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Developmental Changes in Enzyme Activities and in Morphology of Rat Cortex Mitochondria

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Summary: Development of mitochondria in rat brain cortex was investigated in terms of mitochondrial respiratory enzyme activities, and structural and numerical developments of mitochondria. Measurements of succinate-O_2 and NADH-O_2 oxidoreductase activities of mitochondria resulted in simultaneous changes of activities in postnatal rat. Both oxidoreductase activities were still low at 0–5 days old, increased until 15 days, decreased slightly at 21 days and drastically in adult mitochondria. In morphological study, the cross-sectional area of mitochondrion per cell increased gradually until 21 days old, but decreased drastically in adult. The area of a mitochondrion at 5 days increased about 1.5-fold in comparison with that at 0 days, and maintained at 15 and 21 days. However, the values of area of one mitochondrion from 10 days and adult are about half of a maximum value (21 days). Numbers of mitochondrion per cell were still low at 0–5 days, and high constantly (about twice) at 10–21 days. These findings suggest that the organelle division of mitochondria may be carried out at 5–10 days postnatal. The number of adult rat mitochondria decreased slightly. The small and undeveloped mitochondria were observed at 0 day postnatal by use of transmission electron microscopy (TEM). However, during development from 5 days postnatal, larger and elongated mitochondria were observed, and the maximal complexity of structure of cristae is observed at 15 days and 21 days by TEM. In adult cortex, the small mitochondria were also observed with compact and dense cristae. Our results indicate that the changes of activities of mitochondrial respiratory enzymes in rat cortex is good correlated with the structural maturation of mitochondria.

Aerobic electron transport chain (respiratory chain) in mitochondrial inner membrane is a critical enzyme complex for oxidative phosphorylation and energy production. It is a similar situation in the mammalian nerve systems, and it is therefore very important to examine the correlation between nerve cells and their activities of energy producing system for the study of the nerve developmental changes. In fact, human infant with congenital deficiency of respiratory chain have structural abnormality of the brain (Ellaway *et al.*, 1998)[5]. In rat, many references in the mitochondrial energy metabolism during brain maturation have been published. Dahl and Samson reported that the mitochondria from whole brain of rats 1–50 days old were examined, and total protein of mitochondria increased until 21 days and then remains relatively constant (Dahl and Samson, 1959)[4]. The rate of O_2 utilization per protein of mitochondria was constant throughout the range of ages studied. Calculation of the relative amount of mitochondrial protein per cell reveals an increase during the first 21 days. The increase of the total protein was correlated to the growth of brain. Finally, they have suggested that newly formed mitochondria are fully capable of oxidative phosphorylation and O_2 utilization with brain development, which can be accounted for the increase in membrane protein. Gregson and Williams reported that succinate dehydrogenase (SDH) of the adult brain was 3.5 times greater than

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that of the new-born, but the size of mitochondrion from adult rat was similar to that from neonatal (Gregson and Williams, 1969)[7]. Chepelinsky and Rodriguez de Lores Arnaiz reported that great increase of contents of ubiquinone and cytochromes contained in the rat brain respiratory chain was observed between 10 and 20 days, and the contents of ubiquinone and cytochromes remained constant up to 90 days (Chepelinsky and Rodriguez de Lores Arnaiz, 1970)[2]. Mourek *et al.* (1975)[15] investigated the marker enzymes of the inner and outer membranes of mitochondria from cerebral cortex and subcortical material in young (5 days old) and adult rats, and concluded that gradual maturation occurs in the inner membrane during development, but not in the outer membrane.

However, it is not well known the relationship between development of rat brain mitochondria in terms of enzyme activities of the respiratory chain, and fine structural and numberical changes in spite of those works. Therefore, more detail informations gave well understanding of developing mitochondria on rat brain in postnatal stage. In this study, we observed the time course of maturation of rat brain (cortex) mitochondria on morphological and enzymatical view point.

