Comparative development of the pyruvate dehydrogenase complex and citrate synthase in rat brain mitochondria

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1. The enzyme activity of the pyruvate dehydrogenase complex (PDHC) was measured in mitochondria prepared from developing rat brain, before and after steady-state dephosphorylation of the E1α subunit. A marked increase in dephosphorylated (fully activated) PDHC activity occurred between days 10 and 15 *post partum*, which represented approx. 60% of the difference in fully activated PDHC activity measured in foetal and adult rat brain mitochondria. There was no detectable change in the active proportion of the enzyme during mitochondrial preparation nor any qualitative alteration in the detectable catalytic and regulatory components of the complex, which might account for developmental changes in PDHC activity. 2. The PDHC protein content of developing rat brain mitochondria and homogenates was measured by an enzyme-linked immunoadsorbent assay. The development of PDHC protein in both fractions agreed closely with the development of the PDHC activity. The results suggest that the developmental increase in PDHC activity is due to increased synthesis of PDHC protein, which is partly a consequence of an increase in mitochondrial numbers. However, the marked increase in PDHC activity measured between days 10 and 15 *post partum* is mainly due to an increase in the amount of PDHC per mitochondrion. 3. The development of citrate synthase enzyme activity and protein was measured in rat brain homogenates and mitochondria. As only a small increase in citrate synthase activity and protein was detected in mitochondria between days 10 and 15 *post partum*, the marked increase in PDHC protein and enzyme activity may represent specific PDHC synthesis. As several indicators of acquired neurological competence become apparent during this period, it is proposed that preferential synthesis of PDHC may be crucial to this process. 4. The results are discussed with respect to the possible roles played by PDHC in changes of respiratory-substrate utilization and the acquisition of neurological competence occurring during the development of the brain of a non-precocial species such as the rat.

INTRODUCTION

Pyruvate dehydrogenase complex (PDHC) is a multienzyme complex whose known components include E1 (pyruvate dehydrogenase, EC 1.2.4.1), E2 (dihydrolipoamide transacetylase, EC 2.3.1.12) and E3 (dihydrolipoamide dehydrogenase, EC 1.8.1.4). These enzymes collectively catalyse the oxidative decarboxylation of pyruvate and acetylation of CoA. In the mature mammalian brain, the complex assumes unique importance, primarily in glucose utilization (Smith & Sokoloff, 1981), but also in the synthesis of acetylcholine (Tucek & Cheng, 1974). As a consequence of the interposition of the complex between glycolysis and the tricarboxylic acid cycle, the complex is endowed with highly responsive allosteric and covalent regulatory mechanisms (Cate & Roche, 1978; Olson *et al.*, 1981). The complex is inactivated by pyruvate dehydrogenase kinase (EC 2.7.1.99), which phosphorylates the α-subunit of pyruvate dehydrogenase (E1), and is re-activated by pyruvate dehydrogenase phosphatase (EC 3.1.3.43), which dephosphorylates E1 (Randle, 1981; Reed, 1981). The concentration ratios acetyl-CoA/CoA and NADH/NAD^+ modulate both kinase and phosphatase reactions, so that pyruvate flux is responsive to the energetic and synthetic requirements of the cell (Cooper *et al.*, 1975; Olson *et al.*, 1981).

During the normal development of the rat brain, ketone bodies and glucose are respiratory substrates, whereas the energy requirements of the adult rat brain are amost entirely fulfilled by glucose oxidation (Page *et al.*, 1971; Cremer & Heath, 1974). Previous studies in our laboratory have suggested that the acquisition of neurological competence may be a correlate of the development of aerobic glycolytic flux, which may be in turn limited by the developmental increase in PDHC activity (Land *et al.*, 1977; Booth *et al.*, 1980; Leong & Clark, 1984). However, contributions to the increase in PDHC activity may be made by proliferation of mitochondria, an increase in the proportion of active complex or even a change in the subunit composition. In this study, we have investigated these possibilities by enzyme assays, reversible phosphorylation of E1, immunoblotting and quantitative immunochemical assay. The study was carried out on 10- and 15-day-*post-partum* brain homogenates and mitochondria, as previous studies indicated that mitochondrial-bound hexokinase and pyruvate dehydrogenase develop rapidly

Abbreviations used: PDHC, pyruvate dehydrogenase complex; ELISA, enzyme-linked immunoadsorbent assay.

during this time, suggesting that complete aerobic utilization of glucose correlated with the acquisition of co-ordinated motor control and visual perception (Booth *et al.*, 1980). The results of the present study show that there is little or no qualitative alteration in the subunit composition of PDHC or the ability of the complex to be reversibly deactivated by phosphorylation and dephosphorylation. However, immunochemical and enzyme assays suggest that, between days 10 and 15 *post partum*, a specific and preferential increase in the amount of PDHC per mitochondrion results in a marked increase in PDHC activity. The results support the view that synthesis of PDHC correlates with the onset of neurological competence of the 'non-precocial' rat.

MATERIALS AND METHODS

Animals

Rats of the Wistar strain were used. Age was measured in days *post partum*. The age of foetal animals was estimated by counting the numbers of days elapsed since the detection of vaginal plugs. Adult animals were males of 60–70 days of age. Animals of either sex were used at the other ages. Adult rats were fed *ad libitum*, and immature rats were allowed to suckle as normal. The 10-day-old animals were characterized by a lack of motor co-ordination and visual perception; 15-day-old animals had acquired both these apparent indices of neurological competence.

