CHAPTER - III

EFFECTS OF CORTICOSTERONE TREATMENTS ON ACTIVITY AND KINETIC PROPERTIES OF BRAIN MITOCHONDRIAL ATPase DURING DEVELOPMENT.

The F_1-F_0 ATPase/synthase catalyzes electrochemical proton gradient (Δp) - driven ATP syntesis or Δp -generating ATP hydrolysis in the coupling membranes of mitochendria. The peripheral part (F_1) is composed of five different polypeptides in the stochiometry $\alpha_3.\beta_3.\gamma.\delta.\varepsilon$ and is capable of rapid uncoupled ATP hydrolysis when detached from the membrane embedded F_o. The F_o component contains a proton conducting pathway and operating together F_1 -F_0 provide Δp - consuming ATP synthesis or Δp generatiang ATP hydrolysis depending on physiological (in vivo) or experimental (in vitro) conditions. This enzyme in heart and liver mitochondria is known to have three nucleotide binding sites (1-3),out of which one is a catalytic site and the other two are co-operative sites (3).

Glucocorticoids are known to affect the ATPase activity under both in vitro as well as in vivo conditions. Blecher and White (4) have reported that glucocorticoids and variety of other steroids have stimulating effect on ATPase activiy of lymphosarcoma and liver mitochondria. These effects were observed under in vitro conditions and secondly the steroid concentration used was very high (0.6 mM). Hence the physiological relevence of these findings is of dubious nature; the effects could be considered as non-specific pharmacological actions of steroids (5).

Allan et al.(6) studied the in vivo effects of synthetic glucocorticoid, dexamethasone on mitochondrial ATPase activity in liver. In adrenalectomized animals kept for a period of 14 days no significant change in either the total ATPase activity or uncoupler-dependent ATPase activity in noted liver mitochondria. Short-term treatment of dexamethasone significantly increased the ATPase activity in presence of uncoupler (DNP). No effect was observed on the activity of ATPase in absence of uncoupler or in the presence of 1mM Mg^{2+}.

However, glucocorticoid effects on ATPase activity in the brain mitochondria have not been examined. As outlined in the preceeding Chapter (Chapter II) the corticosterone treatments (acute and chronic) significantly decreased the mitochondrial state 3 respiration rates, ADP/O ratios and the ADP phosphorylation rates. Therefore it was of interest to study the in vivo effects of the corticosterone treatments on activity and kinetic properties of ATPase from subsequently isolated brain mitochondria during postnatal development. Studies were hence carried out to examine 1) Mitochondrial ATPase activites under basal conditions and effects of Mg^{2+} and DNP (individually and together) on stimulation of the ATPase activity. 2) Substrate kinetics of ATPase to determine Km and Vmax and 3) Arrhenius kinetics of ATPase to determine phase transition temperature and energies of activation, E_1 and E_2.

MATERIALS AND METHODS

Chemicals

2,4 dinitrophenol (DNP) and adenosine 5'triphosphate (ATP) were purchased from Sigma Chemicals Co. USA and SRL, India respectively.

Sources of other chemicals were the same as described in Chapter II.

Preparation of sonic mitochandrial particles (SMP)

Mitochandrial suspensions in isolation medium were sonicated for two minutes (10 seconds sonication followed by 10 seconds rest interval) using a Branson sonifier. Temperature was maintained at 0 to 4°C during sonication (7).

The sonicated mitochondrial suspension was then subjected to centrifugation at 10,000 g for 10 minutes in a refrigerated sorvall RC 5 centrifuge for pelleting unbroken mitochondria and the supernatant was subjected to centrifugation at 100,000 g for 1 hour at 0 to 4°C in a Sorvall OTD Combi Ultracentrifuge to obtain pellet of SMP. The pelleted SMP were suspended in isolation medium to get about 1 mg of SMP protein/ ml and stored frozen. The samples were used within a week for the studies on substrate and temperature kinetics of ATPase.