Materials and Methods

Transmission Electron Microscopy

All protocols conformed to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the guidelines for the care and used on laboratory animals in Nippon Dental University. Wistar rats were deeply anaesthetized with ether and killed by cervical dislocation. Small blocks of cortex from 0, 5, 10, 15, and 21 days postnatal, and adult (8 weeks of age) rats were fixed in 2% glutaraldehyde-cacodylate buffer (pH 7.2) for 2 hr at 4 °C, and postfixed in a 1% osmic acid for 1 hr at 4 °C. After washing with cacodylate buffer (pH 7.2), they were dehydrated in absolute ethyl alcohol, and then embedded in Epon 812. Sections in about 1 μm thickness were stained with toluidine blue for light microscopic observations. Samples for transmission electron microscopy (TEM) were prepared with an ultramicrotome (Ultracut, Reichert-Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. A Hitachi H-700 transmission electron microscope operating at 80 kV (Hitachi, Ibaraki, Japan) was used for observation. Ten ultramicrographs (magnification × 10,000) were selected randomly from each section. The cross-sectional area (CSA) of cell and mitochondria, and area and number of mitochondrion per one section of cell were measured or counted arbitrarily from several locations by a photo analyzer (Pias LA-500) linked to a personal computer (NEC PC-9801 VX, Tokyo, Japan). A total of 100 cells and 200 mitochondria were measured from each stage.

Isolation of Mitochondria

Mitochondria were isolated by the method of Hogeboom with slight modifications (Hogeboom, 1955)[9]. Immediately following the sacrifice of 0, 5, 10, 15, and 21 days postnatal, and adult (8 weeks of age) Wistar rats after anaesthesia, brain cortices were collected and minced in ice-cold isolation fluid containing 0.1 mM EDTA, 0.25 M sucrose and 5 mM Tris-HCl (pH 7.4). Each sample was homogenized with a Teflon pestle at 1,000 rpm for 10 strokes. The homogenate was brought to about 10 volumes of isolation fluid and centrifuged at 700 × g for 10 min. The supernatant was centrifuged at 7,000 × g for 10 min. The resulting sediment consisted of an upper fluffy layer and low heavy layer. The heavy layer was collected and washed once in isolation fluid and then twice in 0.25 M sucrose in a protein concentration from 0.5 to 1.0 mg/ml and stored at −70 °C until use.

Enzyme Assays

Rat cortex mitochondria (3.0–10 μg of protein) were used for each assay. SDH activity was assayed spectrophotometrically at 25 °C by recording the absorbance change of 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl-2,4-tetrazoluim bromide (MTT) at 570 nm in the presence of phenazine methosulfate (PMS) as an electron mediator. The sample was suspended in a 3 ml reaction-mixture containing 50 mM Tris-HCl (pH 7.4), 2 mM KCN, 0.12 mg/ml PMS and 0.24 mg/ml MTT. NADH-ferricyanide dehydrogenase activity was determined at 25 °C by monitoring the absorbance decrease associated with the reduction of ferricyanide at 440 nm in a 3 ml reaction-mixture containing 50 mM Tris-HCl (pH 7.4), 2 mM KCN and 1 mM $K_3Fe(CN)_6$. Succinate-O_2 and NADH-O_2 oxidoreductase activities were measured polarographically using a Clark-type oxygen electrode (Yellow Spring Ltd. OH, USA). The assay of oxidase activity was performed in 50 mM Tris-HCl (pH 7.5) buffer by monitoring the rate of oxygen uptake at 25 °C in a closed 2 ml-reaction chamber equipped with a magnetic stirrer. Each reaction was initiated by the addition of substrate, succinate (10 mM) or NADH (500 μM). Protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin as a standard (Lowry *et al.*, 1951)[13].

Results

Ultrastructural Observation of Mitochondria

Small and unclear mitochondria with undeveloped cristae were observed in neonatal rat brain cortex (0 day, Fig. 1a). These mitochondria are dispersed in the cytoplasm of cortex cells. During development from 5 days postnatal, large and elongated mitochondria were observed (Fig. 1b). The cristae structure of mitochondria from 10 days or later is more complicated than that from 5 days old (Fig. 1c). The maximal complexity of structure of cristae from 15 days and 21 days old is observed by TEM (Fig. 1d, 1e). In adult cortex, the small mitochondria are also observed with compact and dense cristae (Fig. 1f). These mitochondria (from 5 days to adult) had clearly distinguishable outer and inner membranes with increasing elementary particles which may be F1 part of H^+-F_1F_0ATPase and also observed in cristae.