Chemicals

The sources of chemicals used in the preparation of mitochondria and in enzyme assays were as described previously (Leong & Clark, 1984). [^{32}P]P$_i$ was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Glyceraldehyde 3-phosphate was purchased from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, glycine and Tris were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Acrylamide and SDS were purchased from British Drug Houses, Poole, Dorset, U.K. Ammonium persulphate and *NN'*-methylenebisacrylamide were supplied by LKB, Bromma, Sweden. Pig anti-rabbit immunoglobulin, conjugated to horeseradish peroxidase, was purchased from Dako Ltd., High Wycombe, Bucks., U.K.

Preparation of mitochondria

Non-synaptic mitochondria were prepared from whole brain as described by Lai *et al.* (1977), with the modifications used by Booth & Clark (1978*a*) when preparing synaptosomes. Mitochondrial preparations used for SDS/polyacrylamide-gel electrophoresis and immunoblotting were made 1 mM with respect to phenylmethanesulphonyl fluoride and 5 mM to diaminobenzidine. Long-term storage of mitochondrial protein was at −196°C.

Protein determination

Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma; fraction V) as standard.

Enzyme assays

PDHC activity in mitochondria was measured at 25 °C as described by Reed & Willms (1966), by monitoring the pyruvate-dependent formation of NADH at 340 nm. The pyruvate dehydrogenase activity in rat brain homogenates was measured by coupling with arylamine acetyltransferase (EC 2.3.1.5). The formation of *N*-acetyl-*p*-(*p*-aminophenylazo)benzenesulphonate was monitored as a decrease in A_{460}. This assay gave similar PDHC activities in isolated mitochondria to those recorded by the assay of Reed & Willms (1966). Citrate synthase (EC 4.1.3.7) was measured as described by Clark & Land (1974). Enzyme activities were expressed as nmol/min (munits) per mg of protein.

Reversible phosphorylation

All incubations were carried out at 25 °C. [γ-^{32}P]ATP was generated when [^{32}P]P$_i$ and ATP were incubated for 1 h in the presence of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase (Glynn & Chappell, 1964; England, 1979). The incubation contained 50 mM-Tris/HCl, pH 8.0, 4 mM-$MgCl_2$, 0.8 mM-dithiothreitol, 8 mM-ATP, 8 mM-glycerate 3-phosphate, 3.4 mM-NAD^+, 0.5 mCi of [^{32}P]P$_i$, 700 munits of glyceraldehyde-3-phosphate dehydrogenase and 900 munits of 3-phosphoglycerate kinase. The specific ratioactivity of the resultant [γ-^{32}P]ATP was 113 ± 24 Ci/mmol ($n = 3$) as judged by reverse-phase h.p.l.c. and scintillation counting. More than 75% of the total radioactivity was recovered in the ATP peak.

Before phosphorylation in the presence of [γ-^{32}P]ATP, PDHC was dephosphorylated by incubating mitochondria at a final concentration of 3.5–5 mg/ml for 30 min in KCl medium [125 mM-KCl/20 mM-Tris/HCl 5 mM-KH_2PO_4 (pH 7.2)] plus 5 mM-$MgCl_2$ and 0.5 mM-$CaCl_2$ (Booth & Clark, 1978*b*). Mitochondria were recovered by centrifugation at 10000 ***g*** for 2 min and resuspended to approx. 4 mg of protein/ml in KCl medium containing 1 mM-[γ-^{32}P]ATP, 40 μg of oligomycin/ml, 20 μg of ionophore A23187/ml and 5 mM-K-EGTA. Phosphorylation was allowed to proceed for 15 min, and then the mitochondria were pelleted as described above and washed in KCl medium at 4 °C. Mitochondria were resuspended in KCl medium with the addition of 5 mM-$MgCl_2$ and 0.5 mM-$CaCl_2$. Samples were taken throughout the incubation and prepared for electrophoresis and autoradiography. This indicated that dephosphorylation was achieved within 5 min and that the small amount of radioactivity retained by the complex was not removed by extending the incubation for a further 25 min. In addition the activity measured after 5 min incubation with 5 mM-Mg^{2+} and 0.5 mM-Ca^{2+} was the same as that measured after 30 min incubation with Mg^{2+} and Ca^{2+} before deactivation with ATP.

SDS/polyacrylamide-gel electrophoresis and autoradiography

Samples (0.2 ml), taken during phosphorylation and dephosphorylation incubations, were mixed with 0.5 ml of 10% (w/v) trichloroacetic acid containing 50 mM-sodium phosphate. A sample was also prepared from mitochondria added to a mixture of [γ-^{32}P]ATP and 10% trichloroacetic acid, which was used as a control. Samples were incubated at 4 °C for at least 5 min. The precipitated protein was sedimented and washed six times in trichloroacetic acid/phosphate and then three times in diethyl ether. Samples were resuspended in 10% SDS by sonication and then diluted into electrophoresis sample buffer (Laemmli, 1970). Samples containing

approx. 130 μg of protein were separated on SDS/10%-polyacrylamide slab gels as described by Laemmli (1970). Completed gels were fixed in 50% (v/v) methanol/10% (v/v) acetic acid and stained with 0.1% (w/v) Coomassie Blue in the same solvent. After destaining in methanol/acetic acid, gels were equilibrated with 7% acetic acid, vacuum-dried and exposed to Kodak X-O-Mat film for 3–7 days at room temperature.

