CHAPTER III

DEVELOPMENTAL CHANGES IN THE ENZYMES OF RAT BRAIN MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

INTRODUCTION

The animals born with a relatively mature state of neurological competence are termed as the `precocial species' eg. horse, sheep and other herd animals, and the guinea pig [1]. By contrast, certain other species born with a relatively poor state of neurological development, eg. mouse, rat, rabbit, dog, cat and man are termed as the `non-precocial species' [1]. Associated with this is the concept of critical or vulnerable periods in brain development, during which insults to the brain are much more damaging than in the others [2,3]. The non-precocial species such as rat and mouse thus show a similar type of brain development postnatally, with an immature brain at birth which gradually develops over a period of time to reach its mature and totally functional stage.

In the brain of the fetus, oxidative metabolism makes small contribution to energy requirements [4,5]. The activities of SDH, cytochrome oxidase, NAD^+ - MDH increase from the fetus to the adult. The activity of the TCA cycle enzymes is low at birth and increases to the adult levels during the neonatal period, i.e. the first 3 to 4 weeks. The activity of the dehydrogenases per mitochondria also increases, although the number of mitochondria/gm tissue and distribution of mitochondria within the tissues remains unchanged [4,5].

Although some reports are available on the developmental pattern of respiratory activity in brain mitochondria [6-11], no studies on the developmental profile of the other enzymes related to the energy metabolism have been reported.

As outlined in the "Introduction" section (Chapter I) the liver mitochondrial enzymes are believed to attain the adult levels immediately after birth [12]. However, the studies presented in Chapter II revealed that the liver mitochondrial energy metabolism related enzymes have a specific developmental pattern. The brain attains maturiy much later in life and even after structural maturity is attained, the arborization and dendritic network formation necessary for fucntional maturity still exerts demands on the energy generating machinery of the cell. Thus it is of interest to study the developmental profile of the brain mitochondrial enzymes, vis-a-vis the liver mitochondria.

Studies were hence carried out to observe the developmental profiles of : a) oxidative phosphorylation using different substrates i.e. glutamate, pyruvate + malate, succinate and the artificial electron donor system of ascorbate + TMPD, b) the activity of the primary dehydrogenases: GDH, MDH and SDR, c) the ATPase activity under basal and Mg^{2+} and/or DNP stimulated conditions, d) the substrate kinetics of cytochrome oxidase and e) the substrate and Arrhenius kinetics of SMP ATPase in the brain mitochondria.

MATERIALS AND METHODS

Chemicals :

Sodium salts of pyruvic acid and L-malic acid were purchased from Sigma Chemical Co., USA.

Adenosine-5'-diphosphate (ADP) was from Boehringer Mannheim.

All the other chemicals and reagents were similar to those described in Chapter II.

Animals :

As described in Chapter II.

Isolation of brain mitochondria :

The animals were killed by decapitation and the brain was quickly excised, weighed and placed in a beaker containing chilled (0-4^{o}C) isolation medium (the composition of the isolation medium was similar to that described in Chapter II). The tissues were washed to free them from adhering blood. The number of brains pooled together for preparing a single sample depended on the age group being studied, 5 brains were pooled for 14- and 21-day groups, 4 for 28- and 35-day groups and 2 in case of the adult group. The washed brains were then homogenized to obtain a 10% (w/v) homogenate similar to liver.

The isolation of brain mitochondria was carried out essentially according to the procedure of Ozawa et.al [13] with some modifications as in Bangur et.al [14].

Similar to liver mitochondria, the 10% (w/v) brain homogenate was centrifuged in a Sorvall RC5 centrifuge at 650g for 10 minutes. The post-nuclear supernatant had a fluffy coat of myelin at the top which was carefully discarded. The supernatant was then centrifuged at 10,000 g for 10 minutes to sediment mitochondria. A loose pellet of synaptosomes forms above a tight mitochondrial pellet; this was discarded after gently swirling, to minimize contamination. The mitochondrial pellet was resuspended once again in chilled isolation medium and resedimented once again at 10,000 g for 10 minutes. The pellet obtained is resuspended in the isolation medium to give a final mitochondrial protein content of about 10 to 15 mg/ml. These mitochodria were then used for oxidative phosphorylation studies and other enzyme analyses. All operations were carried out at 0 to 4^{o}C.

The procedure used above has been tested for contamination of the mitochondria with other subcellular components such as synaptosomes (marker enzyme acetylcholinesterase, 0.1 % activity of pure synaptosomes), cytosol (marker enzyme lactate dehydrogenase, 0.2% of the enzyme activity in cytosol), microsomes (NADPH-cytochrome-c-reductase, below detectable limits) and was found to give pure mitochondria [14].

