry, Department of Pediatrics, University of Groningen, Groningen, The Netherlands.

† To whom reprint requests should be addressed.

which may lead to problems of measurement and interpretation. This paper reports experiments using relatively purified mitochondria to study the development of the brain pyruvate dehydrogenase and the mitochondrially associated hexokinase (Bachelard, 1967; Wilson, 1972). The data suggest that both the pyruvate dehydrogenase and the mitochondrial hexokinase develop coincidentally in rat brain at a time somewhat later than the enzymes of the tricarboxylic acid cycle and glycolysis. These results are discussed in the context of the role that these enzymes play in maintaining the high glycolytic flux characteristic of the normal adult mammalian brain.

Experimental

Materials

AMP, ADP, ATP, NAD⁺, NADH, NADP⁺, NADPH, CoA, phosphoenolpyruvate, 2-oxoglutarate, succinate, pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer Corp. (London) Ltd., Lewes, E. Sussex BN7 1LG, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a generous gift from Dr. P. Heytler of E. J. Dupont De Nemours and Co., Wilmington, DE, U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and bovine plasma albumin (fraction V) were obtained from British Drug Houses, Poole, Dorset BH12 4NN, U.K. Atractyloside, acetylthiocholine iodide and Mops* were obtained from Sigma (London) Chemical Co., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K. Pyruvic acid was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and was twice distilled under vacuum and stored at -20°C before use. Ficoll was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. It was prepared before use as a 30% (w/v) solution in double-glass-distilled water and then dialysed against 10 vol. of glass-distilled water in $\frac{3}{4}$ in Visking tubing for 4 h. It was stored at 4°C as a 20% (w/v) solution before use.

Animals

(a) *Adult*. Male rats of the Wistar strain (155–175 g body wt.) were used throughout. 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 of age). After 21 days of age only male animals were used.

* Abbreviations: Mops, 4-morpholinepropanesulphonic acid; ATPase, adenosine triphosphatase.

Mitochondrial preparation

Mitochondria were prepared from rat brain cerebral cortex as described by Clark & Nicklas (1970), except that the initial isolation medium used had the following composition: 225 mM-mannitol, 75 mM-sucrose, 10 mM-Mops and 0.5 mM-EDTA, pH 7.2.

Mitochondrial respiratory studies

Mitochondrial respiration was measured polarographically at 25°C in an incubation medium (final volume 1 ml) consisting of either 100 mM-KCl, 75 mM-mannitol, 25 mM-sucrose, 10 mM-phosphate/Tris, 10 mM-Tris/HCl, 0.05 mM-EDTA, pH 7.4 (100 mM-K⁺ medium), or 5 mM-KCl, 225 mM-mannitol, 75 mM-sucrose, 10 mM-phosphate/Tris, 10 mM-Tris/HCl, 0.05 mM-EDTA, final pH 7.4 (5 mM-K⁺ medium). To the incubation medium was added 0.75–2 mg of mitochondrial protein together with the appropriate substrates. Respiration was stimulated by the addition of 250 μ M-ADP (as 5 μ l of 50 mM) (State 3; Chance & Williams, 1956), and when uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was added it was present at approx. 0.2 nmol/mg of mitochondrial protein.

Mitochondrial incubations

Incubations were carried out in the 100 mM-K⁺ medium (see above) in the presence of 5 mM-MgCl₂, 10 mM-glucose, 1 mM-ADP and a mitochondrial protein concentration of approx. 2 mg/ml at 25°C. Samples were taken at timed intervals and quenched in 6% (v/v) HClO₄. The samples were then centrifuged 15000g for 2 min to remove protein and neutralized with 6 M-K₂CO₃/0.5 M-triethanolamine. The supernatants were kept frozen overnight and analysed spectrophotometrically for glucose 6-phosphate by the method of Lamprecht *et al.* (1974).

Preparation of acetyl-CoA

Acetyl-CoA was prepared from free CoA and freshly prepared acetic anhydride essentially as described by Ochoa (1955).