Preparation of antibodies

Bovine heart PDHC was purified as described by Stanley & Perham (1980), by using poly(ethylene glycol) precipitation but omitting gel filtration on Sepharose CL-2B. The purified complex had a specific activity of 380 munits/mg of protein and was stored at −196 °C. Citrate synthase from pig heart was purchased from Sigma. Polyclonal antibodies to PDHC and citrate synthase were raised in New Zealand White rabbits. The immunogen contained 0.35 mg of antigen/ml in phosphate-buffered saline (0.14 M-NaCl, 2.7 mM-KCl, 1.5 mM-KH_2PO_4, 8.1 mM-Na_2HPO_4), emulsified with 2.5 vol. of Freund's complete adjuvant. Rabbits were injected with 10 mg of antigen into two subcutaneous sites and one intramuscular site. Each rabbit then recieved three booster injections of 50 mg of antigen in incomplete adjuvant at 2-weekly intervals, and were bled 10 days after the last injection. The blood was allowed to clot overnight at 4 °C, and then decanted and centrifuged at 1000 ***g*** for 5 min at 4 °C. Immune serum and pre-immune serum controls were stored in small portions at −20 °C and re-centrifuged immediately before use.

Immunoblotting

After electrophoresis, gels were equilibrated for 45 min with electroblot buffer (10 mM-Tris/192 mM-glycine/20% methanol), and then transferred electrophoretically to nitrocellulose at 13 V/cm for 4 h in electroblot buffer at 4 °C. After transfer, the nitrocellulose sheets were stored overnight at 4 °C in phosphate-buffered saline, containing 3% (w/v) haemoglobin. Each blot was incubated with a 100-fold dilution of antisera for 1 h at room temperature and then washed three times (30 min each) in phosphate-buffered saline containing 0.1% Tween-20. The nitrocellulose sheets were then incubated with a 1:500 dilution of pig anti-rabbit immunoglobulins conjugated to horseradish peroxidase in phosphate-buffered saline/Tween-20 for 1 h. The blots were washed as above and developed by treatment with 1 mM-4-chloronaphth-1-ol in 0.01% (v/v) H_2O_2.

Enzyme-linked immunoadsorbent assay (ELISA)

Rat brain homogenate and mitochondrial protein were made to 1% (w/v) in SDS and then diluted to 50 μg of protein/ml with carbonate buffer (50 mM-Na_2CO_3, pH 9.6). The protein concentration was then re-checked by Folin assay (Lowry *et al.*, 1951). Dilutions of the protein were made in multi-well immunoassay plates (A/S Nunc, Roskilde, Denmark) from 0 to 5 μg of protein/ml in carbonate buffer. The plates were incubated with protein for 2 h, washed with PBT buffer [0.14 M-NaCl, 2.7 mM-KCl, 1.5 mM-KH_2PO_4, 8.1 mM-Na_2HPO_4, 0.5% (w/v) bovine serum albumin, 0.05% (w/v) Tween-20] and then incubated with PBT buffer for a further 2 h. To each well was then added 7.2 μg of anti-PDHC serum in PBT buffer. The wells were washed three times with PBT buffer and then incubated for 2 h with 0.4 μg of pig anti-rabbit immunoglobulins conjugated to horseradish peroxidase/well, diluted into PBT buffer. The wells were then washed as above and developed with 5 mM-*o*-phenylenediamine/10 mM-H_2O_2 in 0.1 M-sodium phosphate/citrate buffer, pH 5.0. For ELISA of mitochondrial protein the reaction was allowed to proceed for 5 min, but was increased to 15 min for ELISA of homogenate protein. The reaction was terminated by the addition of H_2SO_4 (final concn. 0.4 M) and the A_{437} of the reaction product was measured. The relationship between A_{437} and protein concentration was linear for the first 15 min of the reaction and for up to 1 μg of either mitochondrial or homogenate protein per well. The PDHC content was determined in A_{437} units/ng of total protein, by linear-regression analysis. The PDHC content of mitochondria and homogenates prepared from developing rat brain was expressed as a percentage of that measured in the corresponding fraction prepared from adult rat brain.

RESULTS

PDHC activity in mitochondria of developing brain: measurement of active proportion and total activity

The PDHC activity of freshly prepared mitochondria was measured and compared with the activity after steady-state dephosphorylation (Table 1, columns *b–d*). The steady state was established by a time course of enzyme assays of mitochondria incubated with 0.5 mM-Ca^{2+} and 5 mM-Mg^{2+}. These indicated that maximal activity was attained after 5 min and remained unchanged during the next 25 min. Table 1 shows that the PDHC activity of freshly prepared mitochondria increases with age, with a particularly large increment occurring between days 10 and 15 *post partum*. The marked increase in steady-state dephosphorylated (fully activated) PDHC activity occurring between these times accounts for about 60% of the change in activity from foetus to adult. It is also apparent that there is no change in the active proportion of the enzyme during the isolation of mitochondria (see the Discussion section).

Subunit composition of PDHC during development

The presence of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase was demonstrated by reversible phosphorylation in the presence of [γ-^{32}P]ATP. Identification of the E1α subunit was performed by SDS/polyacrylamide-gel electrophoresis, immunoblotting and autoradiography (Fig. 1). Lane (*e*) in Fig. 1 shows an immunoblot of mitochondrial protein incubated under phosphorylating conditions. The antibody reacts most strongly with the E2, E1α and E1β subunits. The E3 subunit and component X (De Marcucci *et al.*, 1985) were not detected. In addition the E2 subunits of rat brain PDHC (Fig. 1, lane *e*) and bovine heart PDHC (Fig. 1, lane *f*) are dissimilar, as reported by Sheu & Kim (1984). This is a species, not tissue, difference (results not shown). In addition to E2 and E1 subunits, the blot shows two components immunochemically stained by PDHC antibody, whose mobility is intermediate between those of E1α and E1β (arrowheads; Fig 1, lane *e*). These components are very minor components of purified bovine PDHC (Fig. 1, lane *f*) which are barely detectable with Coomassie Blue. Their prominence is increased by prolonged storage of

Table 1. **PDHC activity in freshly prepared mitochondria compared with that after steady-state dephosphorylation and rephosphorylation**

In column (*a*) 'Age' F = foetal or 18–21 days gestation; 10 = 10 days *post partum*; 15 = 15 days *post partum*; A = adult. PDHC activity is expressed as mean ± S.D. (for *n* separate experiments), in nmol/min per mg of mitochondrial protein. Column (*c*) shows activity measured after 30 min incubation with 5 mM-Mg^{2+} and 0.5 mM-Ca^{2+}. In column (*d*), active proportion is calculated as 100 × (*b*)/(*c*). Column (*e*) shows PDHC activity after 15 min incubation with 1 mM-ATP. In column (*f*), active proportion is calculated as 100 × (*e*)/(*c*).