Oxidative phosphorylatin :

Oxidative phosphorylation was measured polarographically using a Clark type oxygen electrode as described by Satav and Katyare [15].

The respiration medium was similar to that described in Chapter II.

These studies were carried out with different substrates to check the ativities, at the 3 sites of ATP synthesis, i.e. glutamate (10 mM) and pyruvate + malate (10 mM + 1mM), both of which provide electrons to the first site of ATP synthesis in the ETC, succinate (10 mM), electrons from which enter the 2nd site, while the 3rd site activity was monitored using the artificial electron donor system of ascorbate + TMPD (10 mM + 0.1 mM) with succinate and ascorbate + TMPD, 1 µM rotenone (prepared in absolute alchohol) was also added. Depending on the substrate used 2 to 8 mg of mitochondrial protein was added to the assay system. ADP in small aliquots (80 nmoles of ADP in 10 µl) was added and the state 3 respiration rate was recorded, the respiration rate after ADP depletion represented the state 4 rate. The calculations for respiratory rates, ADP/O ratio, respiratory control ratio (RCR) and ADP- phopsphorylation rate were as described previously [16].

Assays of cytochrome oxidase activity, substrate kinetics and mitochondrial dehydrogenases were as described in Chapter II.

Brain mitochondrial ATPase assay :

This was as described by Satav and Katyare [15]. The reaction mixture contained 250 mM sucrose, 10 mM KCl, 10 mM tris- HCl , pH 7.4; 0.2 mm EDTA and to this 2mM $MgCl_2$ and/or 0.05 mM DNP was added wherever indicated; 0.5 to 1.0 mg of mitochondrial protein was added as the enzyme source. The tubes were then pre-incubated at 37° C for 2 minutes. The reaction was initiated by adding 4.0 mM ATP (final concentration) to the assay system; the final volume was 0.5 ml. The assay was terminated at the end of 10 minutes incubation period by adding 1.0 ml 5% w/v trichloroacetic acid (TCA). The tubes were then kept on ice for 10 minutes to sediment the precipitated proteins and were then centrifuged at 2000 g for 10 minutes. Of the supernatant, 0.5 ml was used to estimate the liberated inorganic phosphate by the method of Fiske and Subba Row as described in Chapter II.

Preparation of sonic mitochondrial particles (SMP) and Arrhenius kinetics of SMP ATPase were carried out by following procedures similar to those employed for liver mitochondria (Chapter II).

SMP ATPase : substrate kinetics :

For the substrate kinetics assay the reaction medium in a final volume of 0.5 ml contained 50 mM tris - HCl, pH 7.4; 75 mM KCl, 0.4 mM EDTA and 6.0 mM $MgCl_2$. 50 mg of SMP protein was pre-incubated with this assay medium for 2 min at 37°C and the reaction was initiated by addition of different concentrations of ATP in a range between 1.0 µM to 7.0 mM, the reaction was carried out at 37°C and at the end of 10 minutes incubation period it was terminated by adding 0.1 ml of 5% (w/v) sodium dodecyl sulfate (SDS) solution.

The liberated Pi was estimated by the Fiske and Subba Row procedure as described in Chapter II.

The Km and Vmax values were determined from the substrate kinetics data using the Lineweaver - Burk and Eadie -Hofstee plots [17]. The values obtained from the two plots were in agreement and hence these were pooled and the averages were obtained.

Protein and inorganic phosphate estimation was similar to that decribed in Chapter II.

RESULTS

The data in tables 1 to 4 represent the respiration rates (state 3 and state 4) and ADP- phosphorylation rates using the different substrates. It can be noted that the rates show a

Table 1

Developmental changes in oxidative phosphorylation in rat brain mitochondria using glutamate as the substrate.

Age	ADP/O ratio	Respiration rate		ADP phosphorylation rate
		State 3	State 4	
14 Days	2.21 ± 0.07^{c}	3.6 ± 0.27^{c}	1.2 ± 0.11^{c}	16.3 ± 1.78^{c}
21 Days	2.20 ± 0.03^{c}	7.6 ± 0.33^{c}	2.9 ± 0.23^{a}	34.5 ± 2.06^{c}
35 Days	2.62 ± 0.06^{c}	15.9 ± 0.69^{a}	3.1 ± 0.13^{a}	85.8 ± 3.19^{b}
Adult	3.03 ± 0.04	19.4 ± 0.69^{a}	4.1 ± 0.45	116.7 ± 9.40

Results are expressed as mean $\pm$ S.E.M of 8 independent observations for individual groups.

