Chapter 8

Effect of dexamethasone treatment on kinetic attributes of brain mitochondrial ATPase during post- natal development.

Introduction

The Fo F1 ATPase catalyzes electrochemical proton gradient Δp-driven ATP synthesis or (Δp)–generating ATP hydrolysis in the coupling membranes of mitochondria(1) The peripheral part is composed of five different polypeptides in the stoichiometry α_3, β_3, γ, δ, ε and is capable of rapid uncoupled ATP hydrolysis when detached from the membrane embedded F_0. The F_0 components contains a proton conducting pathway and operating together F_0-F_1 provide (Δp)–consuming ATP synthesis or (Δp)-generating ATP hydrolysis depending on the physiological (in vivo) or experimental (in vitro) conditions (2-4)

Glucocorticoids are known to affect the ATPase activity under in vitro as well as under in vivo conditions(5) Glucocorticoids have ATPase stimulatory effects in liver as well as lymphosarcoma(6) But the glucocorticoids effects on ATPase activity in brain mitochondria have not been examined Also, the observed effects were in vitro and the concentration used were above the physiological which is above 0 6mM Hence physiological significance of these finding is of dubious nature and the effects could be considered as a pharmacological effects of steroids (5)

The results of the previous Chapters (4 and 6) showed that there was significant changes in the brain mitochondrial oxidative phosphorylation and also changes in the mitochondrial membranes lipid was observed during developmental period after dexamethasone treatment Therefore it is of interest to study the in vivo effects of the dexamethasone treatments on activity and kinetic properties of ATPase from brain mitochondria during postnatal period of development and also in adults Hence studies were carried out to examine 1) Mitochondrial ATPase activities 2) Substrate kinetics of

ATPase to determine Km and Vmax value and, 3) Temperature dependent kinetic proterties

Materials and Methods

The details of chemicals used required as well as method for isolation of mitochondria are as given in previous Chapter 4 of the thesis

Assay of ATPase activity

The ATPase activity in the brain mitochondria was determined in a medium containing 250mM sucrose, 10mM KCl, 0 2mM EDTA, 2 mM ATP, 2mM $MgCl_2$ and/or 50μM DNP (7) After pre-incubating the mitochondrial protein (Ca 100μg) in the assay medium at 37 °C, the reaction was initiated by the addition of ATP at a final concentration of 2 mM (7) The reaction was carried out for 10 min and terminated with 1 1 ml of 5% (w/v) TCA and tubes were immediately kept on ice for 10 min The tubes were centrifuged at 3000 rpm in a table top centrifuge and 1 0ml of aliquots of the supernatant was transferred to another tubes for the estimation of the inorganic phosphorus liberated which was estimated by method of Fiske and Subba Row (8)

For substrate kinetics studies concentration of ATP was varied in the range from 0.04 mM to 2 mM

For temperature kinetics studies, experiments were carried out with fixed ATP concentration of 2 mM and the temperature was varied from 5°-53°C with an increment of 4°C at each step

The data for substrate kinetics were analyzed by the Lineweaver-Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods for the determination of Km and Vmax (9) The values of Km and Vmax obtained by the three methods were in close agreement and were averaged Hill plot analysis was carried out where indicated (9)

The data on temperature kinetics were analyzed for the determination of energies of activation in the high and low temperature ranges (E_1 and E_2 respectively) and phase transition temperature (Tt) according to the method described previously (10)

All the kinetics data were computer analyzed employing Sigma plot version 5 0 (11)

Protein estimation was by the method of Lowry et al with bovine serum albumin used as the standard (12)

Results are given as mean ± SEM

Statistical evaluation of the data was by Students' t-test

Results

The data in the Fig 1 show the age dependent changes in ATPase activity during development As is evident the activity increased in 3 week group, whereas 2 week and 4 week groups resulted decrease in the activity

In the next series of experiments the substrate and temperature dependency of the enzyme activity was examined Fig 2- 6 show the typical graphs for the substrate saturation and the corresponding Eadie-Hofstee plots for 2 to 5 week group and in the adult animals respectively