Assay of brain mitochondrial ATPase

ATPase activities were measured in the freshly isolated intact mitochondria in a medium (final volume 0.5 ml) consisting of 250 mM sucrose, 10 mM tris.HCl pH 7.4, 0.2 mM EDTA; 2 mM $MgCl_2$ and/or 0.05 mM DNP were included wherever indicated. After pre-incubating 0.5 to 1.0 mg of mitochondrial proteins (as a source of enzyme) in the reaction mixture for 2 minutes at 37°C, the reaction was started by adding 4 mM ATP and carried out for 10 minutes (8). At the end of the incubation period, the reaction was terminated by adding 1.0 ml of chilled (0 to 4°C) 5% TCA. Tubes were centrifuged at 3000 rpm in a table top centrifuge. 1.0 ml aliquots of the supernatant were taken for the estimation of phosphorous by the method of Fiske and Subba Row (9).

Kinetic studies on ATPase from SMP

For the Kinetic studies, the assay medium (final volume 0.5 ml) consisted of 50 mM tris.HCl pH 7.4, 7.5 mM KCl, 0.4mM EDTA and 6.0 mM $MgCl_2$. For substrate kinetic studies, the concentration of ATP ranged from 1.0 um to 7.0 mM, while for temperature kinetic studies ATP concentration of was fixed at 6.0 mM and temperature varied from 5°C to 53°C. 50 µg of SMP protein was used as a source of enzyme. At the end of the incubation period, the reaction was terminated by adding 0.1 ml of 5% (w/v) SDS and the liberated inorganic phosphorous was

estimated by the method of Fiske and Subba Row (9).

The values of Km and Vmax were derived from Lineweaver - Burk and Eadie-Hofstee plots and averaged (10). Values of energy of activation in high (E_1)and low (E_2) temperature ranges and phase transition temperature (Tt) were obtained from the Arrhenius plots (11).

Estimation of inorganic phosphorous.

Inorganic phosphorous was estimated according to the method of Fiske and Subba Row (9) with some modifications.

Reagents:

1. Phosphorous standard.

a) Stock standard: Dissolve 35.1 mg of KH_2PO_4 in 50 ml distilled water, then add 1.0 ml of concentrated sulfuric acid and make up the volume to 100 ml to give final concentration of 80 µg phosphorous/ml.

b) Working standard: Dilute the stock standard 1:10 times with distilled water to obtain 8 µg phosphorous/ml.

2. Molybdate II reagent

This was prepared by dissolving 25 g of ammonium molybdate in 200 ml of distilled water, then 300 ml of 10 N sulfuric acid was added and the volume was made upto 1000 ml with distilled water.

3. **ANSA (1-amino-2-naphthol- 4 - sulfonic acid) reagent:**

This was prepared as a triturate and stored in brown colored bottle. Triturate was made by weighing 0.2 ANSA, 1.2 g sodium sulfite and 1.2 g sodium bisulfite; these components were ground in a mortar and pestle to obtain homogeneous mixture.

ANSA reagent was prepared from the triturate freshly by dissolving 40 mg ANSA triturate/ml in distilled water.

Procedure:

Different aliquots of working standard were taken to give concentrations of phosphorous in the range of 1 to 16 µg. For all the standards and samples the volume was made up to 3.5 ml with distilled water; reagent blanks contained 3.5 ml distilled water. After this all the tubes were treated in the same manner: 0.4 ml of molybdate II reagent was added and then lastly 0.1 of ANSA reagent was added. Tubes were vortexed immediately and the blue color developed was read between 8 to 11 minutes (after adding ANSA reagent) in an Erma colorimeter using filter of 660 nm. The standard graph was prepared and slope was calculated and used to find out concentration of liberated inoraganic phosphorous in the samples. This method gives slope of 0.025 (1 µg phosphorous gives 0.025 O.D.).

RESULTS

Effects of corticosterone treatments on brain mitochondrial ATPase activities are summarized in Table 1. In 14- and 21-day-old animals, both acute and chronic treatment decreased basal ATPase activity by 32 to 63%, the extent of decrease being higher in case of chronic treatment. Both the corticosterone treatments had no much effect on basal ATPase activity in mitochondria from, 35 -day -old and adult animals.

Mg^{2+} ATPase activity was also decreased significantly (33 to 60%) by acute treatment in 14 -and 21 -day -old animals. In case of 35 -day -old and adult rats which were unaffected by acute treatment, chronic treatment resulted in significant incerase in Mg^{2+} ATPase activity.

Acute treatment did not have much effect on DNP-ATPase activity but chronic treatment caused significant decrease in 14 -and 21 -day groups; 35 -day -old and adult animals showed 2 to 5 fold decrease in DNP-ATPase activity.