ADP / O ratio - nmoles of ADP phosphorylated / natoms of O_2 consumed.

Respiration rate - nmoles of O_2 consumed / min / mg mitochondrial protein.

ADP - phosphorylation rate - nmoles of ATP formed / min / mg mitochondrial protein.

$^{a}P < 0.05$; $^{b}P < 0.010$; $^{c}P < 0.001$ compared to the adult

Table 2

Developmental changes in oxidative phosphorylation in rat brain mitochondria using pyruvate + malate as the substrate.

Age	ADP/O ratio	Respiration rate		ADP phosphorylation rate
		State 3	State 4	
14 Days	2.01 ± 0.01[c]	4.7 ± 0.11[c]	2.4 ± 0.13[c]	20.6 ± 1.50[c]
21 Days	2.00 ± 0.04[c]	11.1 ± 0.68[c]	5.8 ± 0.23[a]	49.1 ± 3.50[c]
35 Days	2.69 ± 0.05[a]	19.0 ± 1.13[b]	9.9 ± 0.93[a]	103.5 ± 8.09[c]
Adult	2.78 ± 0.09	25.7 ± 1.25	13.1 ± 0.01	156.9 ± 8.81

Results are expressed as mean ± S.E.M. of 8 independent observations for individual group.

Other details are as given in Table 1.

[a] $P < 0.02$; [b] $P < 0.002$; [c] $P < 0.001$ compared to the adults.

Table 3

Developmental changes in oxidative phosphorylation in rat brain mitochondria using succinate as the substrate.

Age	ADP/O ratio	Respiration rate		ADP Phosphorylation rate
		State 3	State 4	
14 Days	0.86 ± 0.04[c]	8.1 ± 0.75[c]	4.0 ± 0.20[c]	13.9 ± 1.48[c]
21 Days	0.81 ± 0.03[c]	21.0 ± 0.85[c]	13.6 ± 0.61[c]	33.6 ± 1.60[c]
35 Days	1.20 ± 0.06[b]	42.7 ± 2.71[c]	20.2 ± 0.61[c]	97.5 ± 7.49[c]
Adult	1.60 ± 0.08	77.6 ± 2.48	40.6 ± 1.21	250.4 ± 18.28

Results are expressed as mean ± S.E.M. of 8 independent observation for individual group.

Other details are as given in Table 1.

[a]$P < 0.05$; [b]$P < 0.002$; [c]$P < 0.001$ compared to the adults.

Table 4

Developmental changes in oxidative phosphorylation in rat brain mitochondria using ascorbate + TMPD as the electron donor system.

Age	ADP/O ratio	Respiration rate		ADP Phosphorylation rate
		State 3	State 4	
14 Days	0.40 ± 0.02	8.0 ± 0.37[c]	3.6 ± 0.18[c]	6.4 ± 0.54[c]
21 Days	0.32 ± 0.02[b]	31.2 ± 2.17[c]	17.2 ± 0.68[a]	19.6 ± 1.37[c]
35 Days	0.52 ± 0.03[b]	79.5 ± 4.11[b]	44.4 ± 1.87[c]	82.8 ± 6.30[c]
Adult	0.41 ± 0.02	151.6 ± 4.54	67.9 ± 4.57	122.8 ± 5.85

Results are expressed as mean ± S.E.M. of 8 independent observations for individual group.

Other details are as given in Table 1.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ compared to the adults.

Table 5

Developmental changes in the activity of rat brain mitochondrial dehydrogenases

Age	Glutamate dehydrogenase	Malate dehydrogenase	Succinate - DCIP reductase
14 Days	1.7 ± 0.08^{c}	11001.0 ± 482.8^{b}	5.0 ± 0.31^{c}
21 Days	3.8 ± 0.07^{c}	9813.3 ± 234.8	18.9 ± 1.01^{a}
35 Days	8.2 ± 0.34^{c}	9620.3 ± 331.5	12.6 ± 0.90^{c}.
Adult	28.1 ± 1.83	8920.5 ± 635.7	22.6 ± 1.10

Results are expressed as mean $\pm$ S.E.M. of 8 independent observations for individual groups.

Activity - nmoles/min/mg mitochondrial protein

$^{a}P < 0.05$; $^{b}P < 0.025$; $^{c}P < 0.001$ compared to the adults.

Table 6

Developmental changes in rat brain mitochondrial ATPase activity.