Enzyme assays

These were carried out at 25°C for comparison with previous results, by using an SP. 800 or SP. 1800 recording spectrophotometer. Enzyme activities were measured in mitochondrial preparations in the presence (where necessary) of sufficient Triton X-100 to release maximal activity (see individual assay). As a routine, pyruvate dehydrogenase and hexokinase activities were measured within 5 h of isolating the mitochondria; samples for other enzyme and protein determinations were frozen at -40°C and assayed the next day. Citrate synthase (EC 4.1.3.7) was measured as described by Clark & Land (1974). Acetylcholinesterase (EC 3.1.1.7) was determined by the method of Ellman *et al.* (1961). NAD⁺-linked

malate dehydrogenase (EC 1.1.1.37) activity was assayed as described by Lai & Clark (1976). Hexokinase (EC 2.7.1.1) was measured essentially as described by Hernandez & Crane (1966). The reaction mixture (1 ml) contained the following (final concentrations): 50mM-Tris/HCl, pH7.5, 5mM-glucose, 8mM-ATP, 10mM-MgCl₂, 1mM-dithiothreitol, 0.5mM-NADP⁺ and 1.75 units of glucose 6-phosphate dehydrogenase. The blank contained no ATP and the assay was linear up to 0.4mg of protein. The presence of 0.1% (v/v) Triton X-100 did not increase the observed rate of activity, illustrating the absence of synaptosomes from the mitochondrial preparation.

Lactate dehydrogenase activity was assayed as described by Clark & Nicklas (1970). Pyruvate dehydrogenase (EC 1.2.4.1) was assayed essentially as described by Reed & Willms (1966). In a final volume of 1ml the reaction mixture contained the following components (final concentrations): 100mM-Tris/HCl, pH8.0, 10mM-NAD⁺, 1 μ M-rotenone, 10mM-oxalate, 2mM-thiamin pyrophosphate, 5mM-MgCl₂, 1mM-dithiothreitol, 1mM-CoA and 0.1% Triton X-100. The reaction was initiated by the addition of 10mM-pyruvate. Active and total pyruvate dehydrogenase were determined as follows. As a routine 5mg of mitochondrial protein was incubated in 700 μ l of (final concns.): 130mM-KCl/20mM-Tris/HCl/5mM-KH₂PO₄, final pH7.2. The mitochondria were maintained in respiratory State 4 (Chance & Williams, 1956) by the presence of 5mM-glutamate and 2.5mM-malate. Two preliminary samples (40 μ l each) were taken and assayed for active pyruvate dehydrogenase as outlined above, and then MgCl₂ and CaCl₂ were added in a minimal volume (10 μ l) to final concentrations of 10 and 1mM respectively. These conditions are known to activate pyruvate dehydrogenase to its fully activated state (R. F. G. Booth & J. B. Clark, unpublished work). Samples (40 μ l) were then withdrawn from the incubation and assayed for pyruvate dehydrogenase activity at 1, 5, 10 and 15min after the addition of the cations. Essentially the pyruvate dehydrogenase was fully activated between 5 and 10min and maintained its activity for the remainder of the incubation period.

Studies of the effect of various compounds on the binding of hexokinase to brain mitochondria

Mitochondria (approx. 5mg of protein) were incubated in 700 μ l of media (see Figure legends). Two initial samples of 100 μ l each were taken and the mitochondria rapidly separated from their suspending medium by centrifugation at 15000g in an Eppendorf 3200 bench centrifuge for 2min. The supernatant was assayed for hexokinase activity. Glucose 6-phosphate was added to the incubation,

and further samples were taken at regularly timed intervals and treated as outlined above. Finally an assessment was made of the total hexokinase in a sample of the incubation medium, and that released by treatment of the mitochondria with the various substances was expressed as a percentage of the total (bound and free) observed activity.

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin as standard.