Measurements in Brain Cell and Mitochondria

In the rat brain cortex, area of a mitochondrion and a cell, and CSA of mitochondrion, and number of mitochondrion per cell from 0–21 days postnatal and adult rat are shown in Figure 2. In cerebral cortex, the area of cell was still low from 0 day to 10 days, increased rapidly at 15 days, and maintained about same value at 21 days, but is low in adult cells (Fig. 2a). The CSA of mitochondrion per cell increased gradually until 21 days old, but decreased drastically in adult (Fig. 2b). The area of a mitochondrion at 5 days increased about 1.5-fold in comparison with that at 0 days, and maintained at 15 and 21 days (Fig. 2c). The values of area of one mitochondrion from 10 days and adult are about half of a maximum value (21 days). The number of mitochondrion per cell was still low at 0–5 days, and high constantly (about twice) at 10–21 days. The number of adult rat decreased slightly (Fig. 2d).

Enzyme Activities of mitochondrion

The changes in specific activities of mitochondrial enzymes from rat cortex are shown in Figure 3. The activity of SDH from the cortex was almost unchanged at 0–10 days postnatal, increased at 15 days postnatal, maintained at 21 days, and then decreased in adult mitochondrion (Fig. 3a). Succinate-O_2 oxidoreductase activities of cortex were still low at 0–5 days, increased until 15 days, and decreased slightly at 21 days and in adult mitochondrion. The values of specific activities and time course of NADH-O_2 oxidoreductase are similar to those of succinate-O_2 oxidoreductase (Fig. 3c, 3d). The activities of NADH-ferricyanide dehydrogenase in the cortex were also still low at 0, 5 and 10 days, increased gradually from 15 to 21 days, and decreased in adult mitochondrion (Fig. 3b). The values of NADH-ferricyanide dehydrogenase are much larger than those of NADH-O_2 oxidoreductase.

Discussion

In this paper, we examined the time courses of the enzyme activities of isolated mitochondrial electron transport chain and morphological studies using TEM in rat brain cortex during development. The developmental changes of specific activities per unit protein content of NADH-O_2 oxidoreductase and succinate-O_2 oxidoreductase have very similar profiles, that is, both these activities remain constant until 5 days postnatal, then increase considerably until 15 days, decrease slightly at 21 days, and finally decrease more at adulthood. Chepelinsky and Rodrigez de Lores Arnaiz suggested that the developmental increases of cytochromes and quinone in the mitochondrial inner membrane may be explained by an increase in the surface area of the mitochondrial inner membrane (Chepelinsky and Rodrigez de Lores Arnaiz 1970)[2]. We supposed that increases of oxidase activities are approximately correlated with the enhancement of complexity of mitochondrial cristae observed by TEM, which is remarkable after 10 days postnatal. The number of mitochondrion per cell nearly double and area of the mitochondrion reduced to one-half between 5 days and 10 days, suggesting that the mitochondrial division may occur in this period. The areas of mitochondrion and cell increase about twice from 10 days to 15 days, stabilize at 21 days, indicating the mitochondria and cell grow synchronously between 10 days and 21 days. As mentioned above, all four enzyme activities, NADH-O_2 oxidoreductase, succinate-O_2 oxidoreductase, NADH dehydrogenase and succinate dehydrogenase, are diminished in adult mitochondria. We do not know the correct occurrence in the development of rat cortex between 21 days and adult, but many reports are published in the developmental changes of rat brain enzymes. The specific activities of sorbitol dehydrogenase whose physiological significance is unknown, increases from birth up to 45 days of age and then decreases in several rat brain regions (Struckhoff, 1993)[16]. The specific activity of β-hydroxybutylate dehydrogenase of mitochondrial inner membrane of rat brain increased gradually up to 30 days, but decreased at 60 days (Gorgani *et al.*, 1986)[6]. In all the rat brain regions studied, glutamate dehydrogenase (mitochondrial matrix enzyme) activity developed