Age	ATPase activity (μmoles of Pi liberated / h / mg protein)			
	Basal	Mg^{2+}	DNP	Mg^{2+} DNP
14 Days	3.6 ± 0.18[a]	13.1 ± 0.53[a]	2.3 ± 0.12	11.6 ± 0.47[a]
21 Days	3.9 ± 0.20[a]	6.4 ± 0.29[a]	2.5 ± 0.17	16.3 ± 0.68
35 Days	2.8 ± 0.17	7.3 ± 0.51	2.2 ± 0.18	10.4 ± 0.51[a]
Adults	2.4 ± 0.18	8.5 ± 0.35	2.8 ± 0.25	16.4 ± 0.43

Results are expressed as mean ± S.E.M. of 8 indepentent observations for individual group.

[a] $P < 0.001$ compared to the adults.

Table 7

Developmental changes in substrate kinetic parameters of rat brain mitochondrial cytochrome oxidase.

Age	Km	Vmax
14 Days	0.33 ± 0.09	19.5 ± 1.59[c]
21 Days	0.42 ± 0.02	162.0 ± 8.36[c]
28 Days	0.51 ± 0.06[a]	447.5 ± 27.10[c]
35 Days	0.34 ± 0.01	341.3 ± 25.76[b]
Adult	0.36 ± 0.02	242.8 ± 11.93

Results are expressed as mean ± S.E.M. of 6 independent observations for individual group.

K_m - mM.

V_{max} - µmoles of O_2 consumed/min/mgmitochondrial protein.

[a] $P < 0.05$, [b] $P < 0.02$; [c] $P < 0.001$ compared to the adults.

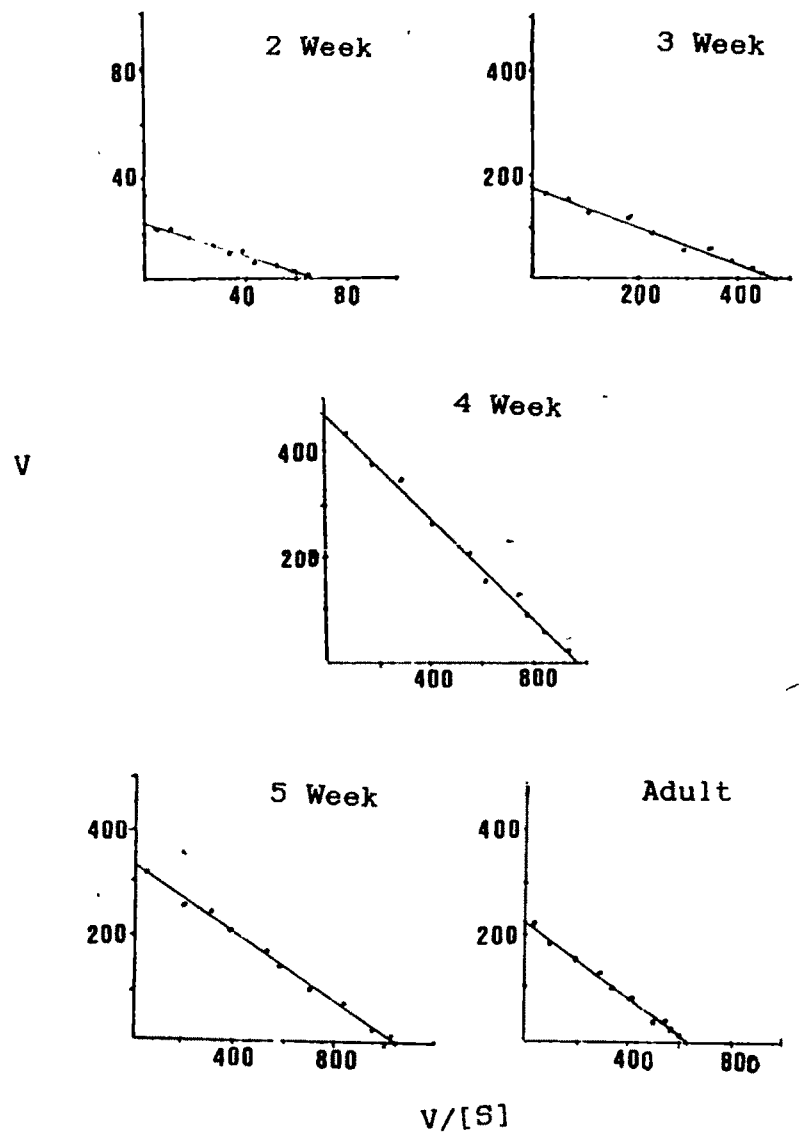

Figure 2

Typical Eadie - Hofstee plots depicting developmental changes in rat brain SMP ATPase. Substrate kinetics.

Table 8

Developmental changes in substrate kinetics parameters of rat brain SMP ATPase.