Fig. 1

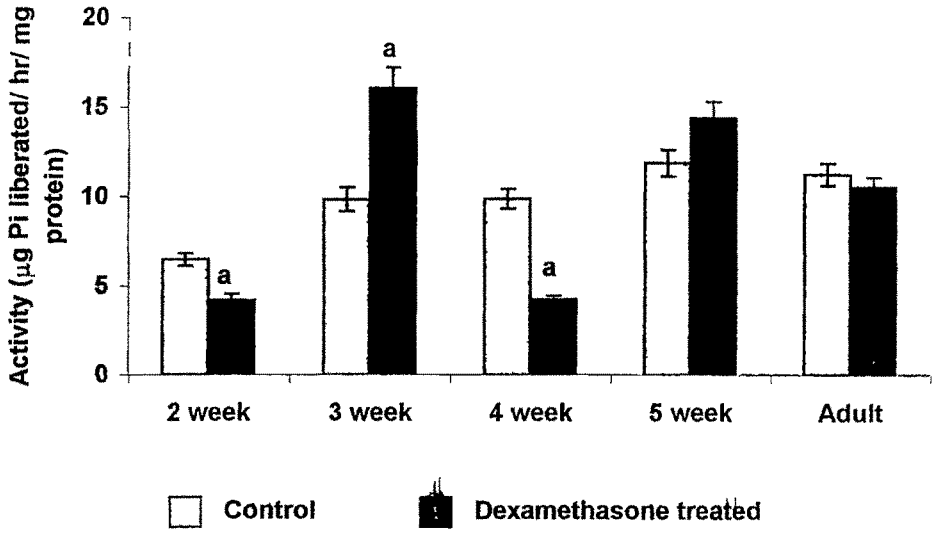
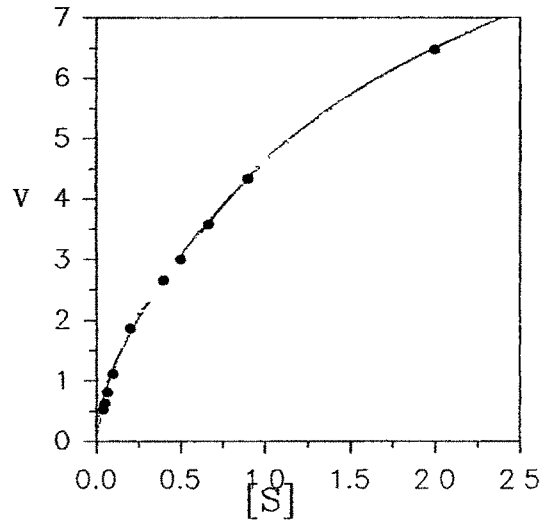

Activity (µg Pi liberated/ hr/ mg protein)
20
15
10
5
0
a
a
2 week
3 week
4 week
5 week
Adult
Control
Dexamethasone treated

Fig. 2 to 6. Typical substrate saturation curves and the respective Eadie-Hofstee plots for brain mitochondrial ATPase in controls and dexamethasone treated animals Experimental deta are as given in the text For determination of substrate kinetics of ATPase, ATP was used as substrate over a concentration range of 0 004 to 2mM The abscissa represents the reacti velocity v, while the ordinate represents [S] For the Eadie-Hofstee plots the abscissa represe the reaction velocity v, while the ordinate represents v/[S] Reaction velocity v == μmol of liberated hr^{-1} mg $protein^{-1}$ V/[S]= reaction velocity divided by the corresponding substra concentration Hill plot analysis were made for sigmoidal curves [log (v/V-v) versus log [S] [S mM