rapidly from birth to a peak at around 30 days post partum and then decreased slightly and leveled off to the adult activity (Leong and Clark, 1984)[12]. Cytochrome c plus c1, cytochrome a, cytochrome b, and ubiquinone of rat brain mitochondria remained constant up to 90 days, following drastic increases between 10 and 20 days (Chepelinsky and Rodriguez de Lores Arnaiz, 1970)[2]. Gregson and Williams reported that the respiratory enzyme content per mitochondrion of the adult brain was 3.5 times greater than that of the neonate (Gregson and Williams, 1969)[7]. Mourek *et al.* (1975)[15] reported that the activity of succinate cytochrome c oxidoreductase of rat cortex of adult is 7-fold greater than that of 5 days old. Pyruvate dehydrogenase complex and citrate synthase, both of which are mitochondrial matrix protein, in rat brain mitochondria increase markedly between 0 day and 15 days, followed by a slight increase up to adult (Malloch *et al.*, 1986)[14]. Dahl and Samson reported that the rate of O_2 utilization per milligram mitochondrial protein of rat brain is constant throughout the age range studied (0 day to 50 days), although the total activity increased (Dahl and Samson, 1958)[4]. These many and different results in references indicated that the rat brain enzyme maturation are individual.

Succinate-ubiquinone oxidoreductase catalyzes oxidation of succinate to fumarate in the tricarboxylic acid (TCA) cycle and transfers reducing equivalents to mitochondrial membrane-bound ubiquinone (Ackrell *et al.*, 1992)[1]. NADH-ubiquinone oxidoreductase also transfers electrons from NADH to ubiquinone (Hatefi, 1985)[8]. Electron from both succinate and NADH are sequentially transported by several enzymes and finally reduce O_2 by cytochrome c oxidase (Kadenbach *et al.*, 1991)[10]. Using isolated mitochondria, we measured SDH and NADH dehydrogenase activities employing PMS plus MTT and ferricyanide as artificial electron acceptors, respectively, and monitoring succinate-O_2 and NADH-O_2 oxidoreductase activities using a oxygen electrode. Therefore, our experiments measured both initial electron transfer events as well as net electron transfer from both succinate and NADH to O_2 in rat brain mitochondria.

Our results showed that the time courses of NADH-oxidoreductase and succinate-O_2 oxidoreductase are almost same, indicating that the rate-limiting step of the electron flow reaction is downstream of the dehydrogenase and may be cytochrome bc1 complex or cytochrome c oxidase. The profile of change of succinate dehydrogenase is similar but not completely same to those of oxidase. The activities of NADH dehydrogenase are quite larger than those of oxidases at every time, and it is very clear that the NADH dehydrogenase does not contain the rate-limiting step of electron flow.

Some scientists reported that the mitochondria of the brain are heterogeneous (Chepelinsky and Rodriguez de Lores Arnaiz, 1970; Leong and Clark, 1984; Lai, 1992; Clark and Nicklas, 1970)[2,3,11,12]. In this paper we could not examined the heterogeneity of mitochondria because in early stage the quantity of mitochondria is not sufficient to prepare separately. In near future we will study the heterogeneity and also prepare separately between glia cell mitochondria and nerve cell mitochondria.