Age	High Affinity Site		Low Affinity Site	
	Km	Vmax	Km	Vmax
21 Days	0.136 ± 0.004[b]	15.5 ± 1.10[b]	1.27 ± 0.06[b]	38.8 ± 1.25[a]
35 Days	0.046 ± 0.007[a]	10.9 ± 2.77[b]	0.79 ± 0.08[a]	21.8 ± 3.47[b]
Adults	0.074 ± 0.002	30.9 ± 1.76	0.47 ± 0.01	53.0 ± 3.50

Results are expressed as mean ± S.E.M of 6 independent observations for individual groups.

K_m - mM.

V_{max} - µmoles of Pi liberated /min/mg mitochondrial protein.

[a] $P < 0.01$; [b] $P < 0.001$ compared to the adults.

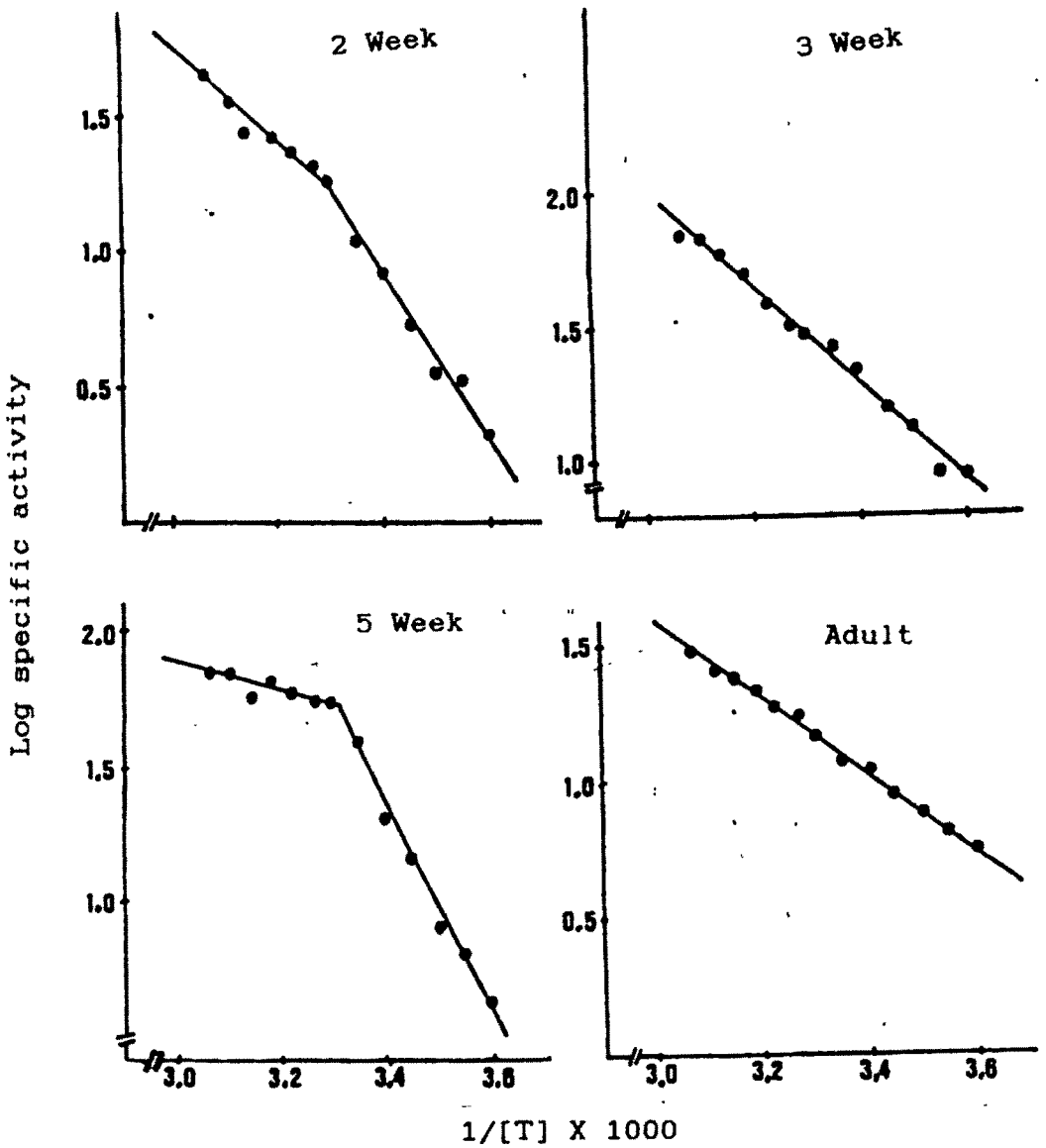

Figure 3

Rat brain SMP ATPase. Typical plots depicting developmental changes in Arrhenius kinetics.