Both the treatments had no effect on Mg^{2+} + DNP ATPase activity in mitochondria from 14- and 35-day-old animals. In case of 21-day-old and adults both the corticosterone treatments lowered the Mg^{2+} + DNP ATPase activity by 25 to 50%; the extent of decrease was higher in chronic treatment group.

Table 1

Effects of corticosterone treatment on brain mitochondrial ATPase activities during development.

Treatment	ATPase activity (μ moles/hr/mg protein)			
	Basal	Mg^{2+}	DNP	Mg^{2+} + DNP
		14-Day-old		
Control	3.40 ± 0.28	12.16 ± 0.61	2.08 ± 0.18	12.11 ± 0.86
Acute	2.06 ± 0.47[a]	8.13 ± 1.47[a]	1.88 ± 0.25	10.13 ± 0.72
Chronic	1.25 ± 0.19[c]	7.68 ± 0.52[c]	1.34 ± 0.16[b]	10.51 ± 0.64
		21-Day-old		
Control	3.45 ± 0.12	6.93 ± 0.46	2.49 ± 0.24	15.90 ± 0.53
Acute	1.97 ± 0.15[c]	2.36 ± 0.46[c]	1.50 ± 0.19[b]	11.76 ± 0.59[c]
Chronic	1.85 ± 0.17[c]	5.75 ± 0.49	1.49 ± 0.13[b]	9.21 ± 0.35[c]
		35-Day-old		
Control	3.26 ± 0.49	7.98 ± 0.69	2.28 ± 0.36	11.36 ± 0.46
Acute	2.96 ± 0.29	6.21 ± 1.16	1.25 ± 0.45	9.70 ± 1.26
Chronic	3.68 ± 0.24	13.23 ± 0.91[c]	4.32 ± 0.14[c]	11.37 ± 0.27
		Adult		
Control	2.19 ± 0.25	8.11 ± 0.53	2.53 ± 0.42	15.40 ± 0.26
Acute	2.05 ± 0.11	8.25 ± 0.43	1.87 ± 0.27	9.63 ± 0.71[c]
Chronic	1.28 ± 0.26[a]	11.22 ± 0.53[c]	16.64 ± 0.99[c]	8.01 ± 1.32[c]

Results are given as mean ± SEM of 8 independent observations in each group.

[a]$P < 0.05$; [b]$P < 0.01$ and [c]$P < 0.001$ compared to the corresponding controls

The substrate kinetics studies have shown that ATPase in brain mitochondria had two catalytic sites: high affinity site and low affinity site. When the data were analyzed by two different plots (i.e. Lineweaver-Burk and Eadie-Hofstee plots) to obtain Km and Vmax, results obtained were quite similar and hence the values were averaged and these are given in Table 2.The typical Eadie - Hofstee plots of ATPase from brain SMP from control and corticosterone treated animals of different age groups are shown in Fig. 1.

Data in Table 2 indicated that in the control animals, Km of the high affinity site decreased with advancement of age ; compared to 21 -day - old rats in 35 -day group it decreased by 66% while adults showed 46% decrease. The Vmax of the high affinity site in adult animals was two times higher than 21 - day group. Similarly the Km of low affinity site for ATP also decreased with age. Compared to 21-day-group , 35-day-old and adults showed 38% and 63% decrease respectively. Adults had Vmax 37% higher than the 21-day group.

Effects of corticosterone treatments on brain SMP ATPase were age-dependent and treatment specific. In 21-day group both the corticosterone treatments led to 32 to 69% decrease in Km for ATP for the high affinity site, but had no effect on Vmax. In case of adults chronic treatment caused 78% increase in Km of high affinity site without affecting the Vmax; acute treatment caused 81% decrease in Vmax but no effect on Km.

Figure 1

Eadie-Hofstee plots of brain SMP ATPase from control and corticosterone-treated animals of different age groups.