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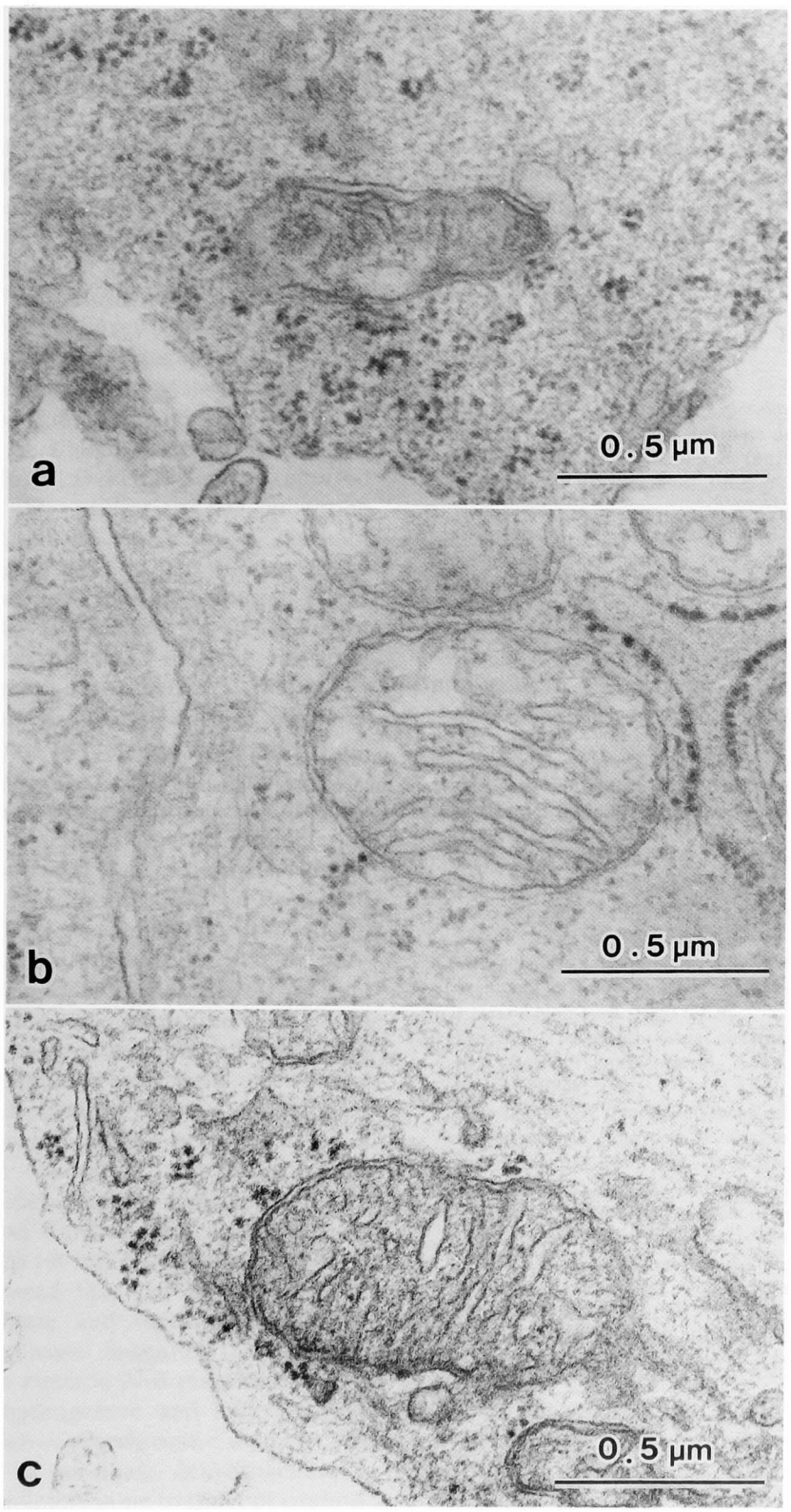