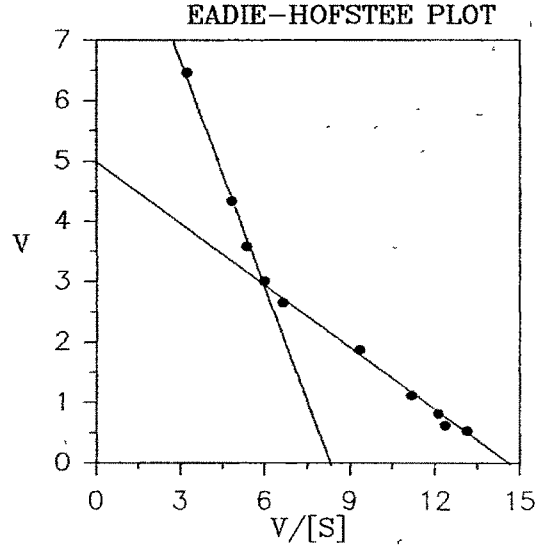
Fig. 2

CONTROL

SUBSTRATE SATURATION CURVE

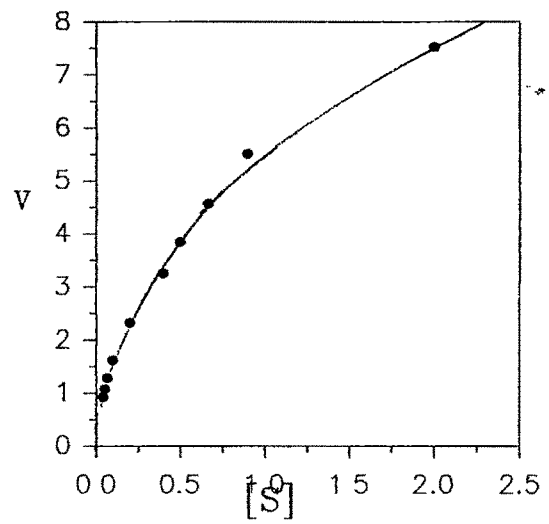


EADIE–HOFSTEE PLOT



DEX TREATED

SUBSTRATE SATURATION CURVE



EADIE–HOFSTEE PLOT

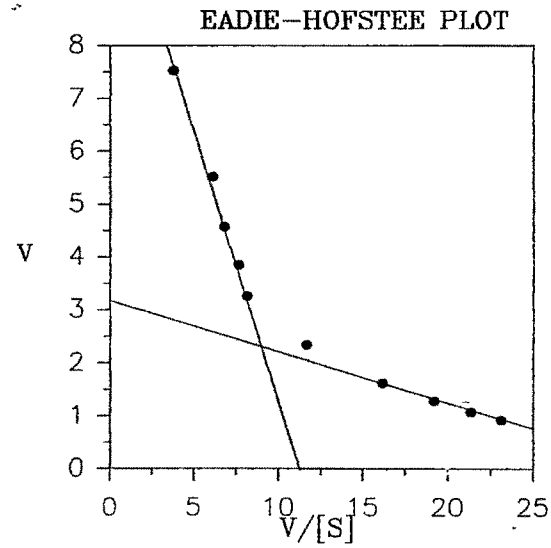


Fig. 3

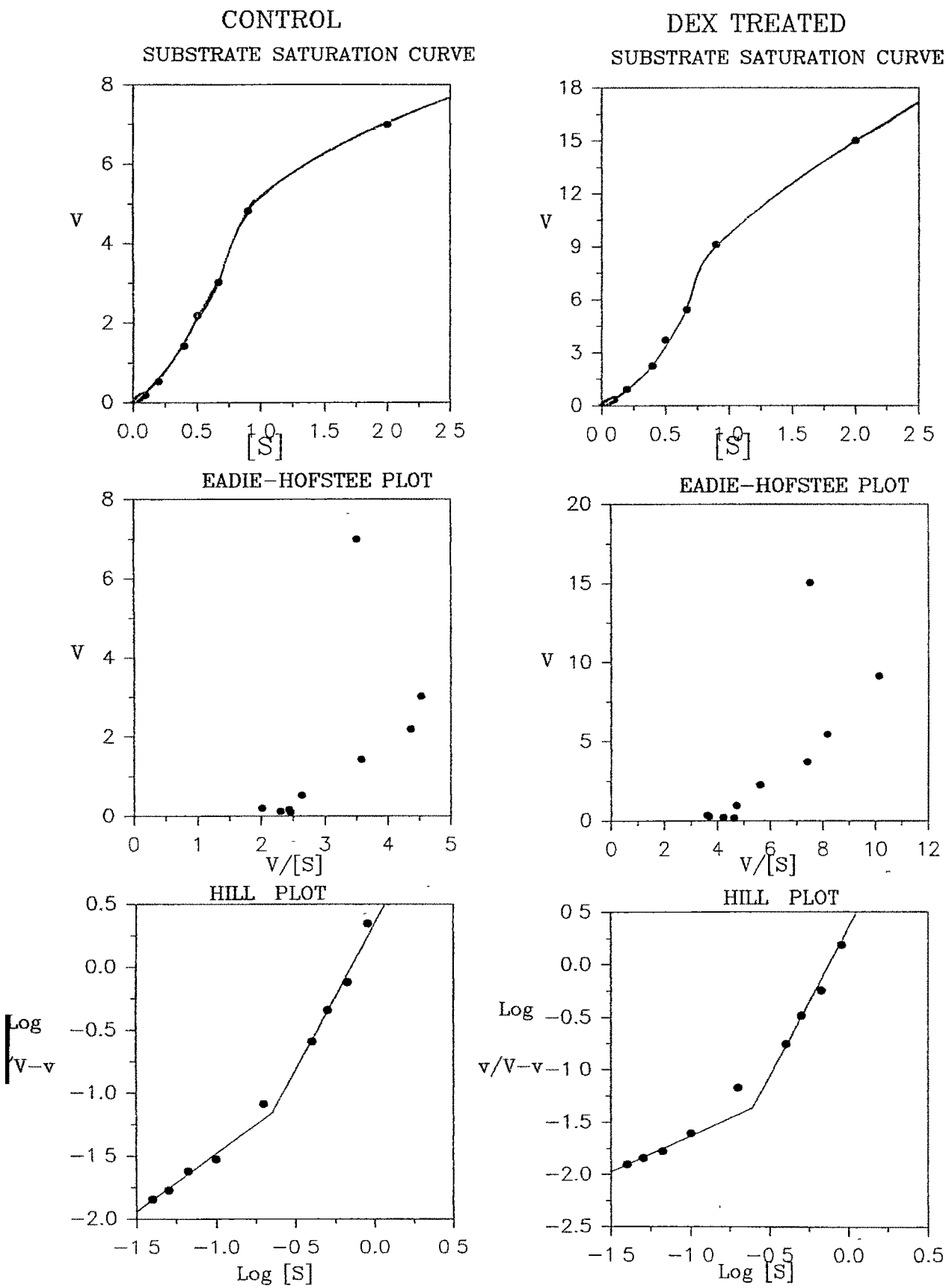

CONTROL
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]
HILL PLOT
Log V–v
Log [S]
DEX TREATED
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]
HILL PLOT
Log v/V–v
Log [S]