Calculation of results

Total tissue activity of pyruvate dehydrogenase was calculated thus:

$$\frac{\text{Total enzyme activity in mitochondrial fraction}}{\text{recovery of citrate synthase in mitochondria (\%)}} \\ = \text{total tissue enzyme activity (\mu mol/min)}$$

Total tissue hexokinase activity was calculated from homogenate activities. Total mitochondrially bound hexokinase activities, expressed per wet wt. of tissue, were corrected for cytoplasmic (synaptoplasmic) contamination by use of the lactate dehydrogenase activity associated with mitochondria as an indicator of cytosolic contamination. Thus, if a = total homogenate hexokinase activity (see Fig. 4) and b = % of lactate dehydrogenase recovered in mitochondrial fraction (see Table 1), then $a \times b$ = contamination of mitochondrial fraction by cytosolic hexokinase (c). Thus, if d = observed mitochondrial hexokinase activity, then $d - c$ = bound mitochondrial hexokinase activity (e). Thus, if f = total bound mitochondrial hexokinase activity/g wet wt. and g = % of mitochondrial yield/g wet wt. based on citrate synthase recoveries (Table 1), then

$$f = \frac{e \times 100}{g} \text{ units/g wet wt.}$$

Thus, if h = total soluble (cytosolic) hexokinase activity per g wet wt., then

$$h = a - f$$

if a is expressed in units/g wet wt. This calculation actually overestimates the amount of contaminating cytosolic hexokinase associated with the mitochondria, as the total homogenate activity is used as the basis of the calculation, and this will include mitochondrially bound hexokinase. The extent of overestimation will increase as the animal gets older and the percentage of the total hexokinase which is mitochondrially bound increases. Thus the estimates of mitochondrially bound hexokinase must be considered as minimal values.

Table 1. *Enzyme and protein recovery in neonatal rat brain mitochondrial preparations*

The brain wt. is the mean $\pm$ s.d. derived from each individual animal (no. of animals = n). The yield of mitochondrial protein/g of tissue is given as a mean together with range of values, since each mitochondrial preparation is derived from several brains (eight to ten). Enzyme activities are measured in units/g wet wt. where 1 unit equals an activity of $1 \mu\text{mol}$ of product formed/min at 25°C . Each value represents the mean of at least three determinations on each mitochondrial preparation and at least three distinct mitochondrial preparations were made for each age (except for acetylcholinesterase, which was measured in triplicate on a single mitochondrial preparation from each age).

Age (days)	Brain wt. (g)	Yield of mito- chondria (mg of protein/g of tissue)	Total recovered enzyme activity									
			Citrate synthase			Lactate dehydrogenase			Acetylcholinesterase			
			Mitochondrial			Mitochondrial			Mitochondrial			
			Homogenate (units/g)	(units/g)	% of homogenate	Homogenate (units/g)	(units/g)	% of homogenate	Homogenate (units/g)	(units/g)	% of homogenate	Homogenate (units/g)
5	0.35 ± 0.06 (n = 36)	0.82 (0.76–0.85)	9.10 ± 0.05	0.69 ± 0.03	7.6	34.6 ± 2.9	0.26 ± 0.06	0.74	1.4	0.01	0.93	
10	0.63 ± 0.11 (n = 35)	1.16 (0.85–1.6)	15.0 ± 1.9	0.85 ± 0.14	5.7	56.8 ± 0.1	0.36 ± 0.03	0.58	2.5	0.46	1.84	
15	1.01 ± 0.03 (n = 30)	0.94 (0.7–1.12)	20.8 ± 3.0	0.83 ± 0.11	4.0	69.6 ± 10.1	0.37 ± 0.01	0.53	3.5	0.03	0.86	
21	1.11 ± 0.01 (n = 17)	1.70 (0.85–2.87)	30.2 ± 1.6	1.65 ± 0.13	5.5	112 ± 1.2	0.37 ± 0.04	0.33	6.2	0.04	0.64	
Adult	1.34 ± 0.01 (n = 16)	1.36 (0.81–1.90)	29.6 ± 1.8	1.42 ± 0.14	4.8	118 ± 7.3	0.14 ± 0.03	0.11	7.2	0.03	0.42	