Age (*a*)	PDHC activity of freshly prepared mitochondria (*b*)	PDHC activity after steady state dephosphorylation (*c*)	Proportion (%) of (%) of fully active complex (*d*)	PDHC activity after phosphorylation (*e*)	Proportion (%) of fully active complex (*f*)
F	39.3 ± 4.0 (*n* = 7)	46.7 ± 4.6 (*n* = 7)	84.1	7.95 ± 1.5 (*n* = 5)	17.0
10	54.7 ± 4.9 (*n* = 8)	63.4 ± 5.0 (*n* = 9)	86.3	10.8 ± 1.7 (*n* = 3)	17.0
15	77.6 ± 11.1 (*n* = 7)	94.2 ± 9.8 (*n* = 15)	82.2	12.8 ± 3.0 (*n* = 6)	13.6
A	81.4 ± 10.5 (*n* = 12)	101.3 ± 5.7 (*n* = 11)	80.3	15.1 ± 3.5 (*n* = 5)	14.9

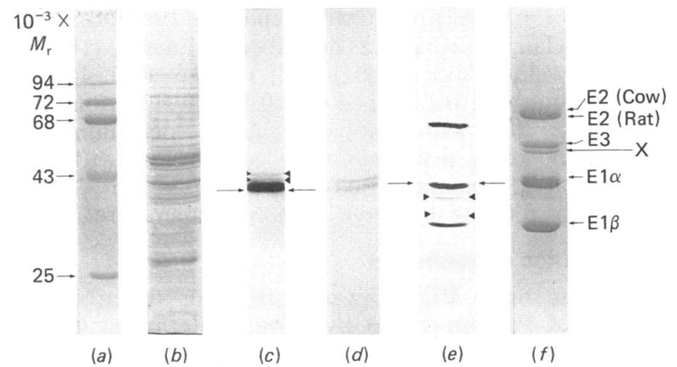

Fig. 1. Identification of E1α subunit of PDHC as the major phosphorylated component of brain mitochondria incubated with [γ-^{32}P]ATP

Adult brain mitochondrial protein (130 μg) was separated by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue (lane *b*). The gels were then vacuum-dried and exposed to Kodak X-O-Mat film. Autoradiographs of mitochondrial protein are shown after incubation with [γ-^{32}P]ATP (lane *c*) or incubation with 0.5 mM-Ca^{2+} and 5 mM-Mg^{2+} after prior incubation with [γ-^{32}P]ATP (lane *d*). After electrophoresis, some gels were used to produce electroblots, which were immunochemically stained for PDHC (lane *e*). Note that the major radioactive protein in phosphorylated mitochondria (lane *c*) has a similar M_r to the E1α component of purified bovine PDHC (lane *f*) and a mitochondrial polypeptide immunochemically stained by using antibodies to PDHC (lane *e*). Note also that the M_r of bovine heart PDHC E2 subunit (lane *f*) is dissimilar to that of rat brain PDHC E2 (lane *e*). M_r (× 10^{-3}) markers are shown in lane (*a*).

mitochondria at −20 °C in the absence of phenylmethanesulphonyl fluoride and diaminobenzidine (G. D. A. Malloch & J. B. Clark, unpublished work) and they may therefore be relatively stable proteolytic degradation products of higher-M_r subunits of the complex. When the radioactivity in small sections of immunoblots of phosphorylated mitochondrial protein was measured, it was found that 85 ± 7% (*n* = 3) of the total radioactivity in the entire length of the blot was located in the band immunochemically stained by PDHC antibody, which also had similar mobility to the E1α subunit of bovine PDHC. This strongly suggests that rat brain PDHC E1α subunit was the major phosphorylated protein of mitochondria in the incubation conditions used. Lane (*c*) in Fig. 1 shows an autoradiograph of mitochondrial protein separated by SDS/polyacrylamide-gel electrophoresis after incubation with [γ-^{32}P]ATP. Two minor polypeptides, of M_r 43500 and 45500 (arrowheads; Fig. 1, lane *c*), were also detected in addition to E1α. The M_r-43500 component is not dephosphorylated during re-activation of PDHC by Ca^{2+} and Mg^{2+}, whereas the M_r-45500 component is dephosphorylated at the same time as E1α (Fig. 1, lane *d*). Although the M_r-45500 component has similar mobility to branched-chain dehydrogenase E1 subunit (Patel & Olson, 1982), no cross-reactivity was noted between PDHC antibody and high loadings of purified branched-chain complex (results not shown). In view of the relative molecular mass of the M_r-45500 component and its phosphorylation properties, which are similar to those of PDHC-E1α, we tentatively