Fig. 4

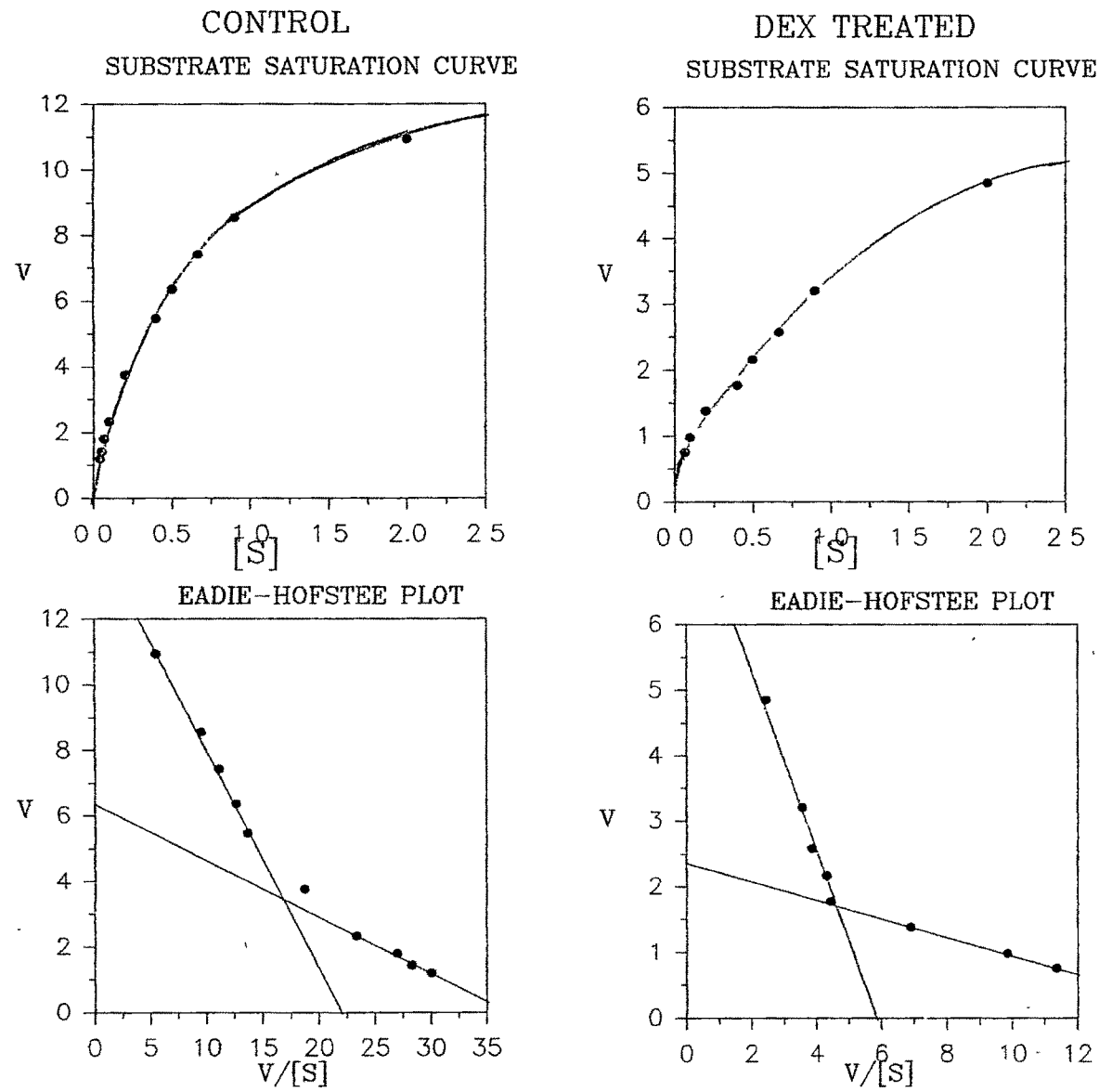

CONTROL
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]
DEX TREATED
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]