Results

Properties of mitochondria from developing rat brain

In view of reports (Holtzman & Moore, 1973) about the purity and integrity of mitochondria from developing rat brain, Tables 1 and 2 have been introduced to provide some evidence of the degree of contamination and the metabolic integrity of mitochondria prepared by the method of Clark & Nicklas (1970) from developing rat brain. Table 1 also provides data on the recovery of citrate synthase, which is necessary for the calculation of total tissue activities of mitochondrial enzymes. There is a marginal increase in the actual yield of mitochondrial protein per g wet wt. of brain as the animal gets older, which relates presumably to the increase in mitochondrial protein, as distinct from mitochondrial numbers, seen during development (see Fig. 1; Gregson & Williams, 1969). Also evident from Tables 1 and 2 are the degrees of cytosolic and membrane contamination of these mitochondria, as judged by the lactate dehydrogenase (Johnson & Whittaker, 1963) and acetylcholinesterase activities (Cotman & Matthews, 1971). Table 2 outlines the specific activities of a number of enzymes associated with the tricarboxylic acid cycle of mitochondria derived from 21-day-old rat brains as compared with adult rat brains. At 21 days the specific activities of both malate dehydrogenase and citrate synthase are essentially the same as that of the adult, whereas that of pyruvate dehydrogenase is approx. 55% of the adult value (see Fig. 1). The 3-hydroxybutyrate dehydrogenase has, however, at 21 days a specific activity almost 3 times higher than the adult value (e.g. Klee & Sokoloff, 1967; Page *et al.*, 1971).

Studies on the O₂ uptake of mitochondria prepared from brains of 21-day-old rats indicated no oxidation in the presence of NADH alone. However, respiratory-control ratios of 5.5 were found with pyruvate and malate as substrates (see Fig. 3) and 4 with 3-hydroxybutyrate and malate (see Fig. 2).

With 10mM-glutamate plus 2.5mM-malate or 10mM-succinate as substrates, oxidation rates and respiratory-control ratios were comparable with those found in mitochondria prepared from the brains of adult rats (Lai & Clark, 1976).

Development of mitochondrial enzyme activities in rat brain

The enzyme studies reported here have been carried out on the purified mitochondrial preparations reported above. Measurement of the 3-hydroxybutyrate dehydrogenase in these mitochondria from brains of different ages confirmed the general pattern of development previously reported by Page *et al.* (1971), namely an activity which increases rapidly to a maximum (1.36 units/g wet wt.) during the period 10–20 days after birth and then declines to a value of approx. 35% of this maximum in the adult (results not shown: see Table 2). In contrast, however, the pyruvate dehydrogenase activity (Fig. 1) is below the limits of detection at day 1, then develops slowly until 15 days, when there is a sharp increase in the rate of development which continues until about 30 days after birth, when the adult values are reached and maintained. The values of the pyruvate dehydrogenase reported in Fig. 1 represent the active portion of the enzyme; it is, however, noteworthy that, until 15 days after birth, almost 100% of the total pyruvate dehydrogenase present is in the active form (R. F. G. Booth, J. M. Land & J. B. Clark, unpublished work). After this period, as the total pyruvate dehydrogenase activity increases, the portion of it that is present in the active form declines to about 70% of the total, which is the value normally found in the mature brain (Siess *et al.*, 1971; Cremer & Teal, 1974). Further, the adult value of the total pyruvate dehydrogenase activity reported here (approx. 3 units/g wet wt. of tissue at 25°C) is approximately 3 times that previously reported (Siess *et al.*, 1971; Cremer & Teal, 1974; Wilbur &

Table 2. *Enzyme activities in brain mitochondria derived from neonatal and adult rats*

Enzymes were assayed as outlined in the Experimental section. The results are the means $\pm$ s.d. of at least three determinations on each mitochondrial preparation, where *n* is the number of separate preparations made at each age.