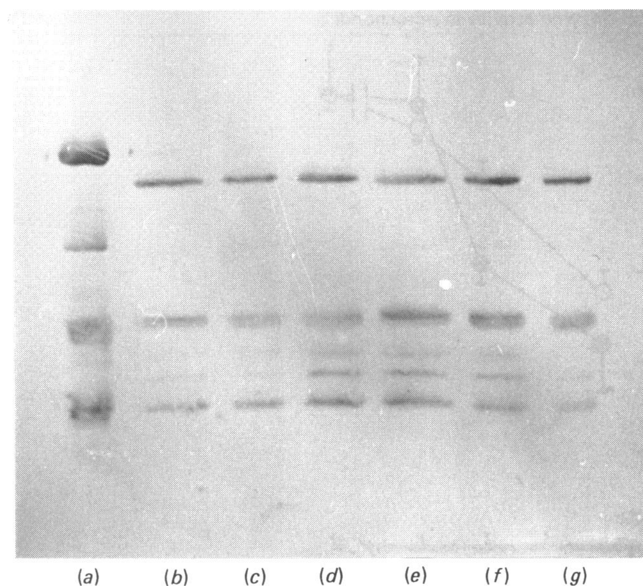

Fig. 2. Immunoblots of developing rat brain mitochondrial protein

Lane (*a*) is 5 μg of purified bovine PDHC. Lanes (*b*)–(*e*) show 50 μg of rat brain mitochondrial protein from foetal (*b*), 10-day (*c*), 15-day (*d*) and adult (*e*) rat brain. Lanes (*e*)–(*g*) inclusive show different loadings of adult rat brain mitochondria, which were 50 μg (*e*), 25 μg (*f*) and 12.5 μg (*g*).

propose that the M_r-45500 component is either the pyruvate dehydrogenase kinase or the E1 subunit of the branched-chain complex.

The demonstration of reversible phosphorylation of E1α is indicative of the presence of both pyruvate dehydrogenase kinase and phosphatase, which were detected at each age studied, with no qualitative age-related change in the results obtained (results not shown). At each age, between 75 and 99% of the total detected radioactivity was associated with E1α. Incubation of mitochondria with ATP also resulted in deactivation of PDHC enzyme activity (Table 1, columns *e* and *f*). The amounts of activity left after 15 min incubation of mitochondria from rat brains at different stages of development were similar, although neither the steady-state extent of phosphorylation nor the kinetics of phosphorylation were measured. Immunoblotting for PDHC in developing rat brain mitochondrial protein demonstrated the presence of all components recognized by the antibody, at each age studied (Fig. 2). These components were the E2, E1α and E1β subunits. The component X (De Marcucci *et al.*, 1985) was clearly identified in 5 μg of purified bovine complex (Fig. 2, lane *a*), but barely detectable among 50 μg or less of brain mitochondrial protein (Fig. 2, lanes *b*–*g*). The E3 component was not recognized in any lane on Fig. 2, which agrees with the low immunogenicity of E3 reported by De Marcucci *et al.* (1985). Careful comparison of Fig. 2 lanes (*b*)–(*e*) also suggests an increase in the amount of PDHC per mitochondrion, consistent with the increase in PDHC activity. This is apparent by comparison of the equal loadings of day-10 and -15 mitochondrial protein (Fig. 2, lanes *c* and *d*) in which the intensity of the bands intermediate between the E1α and E1β bands increases. The intensity of these bands, which are probably proteolytic breakdown products of the PDHC, may be used as a measure of PDHC mitochondrial protein, as indicated in the different loadings of adult mitochondrial protein (Fig. 2, lanes *e*–*g*). Fig. 2 also demonstrates that the apparent M_r of bovine E2 (Fig 2, lane *a*) is greater than that of rat brain E2 (Fig. 2, lanes *b*–*g*).

Measurement of PDHC in developing rat brain mitochondria and homogenates by ELISA

Immunoblotting suggested that increased PDHC activity might be due to an increase in enzyme protein concentration. Thus an ELISA was devised either to confirm or to disprove this. In addition, the development of PDHC in isolated mitochondria was compared with its development in homogenates to estimate the proportion of PDHC activity increase that could be attributed to an increase in mitochondrial numbers, as distinct from an increase in specific enzyme protein per mitochondrion. As the staining pattern of the PDHC did not change during development (Fig. 2), any quantitative change measured by ELISA was unlikely to be due to qualitative differences in the recognition of antigen at different developmental stages.

The PDHC content of homogenates and mitochondria was measured in absorbance units per ng of protein. The PDHC content at each age was then expressed as a

Table 2. Comparative development of PDHC activity and PDHC protein measured by ELISA in rat brain mitochondria and homogenates

For column (*a*), ages are as in Table 1. Column (*b*) shows PDHC activity measured in homogenates by coupled assay to arylamine acetyltransferase. Adult activity is 21 ± 1.1 nmol/min per mg of mitochondrial protein ($n = 4$). Columns (*c*) and (*e*) show PDHC measured by ELISA. Column (*d*) shows PDHC activity measured by pyruvate-dependent reduction of NAD^+ after steady-state dephosphorylation of E1α. Adult activity is 101.3 ± 5.7 nmol/min per mg of mitochondrial protein ($n = 11$). Values are expressed as means ± S.D. for *n* separate experiments.