Table 9

Developmental changes in Arrhenius kinetic parameters of rat brain SMP ATPase.

Age	Phase transition temperature (T_t) ^{o}C	Energy of Activation (KJ/mole)	
		E_1	E_2
14 Days	28.4 ± 0.57[b]	32.6 ± 0.64	53.9 ± 3.03[b]
21 Days	30.5 ± 0.80[b]	9.7 ± 0.90[b]	73.8 ± 3.14[b]
35 Days	------	33.4 ± 0.16[a]	------
Adults	------	30.6 ± 0.57	-------

Results are expressed as mean ± S.E.M of 6 independent observatins for individual groups

[a] $P < 0.005$; [b] $P < 0.001$ compared to the adults.

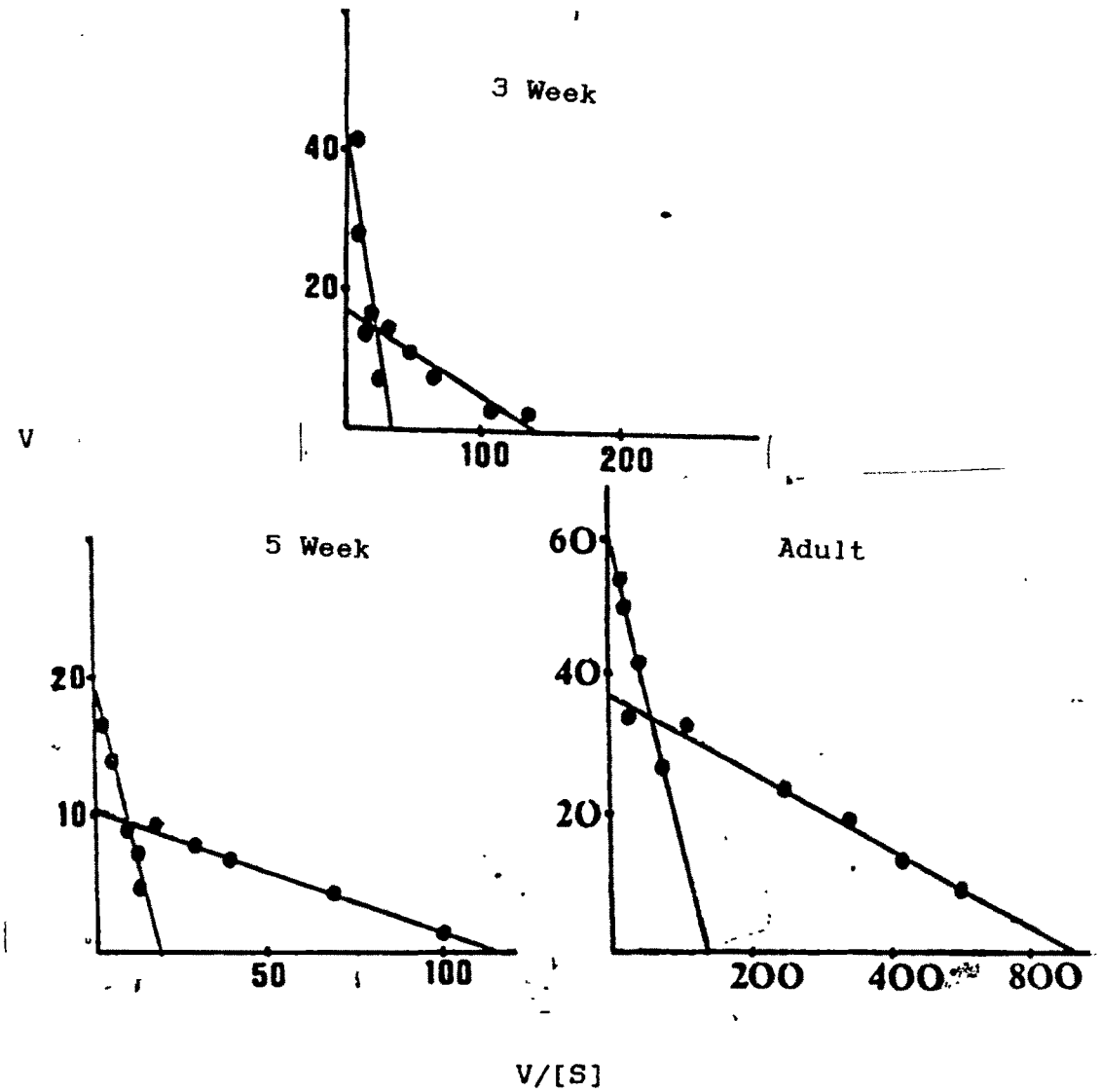

Figure 1

Typical Eadie - Hofstee plots depicting changes in rat brain mitochondrial cytochrome oxidase. Substrate kinetics.

gradually increasing trend with age. However, the extent of increase differed for the four substrates. Thus state 3 rate, for example, increased by about 5.4 fold with glutamate and pyruvate + malate, 9.6 fold with succinate and 19 fold with ascorbate + TMPD. These results thus substantiate the available reports on mitochondrial respiratory activity in different age groups [6-11].