Enzyme	Specific activity (nmol/min per mg of protein)	
	21-day-old	Adult
Pyruvate dehydrogenase	38.3 $\pm$ 2.4 (<i>n</i> = 5)	72.0 $\pm$ 6.1 (<i>n</i> = 14)
3-Hydroxybutyrate dehydrogenase	45.4 $\pm$ 4.1 (<i>n</i> = 3)	16.1 $\pm$ 1.1 (<i>n</i> = 5)
Citrate synthase	1100 $\pm$ 85 (<i>n</i> = 5)	1018 $\pm$ 104 (<i>n</i> = 14)
Glutamate dehydrogenase		
(NAD-linked)	1267 $\pm$ 65 (<i>n</i> = 3)	578 $\pm$ 44 (<i>n</i> = 9)
(NADP-linked)	1115 $\pm$ 88 (<i>n</i> = 3)	490 $\pm$ 39 (<i>n</i> = 4)
Malate dehydrogenase	8359 $\pm$ 140 (<i>n</i> = 5)	8791 $\pm$ 151 (<i>n</i> = 7)
Lactate dehydrogenase	367 $\pm$ 45 (<i>n</i> = 3)	151 $\pm$ 15 (<i>n</i> = 5)
Acetylcholinesterase	42 $\pm$ 4 (<i>n</i> = 5)	27 $\pm$ 3 (<i>n</i> = 5)

Fig. 1. Development of pyruvate dehydrogenase, citrate synthase and protein in rat brain mitochondria

All enzyme values are expressed as percentage of the adult values, which were: pyruvate dehydrogenase (Δ), 2.05 ± 0.20 units/g wet wt. ($n=4$); citrate synthase ($\bullet$), 29.6 ± 1.8 units/g wet wt. ($n=4$). The pyruvate dehydrogenase values represent the active portion of the enzyme (see the Experimental section for assay), and the adult enzyme values are expressed $\pm$ S.D. bars, which are representative of the whole developmental period. Each point represents at least three distinct measurements of the enzyme activity from a mitochondrial preparation derived from eight to ten litter mates. In most cases the values represent a mean from two or three distinct mitochondrial preparations performed at different times on animals of the same age. The mitochondrial protein values ($\circ$) are also expressed as a percentage of the adult value $\pm$ S.D. (28.3 ± 2.4 mg of protein/g wet wt.; $n=4$) and represent a calculated estimate of the total mitochondrial protein present/g wet wt. of tissue, based on the percentage recoveries of citrate synthase shown in Table 1.

Patel, 1974). Fig. 1 also shows the developmental pattern of the citrate synthase in brain mitochondria as well as the development of total brain mitochondrial protein. Citrate synthase has been used throughout this work as a marker mitochondrial tricarboxylic acid-cycle enzyme because of its exclusive mitochondrial location and relative ease of measurement in both homogenates and mitochondria. In common with other enzymes of the tricarboxylic acid cycle (Wilbur & Patel, 1974; MacDonnell & Greengard, 1974; Baquer *et al.*, 1975), it shows a developmental pattern in which the activity increases rapidly at about 7–8 days after birth until 20 days, when the adult value is reached and maintained, a pattern which correlates well with the increase in mitochondrial protein. When pyruvate dehydrogenase is compared with citrate synthase, however, it is evident that the development of the citrate synthase precedes that of the pyruvate dehydrogenase by about 1 week and that the pyruvate dehydrogenase activity is still increasing after development of both citrate synthase and mitochondrial protein has ceased.

Fig. 2. O_2 uptake by rat brain mitochondria, utilizing DL-3-hydroxybutyrate + malate, as a function of age

Rat brain mitochondria were isolated and incubated in a medium containing 100 mM- K^+ (see the Experimental section) and 10 mM-DL-3-hydroxybutyrate + 2.5 mM-malate (State-4 respiration). State 3 was induced by the addition of 250 μ M-ADP. State 3 ($\bullet$) and 4 (Δ) are defined as in Chance & Williams (1956). Respiration rates are expressed as ng-atoms of O/min per mg of mitochondrial protein at 25°C and are mean values of at least two determinations.

Fig. 3. O_2 uptake by rat brain mitochondria utilizing pyruvate + malate as a function of age

All conditions were as in the legend to Fig. 2, except that 5 mM-pyruvate + 2.5 mM-malate was used as substrate.

Figs. 2 and 3, in which the ability of brain mitochondria from rats of different ages to oxidize 3-hydroxybutyrate and pyruvate is recorded, also show that the development of the carbon flux through 3-hydroxybutyrate dehydrogenase and pyruvate dehydrogenase actually mirrors the development of the total enzyme activities measured *in vitro* (Fig. 1, Table 2).