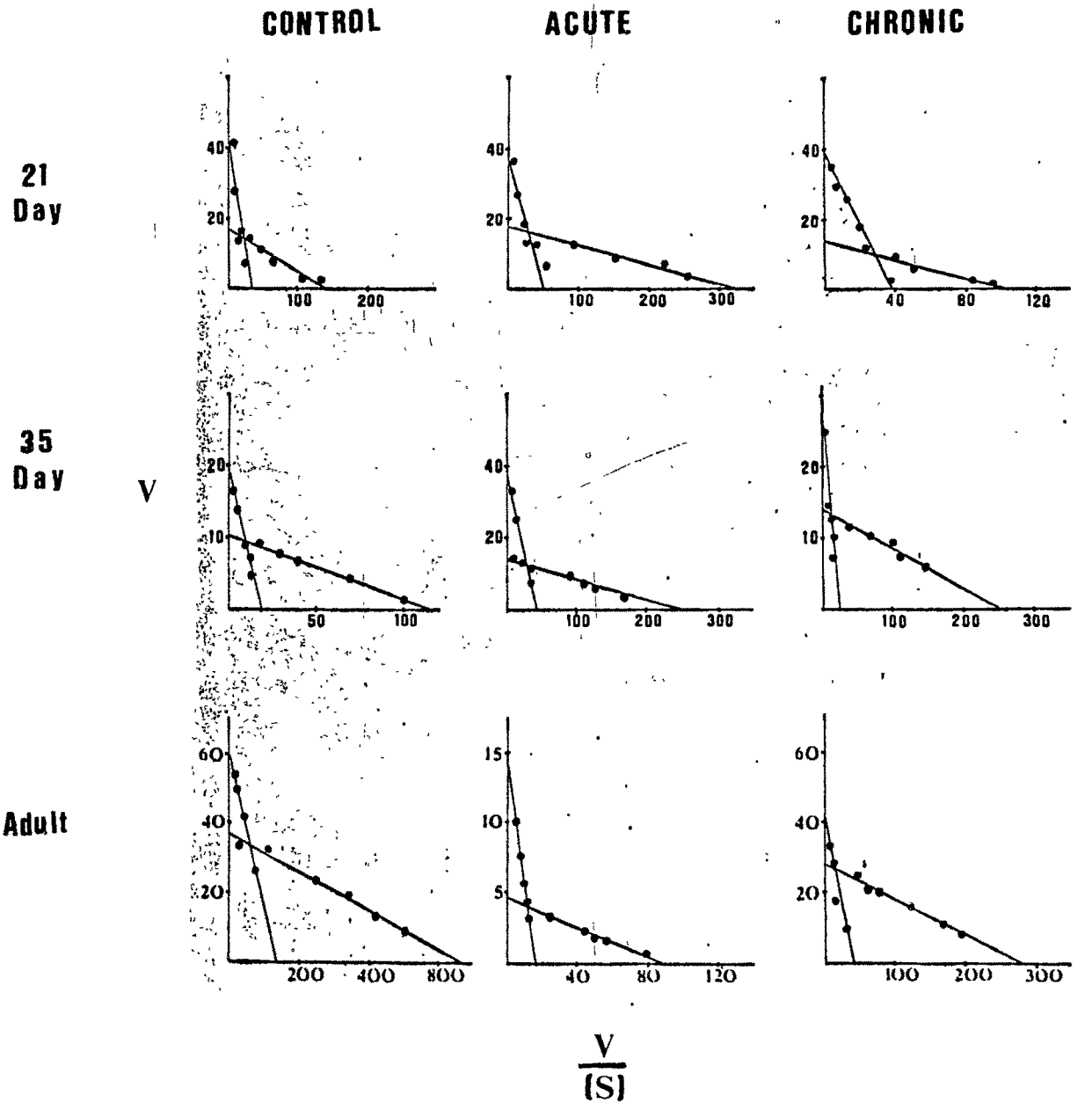

Table 2

Effects of corticosterone treatment on substrate kinetics parameters of brain SMP ATPase during development.