	Homogenates		Mitochondria	
Age (*a*)	% of adult enzyme activity (*b*)	% of adult enzyme protein (*c*)	% of adult enzyme activity (*d*)	% of adult enzyme protein (*e*)
F	14.4 ± 4.0 ($n = 9$)	12.1 ± 4.4 ($n = 4$)	44.2 ± 9.7 ($n = 7$)	46.2 ± 4.5 ($n = 7$)
10	43.5 ± 2.3 ($n = 5$)	44.0 ± 2.3 ($n = 4$)	63.6 ± 4.8 ($n = 8$)	62.9 ± 5.0 ($n = 9$)
15	50.5 ± 4.7 ($n = 4$)	57.2 ± 6.8 ($n = 4$)	96.9 ± 11.0 ($n = 11$)	93.1 ± 9.6 ($n = 15$)

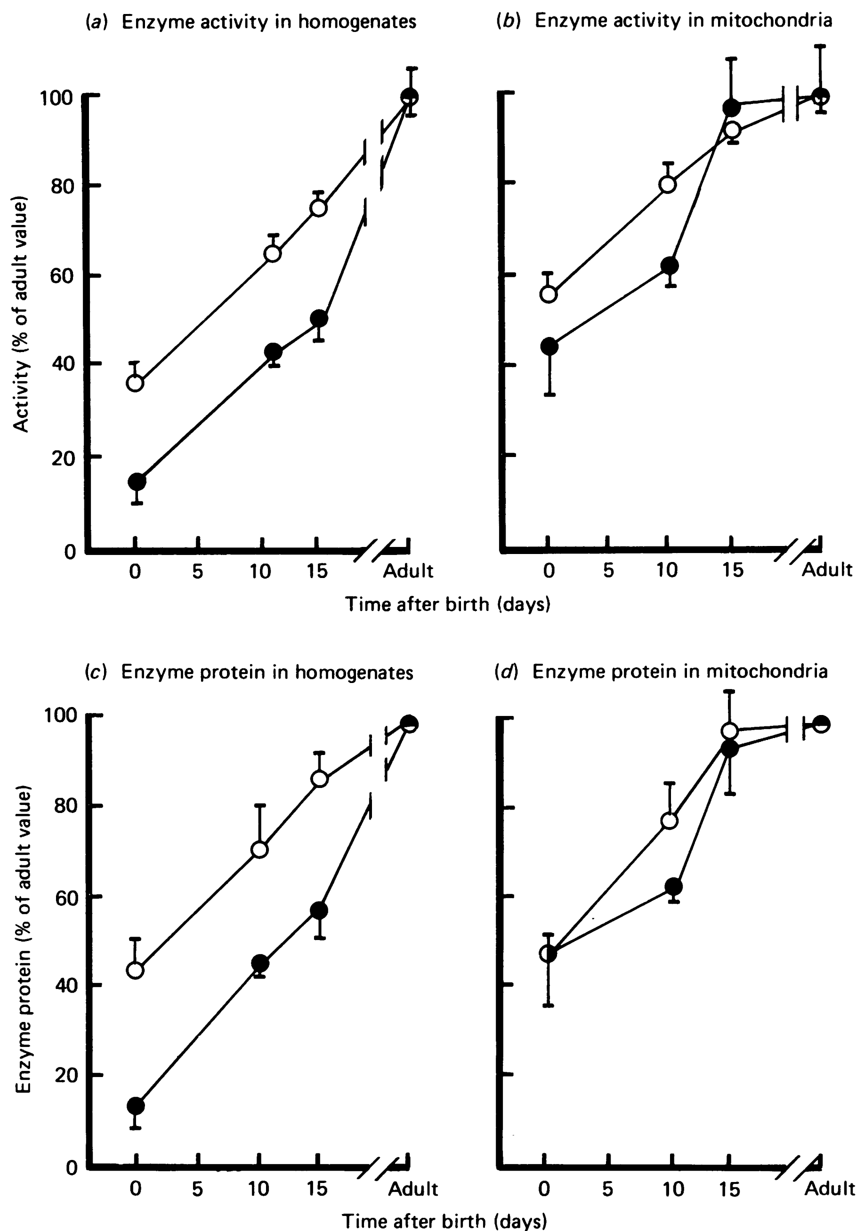

Fig. 3. Comparison of PDHC and citrate synthase development by ELISA

PDHC (●) and citrate synthase (○) in developing rat brain homogenates (panels *a* and *c*) and mitochondria (panels *b* and *d*) are shown on the basis of enzyme activity (panels *a* and *b*) and enzyme-specific protein measured by ELISA (panels *c* and *d*). Other experimental details are given in Table 1, and tabulated values are given in Tables 2 and 3. Error bars indicate ± S.D. when S.D. greater than size of symbol.

percentage of that measured in the corresponding fraction of adult rat brain. In Table 2 the data obtained from this analysis were compared with the development of steady-state dephosphorylated PDHC activity at each age, expressed as a percentage of that measured in the adult. Table 2 shows the close correlation of enzyme activity and enzyme protein in homogenates and mitochondria. This confirms that the measured PDHC activity increases during development, concomitant with an increase in enzyme protein. Comparison of the development of enzyme protein in homogenates and mitochondria suggests that proliferation in mitochondrial numbers contributes to the developmental increase in PDHC. This is particularly true after 15 days *post partum*, when the PDHC activity and enzyme protein content continue to increase in homogenates, but the amount of PDHC per mitochondrion is already at the adult value (Table 2, Fig. 3). There is, however, a large increase in PDHC per mitochondrion between days 10 and 15 *post partum*, whereas the increase in homogenates between these times is relatively limited. This clearly suggests that the measured increase in PDHC activity between days 10 and 15 *post partum* is mainly a product of an increase in the amount of PDHC protein per mitochondrion, as opposed to an increase in mitochondrial numbers.

Table 3. Comparative development of citrate synthase activity and citrate synthase protein measured by ELISA in rat brain mitochondria and homogenates

For column (*a*), ages are as in Table 1. Columns (*b*) and (*d*) show enzyme activity in homogenates and mitochondria measured by spectrophotometric monitoring of CoA-dependent reduction of dithionitrobenzene. Columns (*c*) and (*d*) show enzyme protein measured by ELISA. Other experimental details are as described in Table 1. Adult activities of citrate synthase were 312.2±12.0 nmol/min per mg of protein (n = 5) in homogenates and 1801.8±106.4 nmol/min per mg of protein (n = 5) in mitochondria. Values are expressed as means±S.D. for n separate experiments.