We have also studied the development of the brain hexokinase with respect both to age and to its association with the mitochondria (Fig. 4). The total brain hexokinase activity [mitochondrial (bound) and cytosolic (soluble)] at 5 days is approx. 5 units/g wet wt., which is about 40% of the value of the adult (12 units/g wet wt.). As with the pyruvate

dehydrogenase the main increase in hexokinase activity occurs at approx. 15 days, the adult value being reached at about 25 days. However, when the respective contributions of the bound and soluble hexokinase to the total are assessed, it is evident that the main part of this increase in hexokinase activity is occurring in the bound form of the enzyme. Between 5 and 15 days the bound (mitochondrial) hexokinase remains essentially constant (2–3 units/g wet wt.) at a value approximately of the same order as the soluble (cytosolic) hexokinase (2.8 units/g wet wt.). However, after 15 days the bound hexokinase increased rapidly to values 3–4 times (8.5 units/g wet wt. in the adult rat brain) those found before this period, whereas the soluble (cytosolic) hexokinase increases only very marginally (3.5 units/g wet wt. in the adult rat brain). Further, Fig. 5 shows that the hexokinase is bound to brain mitochondria in a selective fashion (cf. Rose & Warms, 1967; Wilson, 1968). Incubation of brain mitochondria in the presence of high concentrations of KCl or sucrose $\pm$ phosphate only leads to a release of 10–15% of the total hexokinase. If glucose 6-phosphate is added to the incubation in KCl, there is only a small increase in the amount of hexokinase released (approx. 20%

Fig. 4. Development of hexokinase in rat brain

Hexokinase activity was measured in homogenates and mitochondrial fractions as outlined in the Experimental section, and activities are expressed as units/g wet wt. Triton (0.1%) was included in the homogenate assay. Each value is the mean of at least two or three determinations on each preparation. The total soluble activity is calculated from the difference in the total homogenate and total mitochondrial activities. The total mitochondrial activity is assessed by first subtracting the estimated cytosolic contamination and then calculating the total tissue value by reference to the mitochondrial recovery based on the citrate synthase activities (see 'Calculation of results'). ▲, Total hexokinase; ○, mitochondrial (bound) hexokinase; ●, cytosolic (free) hexokinase.

Fig. 5. Release of bound rat brain mitochondrial hexokinase by glucose 6-phosphate

Mitochondria from adult rat brains were prepared as outlined in the Experimental section and then incubated at 25°C with stirring in iso-osmotic media containing 5mM-glutamate, 2.5mM-malate and (a) 0.27M-sucrose, 20mM-Tris/HCl, pH 7.2 (○); (b) 0.26M-sucrose, 20mM-Tris/HCl, 5mM-potassium phosphate, pH 7.2 (●); (c) 0.135M-KCl, 20mM-Tris/HCl, pH 7.2 (□); (d) 0.130M-KCl, 20mM-Tris/HCl, 5mM-potassium phosphate, pH 7.2 (■). Samples (100 μ l) were removed at 2min and 1min before the addition of glucose 6-phosphate and 1 and 2min thereafter. The mitochondria were separated from the incubation medium by centrifugation at 12000g for 2min in an Eppendorf microcentrifuge. The supernatants were then immediately assayed for hexokinase activity as described in the Experimental section. The results are expressed as the amount of enzyme in the supernatant as a percentage of the total (bound+free) enzyme present.

of total hexokinase). However, when glucose 6-phosphate is added to brain mitochondria incubated in a sucrose-containing medium iso-osmotic with the KCl medium but with no phosphate, there is an extensive release of the bound hexokinase which approaches 50% of the total at glucose 6-phosphate concentrations above 0.5mM. If, however, 5mM-phosphate is included in the sucrose medium, the release is inhibited such that only 35–40% of the hexokinase is released at 1mM-glucose 6-phosphate (cf. Tuttle & Wilson, 1970). Also apparent from the data of Table 3 is that the mitochondrially bound hexokinase may affect the respiration of mitochondria by acting as an indirect 'ATPase' and thereby maintaining ADP concentrations. The experiments of Table 3 were carried out in 100mM-K⁺ medium