Fig. 5

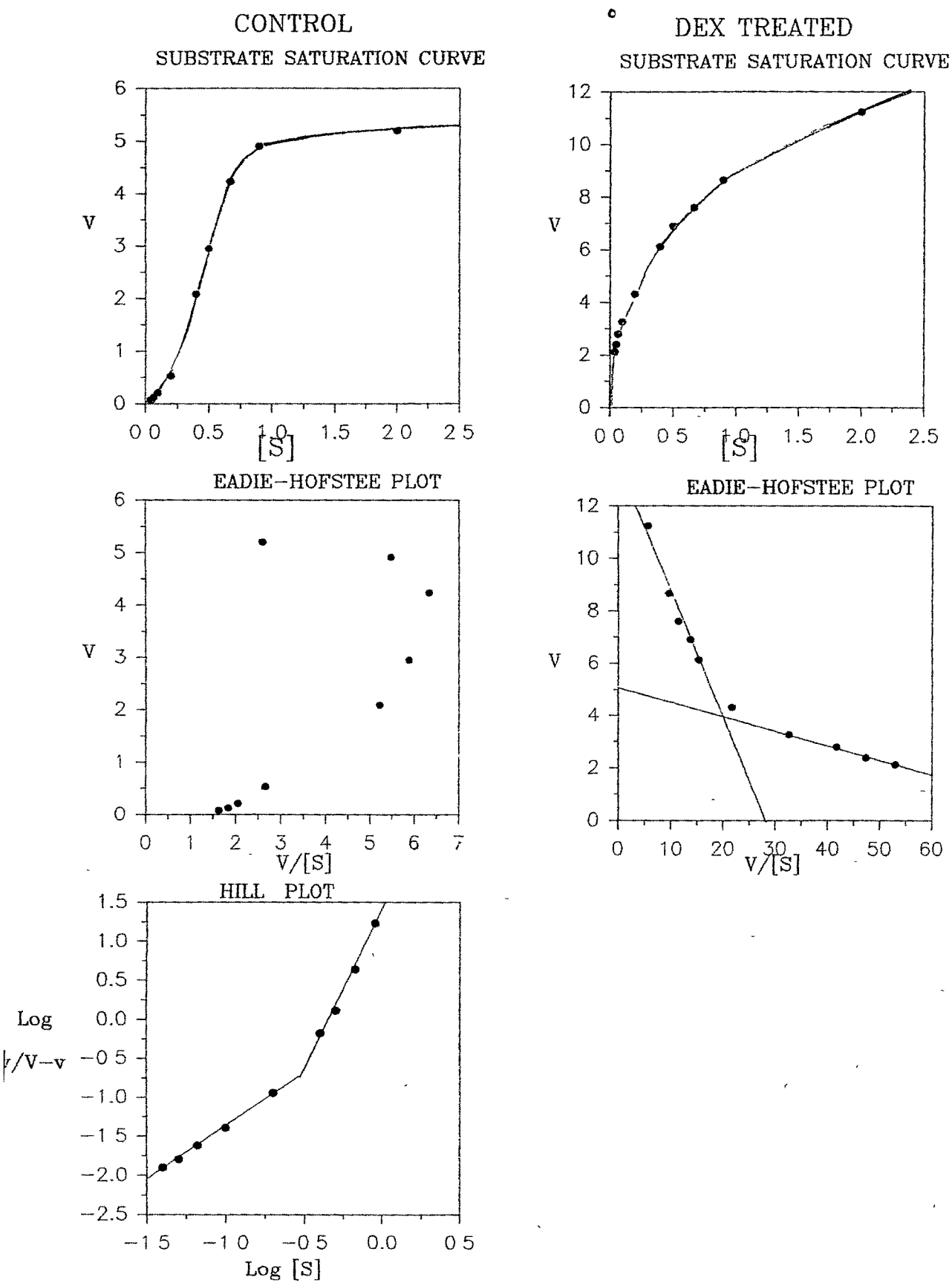
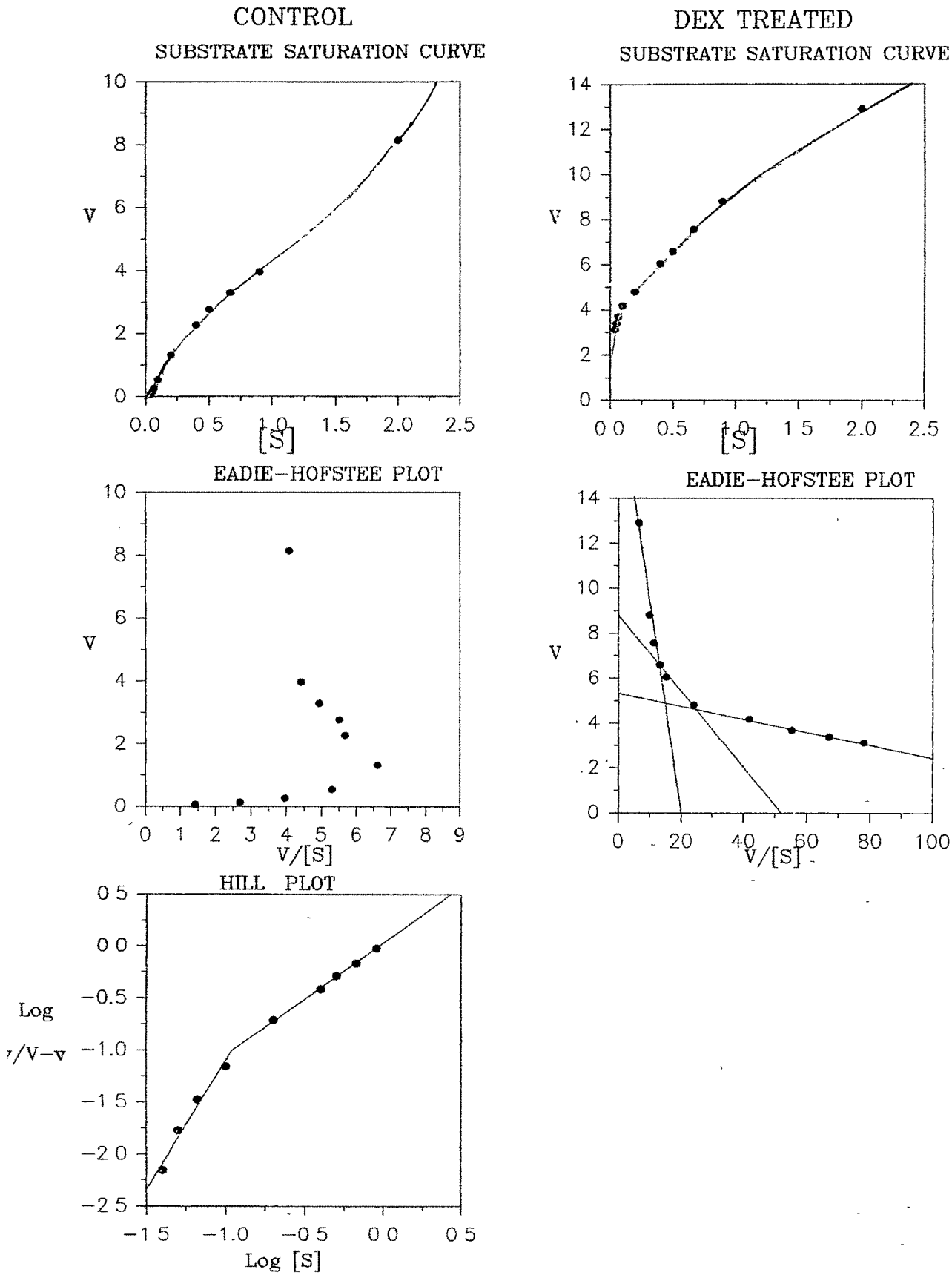