Treatment	High affinity site		Low affinity site	
	Km (mM)	Vmax (μmols/hour/mg protein)	Km (mM)	Vmax (μmols/hour/mg protein)
		21-Day-old		
Control	0.136 ± 0.004	15.48 ± 1.10	1.27 ± 0.06	38.83 ± 1.25
Acute	0.042 ± 0.005[f]	14.14 ± 2.13	0.86 ± 0.04[c]	36.46 ± 1.27
Chronic	0.093 ± 0.008[c]	14.16 ± 0.43	1.10 ± 0.06	41.00 ± 1.15
		35-Day-old		
Control	0.046 ± 0.007	10.85 ± 2.77	0.79 ± 0.08	21.80 ± 3.47
Acute	0.042 ± 0.004	14.27 ± 2.90	0.98 ± 0.08	34.73 ± 3.43[a]
Chronic	0.052 ± 0.002	12.98 ± 0.39	1.55 ± 0.07[d]	28.98 ± 1.01
		Adult		
Control	0.074 ± 0.002	30.93 ± 1.76	0.47 ± 0.01	53.04 ± 3.50
Acute	0.079 ± 0.002	5.84 ± 0.73[f]	0.96 ± 0.09[c]	15.51 ± 0.61[e]
Chronic	0.132 ± 0.016[b]	23.44 ± 2.91	1.00 ± 0.06[d]	44.50 ± 2.94

The data were analyzed by Lineweaver - Burk and Eadie - Hofstee plots and the values of Km and Vmax were averaged.

The results are given as mean ± SEM of 4 independent observations in each group.

[a] $P < 0.1$; [b] $P < 0.05$; [c] $P < 0.02$; [d] $P < 0.01$; [e] $P < 0.002$ and [f] $P < 0.001$ compared to the corresponding controls.

Both the treatments did not have any effect on these kinetic parameters of high affinity site of ATPase in 35-day group.

In 21 -day -old animals only the acute treatment led to 32% decrease in Km for ATP for the low affinity site; Vmax remained unaffected by both the corticosterone treatments. In 35-day group acute treatment significantly increased the Vmax (59% incerase) and chronic treatment increased significantly the Km (96% increase) of low affinity site of brain SNP ATPase. In adults both the corticosterone treatments caused more than 100% increase in Km of low affinity site; Vmax was decreased by 70% upon acute treatment.

Arrhenius kinetics studies of brain SMP ATPase had shown that in control 35-day-old and adult animals, no break in Arrhenius plots were observed. In 14 -day group chronic corticosterone treatment and in case of 35-day-old and adult animals both the corticosterone treatments completely abolished the transition temperature. 21-day group was not affected by corticosterone treatments (Fig. 2). Values of the Arrhenius kinetics parameters of brain SMP ATPase as affected by the two corticosterone treatments are given in Table 3.

Acute treatment to 14-day-old and adult rats caused 22% and 27% decrease reaspectively in the energy of activation E_1. Chronic treatment to 14-day-old animals led to 32% decrease in E_1; 35-day-old and adult groups showed significant (21 to 25%)

Figure 2

Arrhenius plots of brain SMP ATPase from control and corticosterone-treated animals of different age groups.