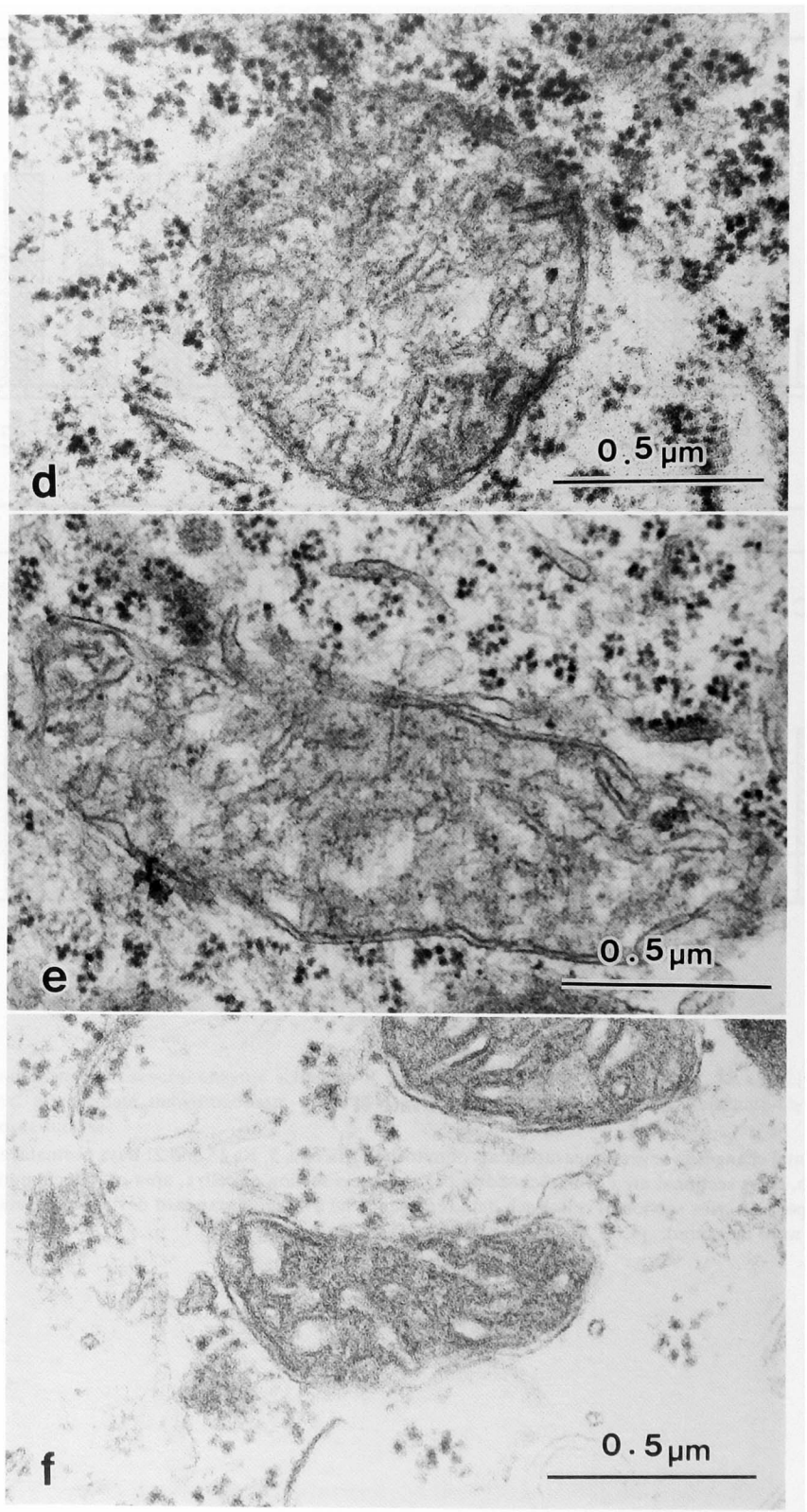
Explanation of Figures