	Homogenates		Mitochondria	
Age (*a*)	% of adult enzyme activity (*b*)	% of adult enzyme protein (*c*)	% of adult enzyme activity (*d*)	% of adult enzyme protein (*e*)
F	37.2±3.4 (n = 8)	41.5±7.3 (n = 4)	54.9±6.5 (n = 7)	45.2±10.0 (n = 4)
10	66.0±3.6 (n = 9)	69.5±9.8 (n = 4)	80.1±4.7 (n = 8)	78.1±7.2 (n = 4)
15	76.0±2.0 (n = 5)	85.5±6.6 (n = 4)	94.0±2.2 (n = 5)	96.7±8.6 (n = 4)

Comparative development of PDHC and citrate synthase

The development of citrate synthase was measured in homogenates and mitochondria, by enzyme assay and ELISA (Table 3). The enzyme activity increases in proportion to the enzyme-specific protein in both fractions, as demonstrated for PDHC. Subtraction of the change in the development of citrate synthase protein and enzyme activity in mitochondria from that measured in homogenates also suggests that mitochondrial proliferation contributes to the increase in citrate synthase activity. However, whereas PDHC enzyme activity and protein content undergo a marked increase per mitochondrion between days 10 and 15 (Table 2), this cannot be demonstrated for either the development of citrate synthase activity or protein content of mitochondria (Table 3). This suggests that the increase in citrate synthase activity measured between days 10 and 15 may be mainly due to mitochondrial proliferation. The combined data of Tables 2 and 3 suggest that the development of PDHC lags behind that of citrate synthase and that the increase in PDHC per mitochondrion occurring between days 10 and 15 *post partum* is specific for PDHC. This is most clearly demonstrated in Fig. 3. Whereas citrate synthase has acquired about 80% of the adult activity in 10-day-old rat brain mitochondria, PDHC has acquired only about 60% of the activity of the adult mitochondrion by 10 days. In addition, whereas citrate synthase activity increases by less than 20% during days 10–15 post-partum, PDHC increases by more than 50%. Therefore the amount of PDHC per mitochondrion, and the consequent enzyme activity of the complex, increases more than that of citrate synthase immediately before and during the period at which the obvious indicators of acquired neurological competence become apparent.

DISCUSSION

This study has examined the development of PDHC in the rat brain to ascertain whether the increase in enzyme activity measured in this and previous studies (Land *et al.*, 1977; Booth *et al.*, 1980) was due to (*a*) a change in the active proportion of the enzyme during mitochondrial isolation, (*b*) a qualitative alteration of the subunit composition or (*c*) an increase in enzyme protein. It is clear that neither of the first two possibilities are likely. The complex was present in approx. 80% of its fully active form at each age studied, suggesting that previous studies (Land *et al.*, 1977; Booth *et al.*, 1980), in which only the active proportion of PDHC activity was measured, gave an accurate reflection of the developmental profile of the increase in total enzyme activity. However, this active proportion is unlikely to reflect the active proportion of the complex *in vivo*, as no steps were taken to preserve the phosphorylation state of the complex during preparation of mitochondria. Jope & Blass (1976) have shown that the proportion of active pyruvate dehydrogenase increases quickly to become equal to the total activity within 1 min of the onset of ischaemia. Furthermore, Booth & Clark (1981) have demonstrated a decrease in the active/inactive ratio of PDHC in neonatal-rat brain mitochondria, but not in adult, when incubated with ketone bodies.

Immunoblotting and reversible phosphorylation were used to demonstrate the presence of E2, E1α, E1β, pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase. The E3 component was not recognized by our antibody, and component X (De Marcucci *et al.*, 1985) of rat brain PDHC was only just detectable. However, all other components were identified by immunoblotting of mitochondria at each age studied. Reversible phosphorylation demonstrated the presence of highly specific and responsive phosphatase and kinase activity at each age studied. This demonstrates that PDHC retains the potential to regulate pyruvate flux when alterations of substrate utilization occur during development. The combination of the immunoblotting and reversible-phosphorylation data suggests that, at each age studied, all of the detectable regulatory and catalytic components of the complex are present, demonstrating that the increase in PDHC activity and the amount of PDHC protein measured by ELISA are not due to qualitative alteration of the subunit composition of the complex. The development of PDHC enzyme activity and PDHC antigen exhibit close correlation, which indicates that the increase in activity is probably a result of an increase in PDHC protein. This in turn means that PDHC synthesis must exceed turnover, and suggests that developmental regulation of PDHC activity is determined by simultaneous synthesis and assembly of the subunit proteins.

Comparison of the development of PDHC and citrate

synthase in isolated mitochondria and brain homogenates provided evidence that the developmental increase in enzyme activities and protein consists of two components. One of these components appears to be an increase in mitochondrial numbers and the other an increase in amount of enzyme protein per mitochondrion. Mitochondrial proliferation was assessed by comparing the development of PDHC and citrate synthase in homogenates and mitochondria. Both enzyme assays and ELISA confirm that there is a developmental increase in PDHC and citrate synthase enzyme activity and protein in homogenates, which is greater than the corresponding increase in activity and protein in the isolated mitochondrial fraction. The difference in the development of enzyme activities and enzyme protein is probably the contribution from mitochondrial proliferation. This is consistent with the increased yield of mitochondrial protein per g wet wt. of brain, which increased from 0.7 mg/g wet wt. in the foetus to 1.1 mg/g wet wt. at 15 days and 1.9 mg/g wet wt. in the adult. This was also observed by Land *et al.* (1977) when preparing mitochondria from developing rat forebrain. Most of the detectable increase in citrate synthase activity and protein (56%) occurred between birth and 10 days *post partum*. For PDHC about 60% of the increase in PDHC activity and protein occurs 10–15 days *post partum*, during which time there is only a small increase in citrate synthase activity and protein. This period of rat brain development is particularly significant. During this period there is a particularly rapid rise in oxygen consumption and decreased ability to survive anoxia (Fazekas *et al.*, 1941; Samson & Dahl, 1957). This period also corresponds with the time of emergence of electrical excitability in the cerebral cortex (Millichap, 1957), a pronounced change in neuro-muscular co-ordination, the acquisition of visual perception and a marked increase in the swimming ability of the animal (Schapiro *et al.*, 1970).