CONTROL
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]
HILL PLOT
Log v/V–v
Log [S]
DEX TREATED
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]

Fig. 6


CONTROL
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]
HILL PLOT
Log
r/V–v
Log [S]
DEX TREATED
SUBSTRATE SATURATION CURVE
V
[S]
EADIE–HOFSTEE PLOT
V
V/[S]

200

As can be seen in the control group, normal substrate saturation pattern was noted for 2 and 4 week animals while in the 3 week, 5 week and adult animals the pattern was allosteric i e the curve was sigmoidal After dexamethasone treatment, in the 5 week group and in adults the allostericity was abolished and normal substrate saturation pattern was seen

For determination of Km and Vmax values, data were further analysed in terms of Lineweaver Burk, Eadie-Hofstee and Eisenthal Cornish-Bowden plots (9) wherever applicable For sake of brevity only the respective Eadie-Hofstee plots are shown in Fig 2-6 The Km and Vmax values determined by the three methods were in close agreement and were averaged Thus the results are given as mean $\pm$ SEM of the averaged values (Table 1)

The Hill plot analysis was performed in the group which showed allosteric sigmoidal plots and Hill coefficients (n) calculated as well as $S_{0.5}$ values were determined These data are given in Table 2 As can be seen from the data in Table 1, the 2 week and 4 week animals, both control as well as dexamethasone treated showed presence of two component of ATPase depicting high affinity and low capacity, and low affinity and high capacity binding In the 2 week animals, dexamethasone treatment resulted in lowering the Km of component I to one third value while the Vmax decreased by 40% No change in component II was observed after dexamethasone treatment The 4 week group showed significant decrease in Vmax of component I without decrease in Km whereas Km of component II increased 3 fold and the Vmax value decreased by a similar factor after dexamethasone treatment

Table 1. Effect of dexamethasone treatment on substrate kinetics properties of rat brain mitochondrial ATPase.

		Component I		Component II		Component III	
Group	Treatment	Km $_1$	V *max* $_1$	Km $_2$	V *max* $_2$	Km $_3$	V *max* $_3$
2 week	Control	0 303±0 056	4 88±0 658	1 08±0 131	11 87±1 282	--	--
	Dex	0 092±0 016 [b]	3 03±0 601	0 87±0 054	10 03±1 115	--	--
3 week	Control	--					
	Dex	--					
4 week	Control	0 146±0 027	6 83±0 949	0 58±0 055	16 29±1 587		
	Dex	0 154±0 023	1 66±0 081 [a]	1 571±0 153 [a]	6 85±0 503 [a]		
5 week	Control	--	--				
	Dex	0 091±0 010	5 87±0 789	0 457±0 077	14 76±0 917		
Adult	Control	--	--				
	Dex	0 036±0 009	5 64±0 305	0 206±0 022	9 02±0 048	1 205±0 158	19 16±0 73

The experimental details are as given in the text

The results are given as mean ± SEM of the 8 independent observations For the substrate ATP was used in the concentration range of 0 004 to 2 mM

Km, mM,

Vmax , µmole of Pi liberated /hr/ mg protein

The Km and Vmax values were calculated by three different methods of analysis as described in the text using Sigma Plot version 5 0 and averaged for calculating the mean ± SEM values

[a] $p< 0\ 002$, and [b] $p<0\ 001$ compared with the corresponding control

Following dexamethasone treatment the 5 week animals displayed presence of two ATPase components with kinetic attributes (Km and Vmax) comparable to 4 week animals In the adults following dexamethasone treatment, ATPase activity resulted in three components The 3, 5 week and adults (both control and dexamethasone treated groups) showed allosteric pattern (Fig 2,3 and 5). From comparing Hill plots (Fig 2,3,5), the Hill coefficients n_1 and n_2 and $S_{0.5}$ were calculated (Table 2)