Table 3. *Effect of bound hexokinase on brain mitochondrial respiration*

O₂-uptake studies were carried out in the 100mM-K⁺ medium (see the Experimental section) in the presence of 5mM-Mg²⁺ at 25°C at a protein concentration of approx. 0.5mg of mitochondrial protein/ml; 10mM-glucose was either present or absent as indicated. Pyruvate (5mM) and malate (2.5mM) were added as substrates and 250μM-ADP to induce State 3 [State 3 and 4 are as defined by Chance & Williams, (1956)]. The rates are measured in ng-atoms of O₂/min per mg of mitochondrial protein, and are expressed ±s.d., each being the means representative of at least three experiments.

Medium	Additions	Respiration rate (ng-atoms of O ₂ /min per mg of protein)
+Glucose	Pyruvate+malate	53 ± 12
	+ADP (State 3)	114 ± 11
	State 4	88 ± 8
-Glucose	Pyruvate+malate	56 ± 7
	+ADP (State 3)	116 ± 0.3
	State 4	64 ± 5

in the presence of Mg²⁺. This accounts for the relatively high State-4 (i.e. in the absence of ADP) respiration rate and the low respiratory-control ratio. In the absence of glucose (i.e. hexokinase inactive) the State-4 rate before and after ADP stimulation is not markedly different, when glucose is present (i.e. hexokinase active) the State-4 rate after ADP addition is almost 70% higher than that before ADP addition. This suggests that the hexokinase activity is maintaining a high intramitochondrial ADP concentration and hence a relatively high rate of respiration as compared with the normal State 4. This is supported by the data of Fig. 6, which show the rate of glucose 6-phosphate production under the conditions of Table 3. Glucose 6-phosphate production is linear with time for the first 3 min (36nmol/min per mg of protein), after which time O₂ may become rate-limiting. However, in the presence of atractyloside (final concentration 33μM), the rate of glucose 6-phosphate production was inhibited by 50% and was linear for the entire 6min incubation period.

Discussion

Although Hawkins *et al.* (1971) demonstrated considerable plasma concentrations of glucose circulating in the suckling rat, the large negative arteriovenous difference for lactate across the young brain suggests that the oxidative utilization of glucose in the brain of suckling rats is limited (Cremer & Heath, 1974). The reason for this does not appear to be a low tricarboxylic acid-cycle activity, since all the enzymes of the cycle so far studied develop

Fig. 6. *Glucose 6-phosphate production by hexokinase bound to rat brain mitochondria*

Mitochondria from adult rat brains were prepared as outlined in the Experimental section. These were incubated at a concentration of 2mg/ml in the 100mM-K⁺ medium (see the Experimental section), which had been oxygenated by bubbling with O₂/CO₂ (19:1) and contained 5mM-Mg²⁺, 10mM-glucose, 1mM-ADP, 5mM-pyruvate and 2.5mM-malate at 25°C. Samples (0.4ml) were removed at 0, 1, 2, 3, 4 and 6 min, and quenched, neutralized and assayed for glucose 6-phosphate as outlined in the Experimental section. Atractyloside when present (○) was added at a final concentration of 33μM, 1min before the reaction was initiated with ADP. ●, Control.

early after birth, and ketone bodies are metabolized quite actively (Benjamins & McKhann, 1972; Williamson & Buckley, 1973). A possible explanation lies in the relatively late development of the pyruvate dehydrogenase complex (see Fig. 1), such that at 20 days of age (the usual weaning age of rats) the pyruvate dehydrogenase has attained only 50–60% of its adult activity, whereas the tricarboxylic acid-cycle and ketone-body-utilizing enzymes are fully developed. Thereafter the pyruvate dehydrogenase activity increases to its adult value (Fig. 1), the tricarboxylic acid-cycle enzymes remain constant (Benjamins & McKhann, 1972; MacDonnell & Greengard, 1974; Baquer *et al.*, 1975), but the ketone-body-utilizing enzymes decrease in activity (Williamson & Buckley, 1973). In addition to the relatively low pyruvate dehydrogenase activity, the