The activities of primary dehydrogenases are given in Table 5. GDH activity increased gradually from 14 day onwards and the final increase in the adults was 17 fold. MDH activity on day 14 was about 12 % higher than the adults, but gradually declined with age. SDR, similar to GDH showed the least activity in the 14 day group (22% of the adult) but gradually increased by 5 fold in the adult.

The mitochondrial ATPase activity (Table 6) was different in the different age groups depending on the stimulatory condition. Basal activity was the highest in the 14-day group but gradually decreased till the adult stage. Mg^{2+} ATPase activity in the 21-day-group decreased by about 50% compared to the 14-day-old group but reached a steady state value by day 21. The DNP stimulated ATPase activity was constant irrespective of the age group. Mg^{2+} + DNP ATPase activity was highest in the 21-day-group and the adult.

Figure 1 shows the typical Eadie-Hofstee plots for the substrate kinetics parameters of cytochrome oxidase in

different age groups. The values of Km and Vmax are given in Table 7. The Km in all age groups, except 28 days, was comparable to the adults; in the 28 day group the value was almost double. In case of Vmax the value was the lowest on the 14 day but registered a 23 fold increase on day 28, thereafter the activity gradually decreased with age till in adults it reached about 50% of the 28-day value.

The substrate kinetics studies for brain SMP ATPase revealed presence of two catalytic sites, a high affinity site and the other, a low affinity site. Typical Eadie - Hofstee plots are shown in Figure 2 and the data on K_m and V_{max} are given in Table 8. The Km of the high affinity site decreased on day 35 but increased again in the adults. While in the low affinity site the K_m gradually decreased from day 21 to the adult. The V_{max} of the high affinity site decreased by about 30% from day 21 to day 35 and then doubled in the adults. The Vmax for the low affinity site also displayed a similar pattern.

The studies on brain SMP ATPase could not be carried out on the 14-day-old rats due to the unavailability of sufficient amount of mitochondrial protein. The brain SMP ATPase in the adult and the 35-day-group did not exhibit phase transition, which was seen in both the 14- and 21-day-groups. The typical Arrhenius plots are shown in Figure 3. It is evident from the data in Table 9 that the T_t values did not differ

much in the latter two groups. However the E_1 value on day 21 was only 31% of the adult. The E_2 value on day 14 increased by about 20 KJ by day 21.

DISCUSSION

The observations on age dependent changes in the oxidative phosphorylation are in close agreement with the earlier reports [6-11]. Thus this gradual increase in the rate of respiration and oxidative phosphorylation would provide the increased amount of ATP that the brain requires for achieving its functional and structural maturity after birth.

GDH showed an increase in activity with development and this was mirrored in the increase in the glutamate stimulated respiration. However, MDH, also a soluble dehydrogenase like GDH, actually showed a decreasing trend in activity. However, activity in the adults was about 350 fold higher than the corresponding state 3 respiration rate. Thus even if there is a decrease, the final activity is still more than sufficient to channel electrons into the ETC; as outlined (Chapter II), the dehydrogenases are never rate limiting. SDR, similar to GDH showed increased activity, which paralleled the increase in succinate stimulated respiration.

Earlier reports by Land et.al [18] had shown a 2 fold decrease in GDH and a slight increase in MDH in rat brain from 21-day and adult rats, while a study on human neocortex

enzymes involved in energy metabolism [19] has reported an age - dependent increase in the GDH and SDH activity from 8 week old fetus to adults. Wilbur and Patel [3] also had shown an increased NAD^+ - MDH activity with age.

The mitochondrial ATPase, the 5th complex of the ETC studied under basal condition showed a drop in activity from the 21-day-group to the adults. This would thus mean that the production of ATP *in situ* would decrease after 21 days. However the ADP - phosphorylation rate, as can be seen from the results, increased gradually, thus these two aspects do not complement each other. Also the fact, that the dendritic network formation and arborization is still continuing even after 21 days would make these results difficult to understand. However, by day 21 the more energy intensive process of structural maturation is complete and the energy requirement for the process of arborizaton would not be as high as that of the previous process and hence the ATP synthase activity could have decreased concomitant to the decreased ATP demand, while the high ADP - phosphorylation rate could be pointing towards the high inherent capacity of the brain mitochondria to carry out coupled phosphorylation of ADP.