Plate I

Fig. 1. Electron micrographs of rat cortex mitochondria. Small mitochondria with a few undeveloped cristae in neonatal rat (a); mitochondria with developed cristae at 5–21 days postnatal (b–e); small mitochondria with well-developed cristae of adult (f).

Plate I

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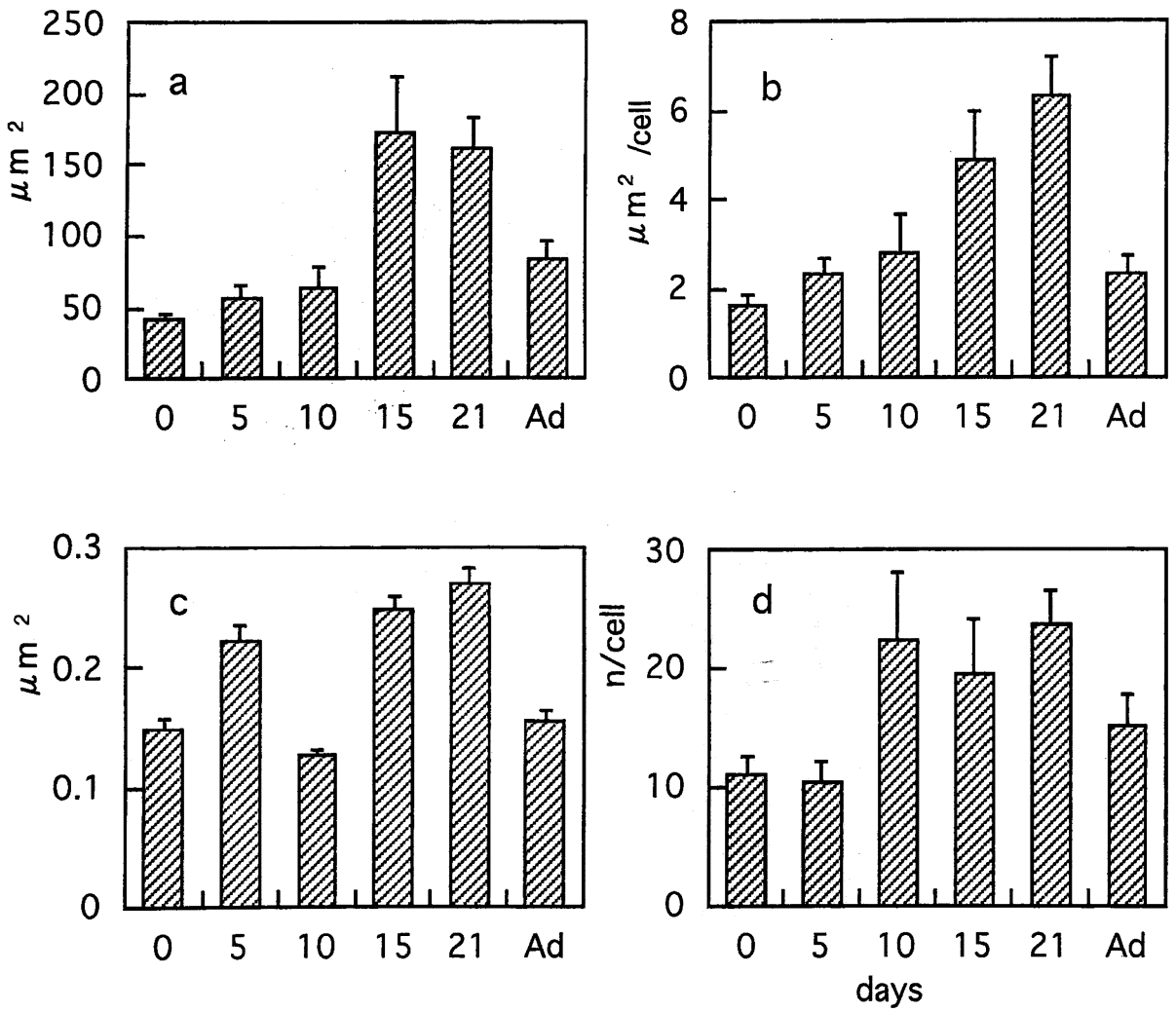

Plate II

Fig. 2. Developmental changes in several measurements of mitochondria of 0, 5, 10, 15 and 21 days postnatal and adult rat cortex. a, area of cell; b, cross sectional area of mitochondria per one cross section of cell; c, area of mitochondrion; d, number of mitochondrion per one cross section of cell. Each data represents the average + standard deviation. A total of 200 mitochondria and 100 cells were measured.