brains of suckling rats also possess a low hexokinase activity whose total activity does not develop significantly until the latter part of the suckling period (15 days; see Fig. 4). As in the heart (Font *et al.*, 1975), the brain hexokinase is distributed between the cytoplasmic and mitochondrial compartments, and it appears to be the mitochondrially bound form whose activity increases during the postnatal period (Fig. 4). During the neonatal period and in adulthood the cytoplasmic hexokinase activity appears to remain essentially constant (approx. 3.5 units/g wet wt.). The low activity of hexokinase present during the suckling period is presumably concerned with providing substrate for the pentose phosphate pathway, which, together with fatty acid and myelin synthesis, is relatively high in activity during this period as compared with the adult (Kuhlman & Lowry, 1956; Winick, 1970; Volpe & Kishimoto, 1972; Baquer *et al.*, 1975). Additionally, a certain amount of pyruvate from glycolysis will be required as a substrate for pyruvate carboxylase in an 'anaplerotic' role to maintain the tricarboxylic acid-cycle carbon pool lost to the cytosol in biosynthetic activities in the young brain (e.g. lipid and transmitter synthesis). Evidence in support of this comes from the developmental pattern of brain pyruvate carboxylase, which shows a maximum activity at 20 days (0.5–0.6 unit/g wet wt.) and then declines in adulthood to about 50% of this value (Land & Clark, 1975). It is, however, only when glucose becomes the predominant energy fuel for brain that the necessity for the increase in hexokinase (and pyruvate dehydrogenase) activity becomes mandatory. Additionally, the timing of the increases in hexokinase and pyruvate dehydrogenase may also be significant, since it is at about 15 days in the rat that a marked increase in sensory responsiveness and elicited co-ordinated motor activity becomes apparent.

The coincidence of these developmental patterns raises the question as to whether there is any significance in the association of hexokinase with brain mitochondria. Insight into this phenomenon may be made by reference to work on energy metabolism in the heart (Jacobus & Lehninger, 1973; Saks *et al.*, 1976) and the association of creatine kinase with heart mitochondria. A somewhat analogous situation may be true for brain mitochondria and hexokinase. The normal adult brain, unlike the brain from the suckling rat and the heart, is almost 100% dependent on glucose oxidation for its energy requirements. Hence, for efficient brain function, not only is it necessary to provide a continual ATP supply for the maintenance of the state of polarization of the nerve membrane but also for a continual phosphorylation of glucose for glycolysis. Thus the association of the brain hexokinase with the mitochondria may be a device for providing ATP preferentially for glucose

phosphorylation over other less essential ATP-requiring processes. Preliminary observations have suggested that brain mitochondria also possess a bound creatine kinase (Jacobs & Klingenberg, 1964; Jacobus & Lehninger, 1973; R. F. G. Booth, unpublished work). Thus in the adult brain both the bound hexokinase and creatine kinase would be competing for ATP produced by oxidative phosphorylation, and the fine control of ATP utilization by these reactions may be influenced by the relative ratio of the hexokinase and creatine kinase activities bound to the mitochondria.

The efficient and continuing production of ATP from glycolysis and the tricarboxylic acid cycle requires, in addition to active glucose phosphorylation, that pyruvate be efficiently converted into acetyl-CoA by the pyruvate dehydrogenase complex. This enzyme, as isolated from a number of tissues, including brain (this paper; Hucho, 1975) is controlled in part by a phosphorylation (enzyme-inactivated)/dephosphorylation (enzyme-activated) process involving ATP. Further, in isolated hepatocytes, Siess & Wieland (1976) have shown a close correlation between the intramitochondrial phosphate potential ($[ATP]/[ADP] + [P_i]$) and the activation stage of the pyruvate dehydrogenase. Thus it seems probable that the strategic positioning of hexokinase and other kinases on the brain mitochondrial membrane may influence the intramitochondrial phosphate potential, such that the pyruvate dehydrogenase is maintained in the correct state of activation to satisfy the brain's energy requirements.

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