The typical Hill plots for the 3 week (control and dexamethasone treated), 5 week (control) and adult (control) are shown in the Fig 3, 5 and 6 It is interesting to note that the pattern was reversed in the adults The data on Hill plot analysis are given in Table 2 Thus in the 3 week group the values of n and $S_{0.5}$ were comparable for control as well as dexamethasone treated group over the entire concentration range of ATP In the lower concentration range the enzyme bound 1 ATP molecules, in high concentration range 2 ATP molecules were bound $S_{0.5}$ values in high concentration range were lower than those in the low concentration range Practically same pattern was seen for 5 week control except that the enzyme bound 2 and 3 molecules of ATP respectively in low and high concentration range For the adult the pattern was reversed as pointed out above As a consequence the enzyme bound 3 molecules of ATP in low concentration range and 1 in high concentration range Also the value of $S_{0.5}$ in low concentration range was significantly low

The dexamethasone induced changes were also reflected in the temperature kinetics of the enzyme The typical plot depicting the temperature dependent changes in velocity are shown in Fig 7-11 The corresponding Arrhenius plot are also included It is of interest to

Table 2. Effect of dexamethasone treatment on Hill plot analysis of rat brain mitochondrial ATPase.

Group	Treatment	n_1	$S_{0.5\,(1)}$	Transition mM	n_2	$S_{0.5\,(2)}$
3 week	Control	0 934±0 061	1 940±0 25	0 233±0 014	1 795±0 082	0 832±0 026
	Dex	0 827±0 11	1 520±0 22	0 180±0 002	1 811±0 194	0 807±0 037
5 week	Control	1 218±0 18	0 829±0 23	0 285±0 082	2 620±0 410	0 530±0 064
Adult	Control	0 837±0 039	1 124±0 058	0 101±0 013	2 781±0 479	0 283±0 069

The experimental details are as given in the text

The results are given as mean ± SEM of the 8 independent observations. For the substrate ATP was used in the concentration range of 0 004 to 2 mM

$S_{0.5(1)}$ and $S_{0.5\,(2)}$, mM, n_1 and n_2 are Hill co-efficients

Fig. 7 to 11. Typical temperature curves and the respective Arrhennius plots for rat br mitochondrial ATPase in controls and dexamethasone treated animals Experimental details as given in the text The ATPase activity was determined with 2 mM ATP The absci represents the reaction velocity v, while the ordinate represents the temperature in °C React velocity v = µmol of Pi liberated hr^{-1} mg $protein^{-1}$ For the Arrhennius plots abscissa represe the log of reaction velocity v, while the ordinate represents reciprocal of absolute temperat T*1000 Reaction velocity v = µmol of Pi liberated hr^{-1} mg $protein^{-1}$ Absolute temperature °Kelvin

Fig.7.