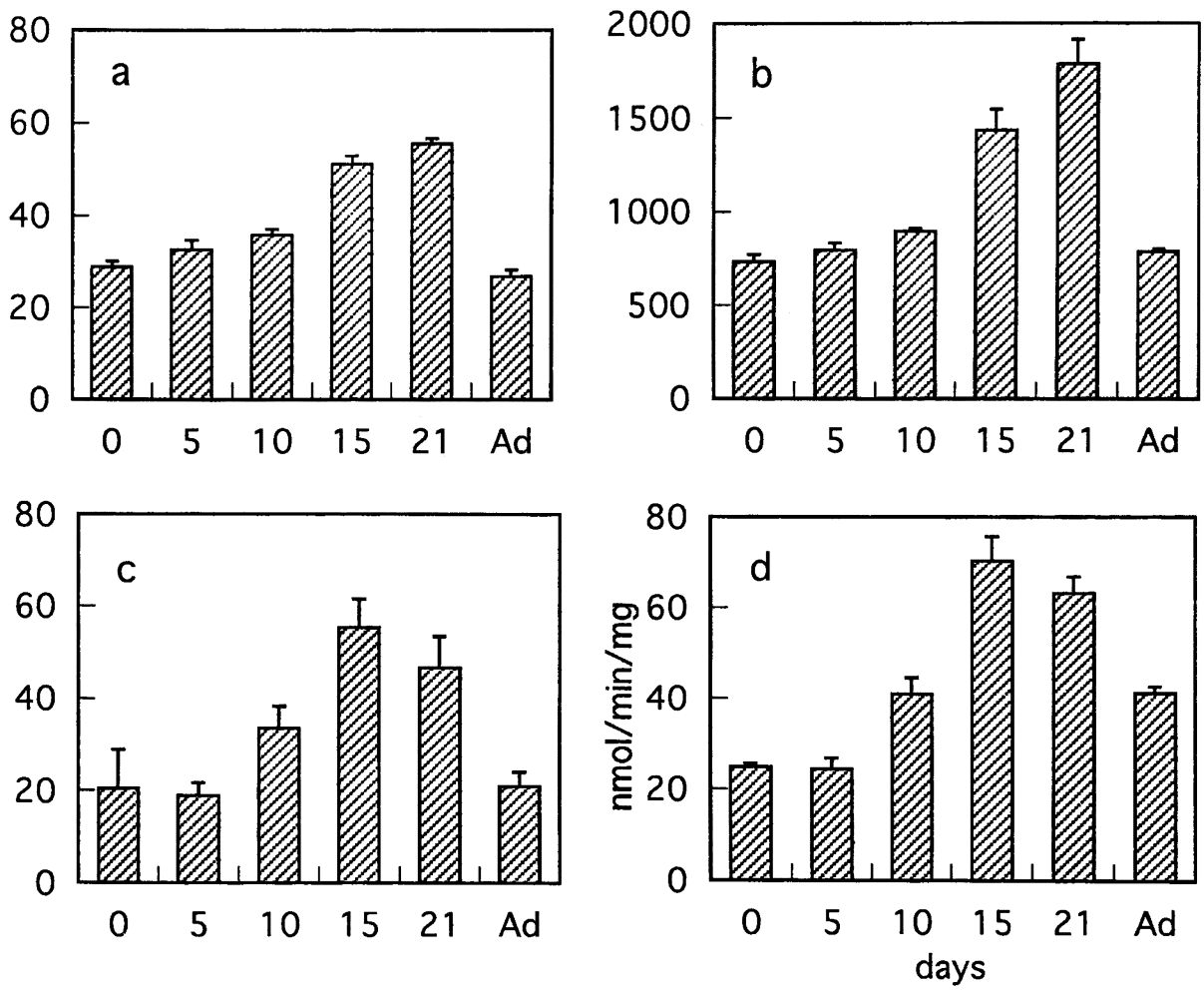

Plate III

Fig. 3. Developmental changes in several enzyme activities of isolated mitochondria from 0, 5, 10, 15 and 21 days postnatal and adult rat cortex. a, succinate dehydrogenase; b, NADH-ferricyanide dehydrogenase; c, succinate-O_2 oxidoreductase; d, NADH-O_2 oxidoreductase.