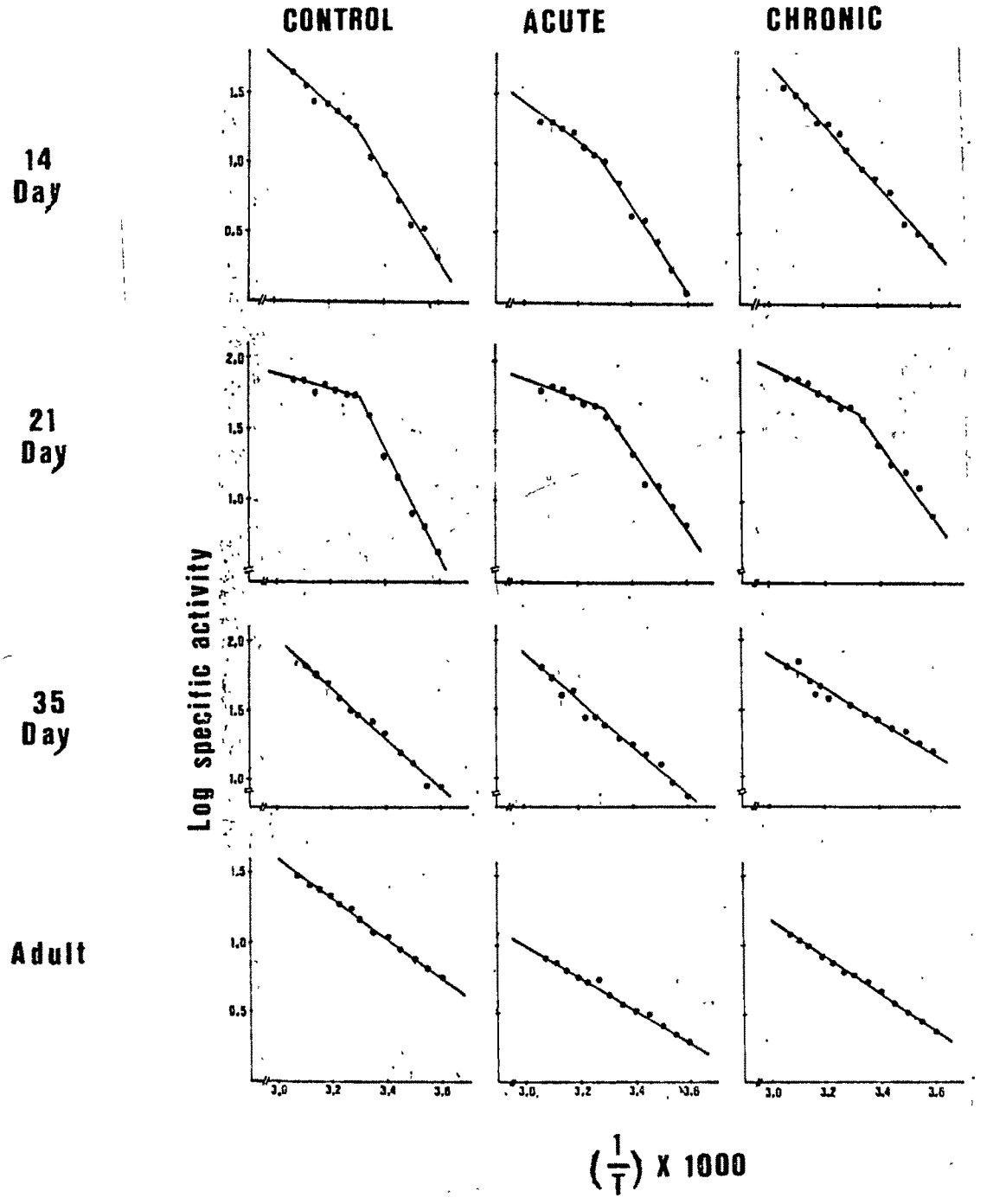

Table 3

Effects of corticosterone treatment on Arrhenius Kinetics parameters of brain SMP ATPase during development.

Treatment	Phase transition temperature (Tt),°C	Energy of activation (KJ/mol)	
		E_1	E_2
	14-Day-old		
Control	28.4 ± 0.57	32.6 ± 0.64	53.9 ± 3.03
Acute	30.1 ± 1.35	25.4 ± 0.79[d]	53.9 ± 3.19
Chronic	———	43.0 ± 2.65[b]	———
	21-Day-old		
Control	30.5 ± 0.80	9.7 ± 0.90	73.8 ± 3.14
Acute	31.4 ± 0.80	10.5 ± 1.88	58.1 ± 0.39[c]
Chronic	29.8 ± 1.45	14.9 ± 2.43	56.8 ± 0.39[c]
	35-Day-old		
Control	———	33.4 ± 0.16	———
Acute	———	32.8 ± 0.12	———
Chronic	———	25.0 ± 0.94[d]	———
	Adult		
Control	———	30.6 ± 0.57	———
Acute	———	22.4 ± 1.70[c]	———
Chronic	———	24.1 ± 2.00[a]	———

Results are given as mean ± SEM of 4 independent observations in each group.

[a]$P < 0.1$; [b]$P < 0.05$; [c]$P < 0.02$ and [d]$P < 0.01$ compared to the corresponding controls.

decrease in E_1 of brain SMP ATPase.

Activation energy E_2 of brain SMP ATPase decreased significantly (21 to 23% decrease) upon both the corticosterone treatments to 21 -day -old rats.

DISCUSSION

From the data presented in this chapter it is clear that ATPase activity did not show much change during development. Brain mitochondrial ATPase activity in other age groups was comparable to that of the adults. Thus, the ATPase activity does not seem to be the limiting factor for ATP synthesis; the ADP -phosphorylation rates increased by several folds during development (e.g. see Chapter II). The ATP synthesis in brain mitochondria from young animals could be limited by the mitochondrial respiration, as the mitochondrial state 3 respiration rates were substantially low in young animals compared to the adults (chapter II).