The cytochrome oxidase substrate kinetic parameters have a pattern similar to that of liver; the maximum Km and Vmax values are in the 28-day-group while the adults have the lowest Km and an intermediate Vmax. The factors likely to

affect the cytochrome oxidase kinetic parameters have already been discussd (Chapter II). The same considerations and conditions would also apply for the brain cytochrome oxidase Km and Vmax. The developmental changes in brain mitochondrial lipids are reported in Chapter IV of the thesis. However, although DPG is a essential requirment for the proper functioning of C.O., the changes seen in the DPG content do not match the changes in the K_m and V_{max} of C.O. in the corresponding age groups.

The substrate kinetics parameter studies of SMP ATPase showed presence of two affinity sites, a high affinity and a low affinity site. The high affinity site had about 6 to 10 fold lower Km than the low affinity site. While the Vmax also was about half in the same age group. In case of the high affinity site the adults had the highest Vmax and an intermediate Km while in the low affinity site the adult age group was the most efficient since the Km was the least and the Vmax highest in this group.

The Arrhenius kinetics of SMP ATPase shows absence of phase transition in the older age groups i.e. 35-day and adults and correspondingly the E_2 in both these cases is also absent . The lipids in these age groups do not seem to change their characteristics within the temperatures studied. The enzyme seems to be more strongly affected by the rise in temperature in the earlier age groups and with maturation this response decreases. In membrane - bound enzymes, there is a

sudden change in activation energy of the enzyme at a particular temperature which may be the crystalline to liquid-crystalline state transition temperature of the membrane [20]. This difference in the thermal behavior could be attributed either to any one or a combination of the following :

a) The differences in fatty acid composition of the PLs between the immature and adult brain mitchondria; since this composition is known to change during brain development [21] and the changes in the fatty acid composition are known to alter energies of activation [22]. Since this enzyme displays specific requirements for PLs the changes in PL compounds themselves would also affect the transition temperatures.

b) Possible differences in the mode of incorporation of the enzyme into PL bilayers resulting in difference in the type of lipid - protein interaction during brain development. Linear Arrhenius plots have been obtained for lipid requiring enzymes such as $Na^+ - K^+$ ATPase and succinate oxidase when treated with detergents and phospholipases [23,24]. Studies on enzyme -PL interaction have shown that at low levels of detergent incorporation into the PL membrane the detergent does not display this property [25] and hence the apparent lack of a break in the mature rat brain SMP ATPase Arrhenius plots could be due to the differences in the mode of lipid - protein interaction [26].

c) Another possible explanation could be due to cholesterol (CHL) action . During development CHL concentration is known

to increase in rat brain [27]. The role of CHL as a membrane component has been studied rather extensively with PL mono- and bilayers [28]. As can be seen from the results (Chapter IV) the CHL content was high in the 35-day and adult rat brain mitochondria compared to the younger age groups and hence this could lead to increased condensation and hence loss of phase transition .

Studies on other membrane-bound enzymes such as Na^+ K^+ ATPase and acetylcholinesterase have shown differences in their Arrhenius plots with development and [26] hence the alteration observed with development in case of ATPase could also be explained on the basis of either one or more of the above conditions working in tandem.

SUMMARY

Developmental changes in the rat brain mitochondrial enzymes of the ETC were studied.

All the oxidative phosphorylation parameters with the different substrates showed a developmental pattern with an increasing trend in activity.

Both the GDH and SDR activities incresed from the young to the adults, by contrast MDH activity decreased. The basal ATPase activity was the highest on day 21 and then decreased, while the stimulation of ATPase by DNP was absent in all the age groups.

The values of Km and Vmax for cytochrome oxidase were maximum in the 28-day old animals, while in all the other age groups the Km did not differ much. The Vmax in adults was intermediate between the values on day 14 (lowest) and on day 28 (highest).

Two substrate (ATP) binding sites: high affinity and low affinity, were evident from SMP ATPase studies. The Km was about 6 to 10 times lower for the high affinity site compared to the low affinity site, while the Vmax was about half in the high affinity site for all the corresponding age groups.

In animals belonging to the older age groups, i.e. 35-day and adult no phase transition and E_2 were evident. In the younger groups (14- and 21-day old) E_1 and E_2 differed by about 20 KJ but the T_t remained almost the same. The 21 day group had only 30 % E_1 of the other age groups.

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