There is, in addition, evidence to suggest that the enzymes associated with the complete oxidation of glucose correlate with this onset of neurological competence (Booth *et al.*, 1980). In the 'precocial' guinea pig, which is born in a relatively advanced stage of development, the enzymes associated with the utilization of glucose develop before birth, whereas in the 'non-precocial' rat they develop after birth. Previous studies in our laboratory have indicated that the development of PDHC may lag behind other enzymes of energy metabolism (Land *et al.*, 1977; Booth *et al.*, 1980; Leong & Clark, 1984). This implicates PDHC development as an important influence in brain maturation, as the complex connects the flux of glycolytic products from glucose to the tricarboxylic acid cycle, which becomes the predominant pathway for energy metabolism in the normal mature brain. The relatively earlier development of citrate synthase. however, is consistent with the need for tricarboxylic-acid-cycle enzyme activity during the neonatal period of ketone-body utilization. The relatively lower content of PDHC per mitochondrion from birth to 10 days *post partum* is also consistent with the relatively higher arteriovenous difference for lactate in the neonatal rat brain (Hawkins *et al.*, 1971).

This study has shown conclusively that, during the onset of neurological competence, there is a relatively large increase in the amount of PDHC per mitochondrion, whereas, for another mitochondrial enzyme, citrate synthase, this is not the case. This is further evidence for the view that the development of a fully active aerobic glycolysis is a crucial factor in the development of neurological competence, and that PDHC is a key element in this process.

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REFERENCES

Booth, R. F. G. & Clark, J. B. (1978*a*) Biochem. J. **176**, 365–370
Booth, R. F. G. & Clark, J. B. (1978*b*) J. Neurochem. **30**, 1003–1008
Booth, R. F. G. & Clark, J. B. (1981) J. Neurochem. **37**, 179–185
Booth, R. F. G., Patel, T. B. & Clark, J. B. (1980) J. Neurochem. **34**, 17–25
Cate, R. L. & Roche, T. E. (1978) J. Biol. Chem. **253**, 496–503
Clark, J. B. & Land, J. M. (1974) Biochem. J. **140**, 25–29
Cooper, R. H., Randle, P. J. & Denton, R. M. (1975) Nature (London) **257**, 808–809
Cremer, J. E. & Heath, D. F. (1974) Biochem. J. **142**, 527–544
De Marcucci, O. L., Hunter, A. & Lindsay, J. G. (1985) Biochem. J. **226**, 509–517
England, P. J. (1979) Anal. Biochem. **93**, 272–274
Fazekas, J. F., Alexander, F. A. D. & Himwich, H. E. (1941) Am. J. Physiol. **134**, 281–287
Glynn, I. M. & Chappell, J. B. (1964) Biochem. J. **90**, 147–149
Hawkins, R. A., Williamson, D. H. & Krebs, H. A. (1971) Biochem. J. **122**, 13–18
Jope, R. & Blass, J. P. (1976) J. Neurochem. **26**, 709–714
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lai, J. C. K., Walsh, J. M., Dennis, S. C. & Clark, J. B. (1977) J. Neurochem. **28**, 625–631
Land, J. M., Booth, R. F. G., Berger, R. & Clark, J. B. (1977) Biochem. J. **164**, 539–544
Leong, S. F. & Clark, J. B. (1984) Biochem. J. **218**, 139–145
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Millichap, J. G. (1957) Proc. Soc. Exp. Biol. Med. **96**, 125–129
Olson, M. S., Scholz, R., Buffington, C., Dennis, S. C., Padma, A., Patel, T. B., Waymack, P. & DeBuysere, M. S. (1981) in The Regulation of Carbohydrate Formation and Utilization in Mammals (Veneziale, C. M., ed.), pp. 153–189, University Park Press, Baltimore
Page, M. A., Krebs, H. A. & Williamson, D. H. (1971) Biochem. J. **121**, 49–53
Patel, T. B. & Olson, M. S. (1982) Biochemistry **21**, 4259–4265
Randle, P. J. (1981) Curr. Top. Cell. Regul. **18**, 107–129
Reed, L. J. (1981) Curr. Top. Cell. Regul. **18**, 95–106
Reed, L. J. & Willms, C. R. (1966) Methods Enzymol. **9**, 247–265
Samson, F. E. & Dahl, N. A. (1957) Am. J. Physiol. **188**, 277–280
Schapiro, S., Salas, M. & Vokovich, K. (1970) Science **168**, 147–150
Sheu, K.-F. R. & Kim, Y. T. (1984) J. Neurochem. **43**, 1352–1358
Smith, C. B. & Sokoloff, L. (1981) in The Molecular Basis of Neuropathology (Davison, A. N. & Thompson, R. H. S., eds.), pp. 104–131, Edward Arnold, London
Stanley, C. J. & Perham, R. N. (1980) Biochem. J. **191**, 147–154
Tucek, S. & Cheng, S. C. (1974) J. Neurochem. **22**, 893–914

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