In brain mitochondria from 14-and 21-day-old animals, in general, both the corticosterone treatments caused significant decrease in ATPase activity under basal as well as stimulated / uncoupled conditions. This could possibly be one of the factor responsible for significant reduction in ADP-phosphorylation rates in brain mitochondria from these groups mentioned in Chapter II.

Surprisingly, acute treatment had no effect on ATPase activity in 35-day group and chronic treatment led to significant increase in Mg^{2+} as well as DNP- stimulated ATPase activity; although both the corticosterone treatments adversely affected brain mitochondrial respiration and oxidative phosphorylation in this age group (chapter II). The observed increase in ATPase activity could possibly be a compensatory mechanism to overcome the decreased ADP – phosphorylation rates in this group. Adults also showed similar increase in Mg^{2+} and DNP -stimulated ATPase activity upon chronic treatment, but the basal and Mg^{2+} + DNP - ATPase activities were decreased significantly.

Mitochondrial ATPase from heart and liver is known to exibit three components possessing different kinetic properties (1-3). Substrate kinetic studies of brain SMP ATPase revealed that ATPase in brain mitochondria has only two components. The high affinity site is characterized by lower values of Km and Vmax whereas low affinity site of ATPase has higher Km and Vmax. The Km and Vmax of both the sites of ATPase showed specific developmental changes.

Corticosterone treatments in age-dependent and treatment specific manner altered the kinetic properties of brain SMP ATPase. The corticosterone treatments mainly affected the Km for ATP of both the sites without having much effects on Vmax. In 21-day group, Km of both the sites was decreased whereas in

adults the Km value showed significant increase upon corticosterone treatment. 35-day-group was least affected by the corticosterone treatments.

Mitochondrial ATPase which is localized in the inner membrane has specific requirement for negatively charged phospholipid for its catalytic fonction (12,13). The distribution of phospholipid classes within the membrane plays an important role in modulating ATPase function (12). The changes in membrane phospholipids and ionic interactions between enzyme protein and lipid matrix could affect the interactions of substrate with the catalytic site. The observed changes in Km and Vmax of ATPase upon corticosterone treatments could be attributed to this factor as both the corticosterone treatments significantly attered the membrane lipid/phospholipid environment of brain mitochondria (chapter IV).

Arrhenius kinetics studies have shown that in control and corticosterone treated animals of 35-day group and adults, and also the chronically treated 14-day-old rats, the break in Arrhenius plots of brain SMP ATPase was completely abolished. Cholesterol has been known to abolish the transition temperature in Arrhenius plots (14). The cholestrol content of brain mitochondria from these groups was higher than those showing a break in the Arrhenius plots (chapter IV) hence the

observed changes in transition temperature in Arrhenius plots could be attributed to this factor.

As a result of the alterations in the lipid environment of brain mitochondria,the energy of activation E_1 and E_2 of brain SMP ATPase also showed significant changes upon corticosterone treatments in age-depandent manner.

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SUMMARY

1. ATPase activity in brain mitochondria did not show much change during development.

Corticosterone treatments to animals at pre-weanling stage decreased significantly the basal and Mg^{2+} as well as DNP - stimulated ATPase activity.

In case of 35-day-old and adult rats the corticosterone treatments in general increased the ATPase activity under stimulated conditions but the basal ATPase activity had decreased significantly.

2. Substrate kinetics studies revealed that the brain SMP ATPase has two different catalytic sites: a high affinity site and a low affinity site.

Effects of corticosterone treatments on the kinetic properties of these two different catalytic sites of brain SMP ATPase were age-dependent and treatment specific.

The major effects of corticosterone treatments were on Km rather than Vmax. In young animals it decreased the Km of both the sites but in adults the Km was increased. 35-day group was least affected by corticosterone treatments.

3. Arrhenius kinetics studies showed that in 14-day group upon chronic treatment and in 35-day-old and adult animals, control as well as corticosterone treatment groups the transition temperature was completely abolished. These changes correlated well with the cholesterol content of brain mitochondria in these groups.

The energies of activation E_1 and E_2 of brain SMP ATPase also showed significant alterations upon corticosterone treatments in age-dependent manner.