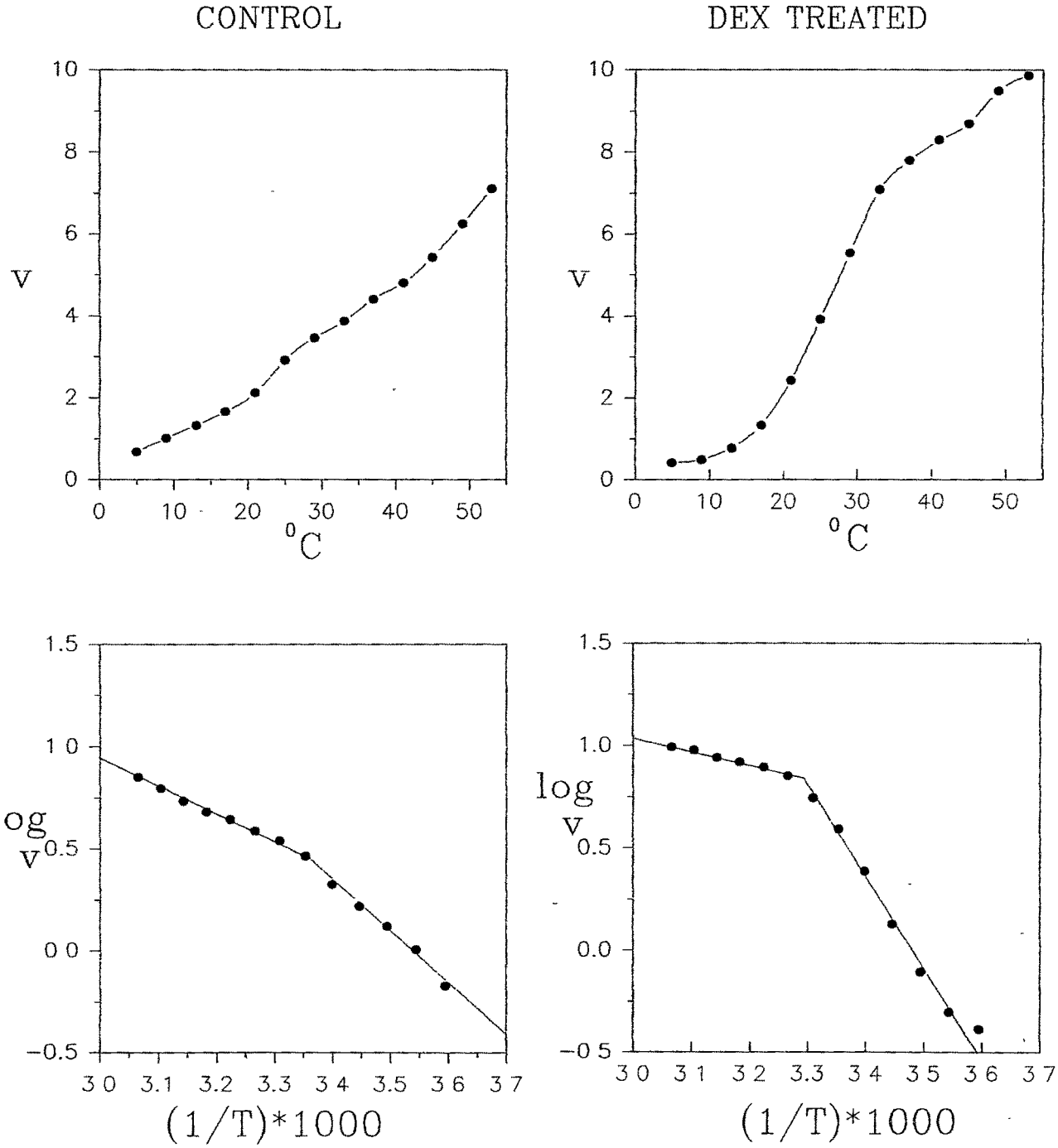


Fig.8.

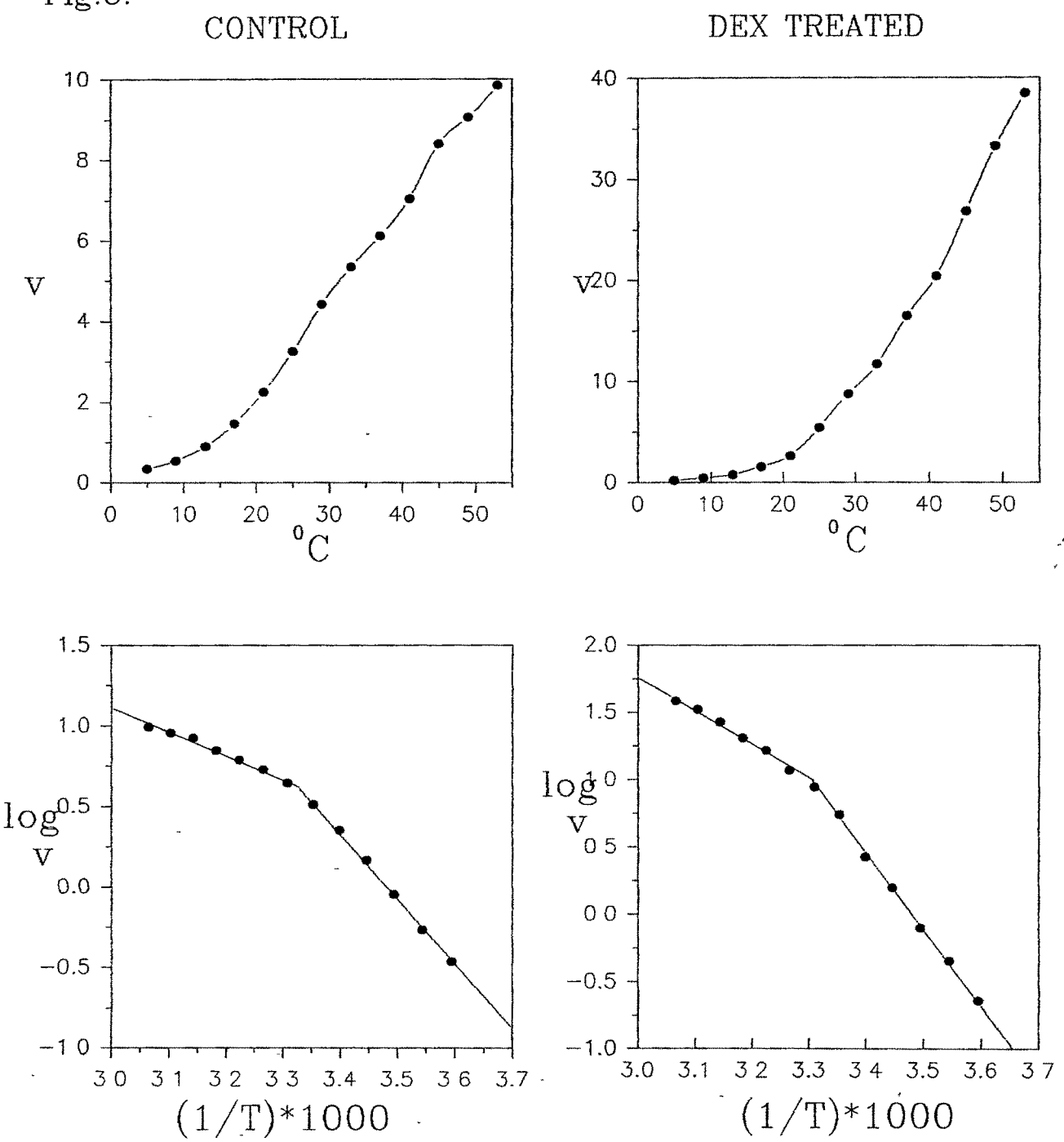


Fig.9.

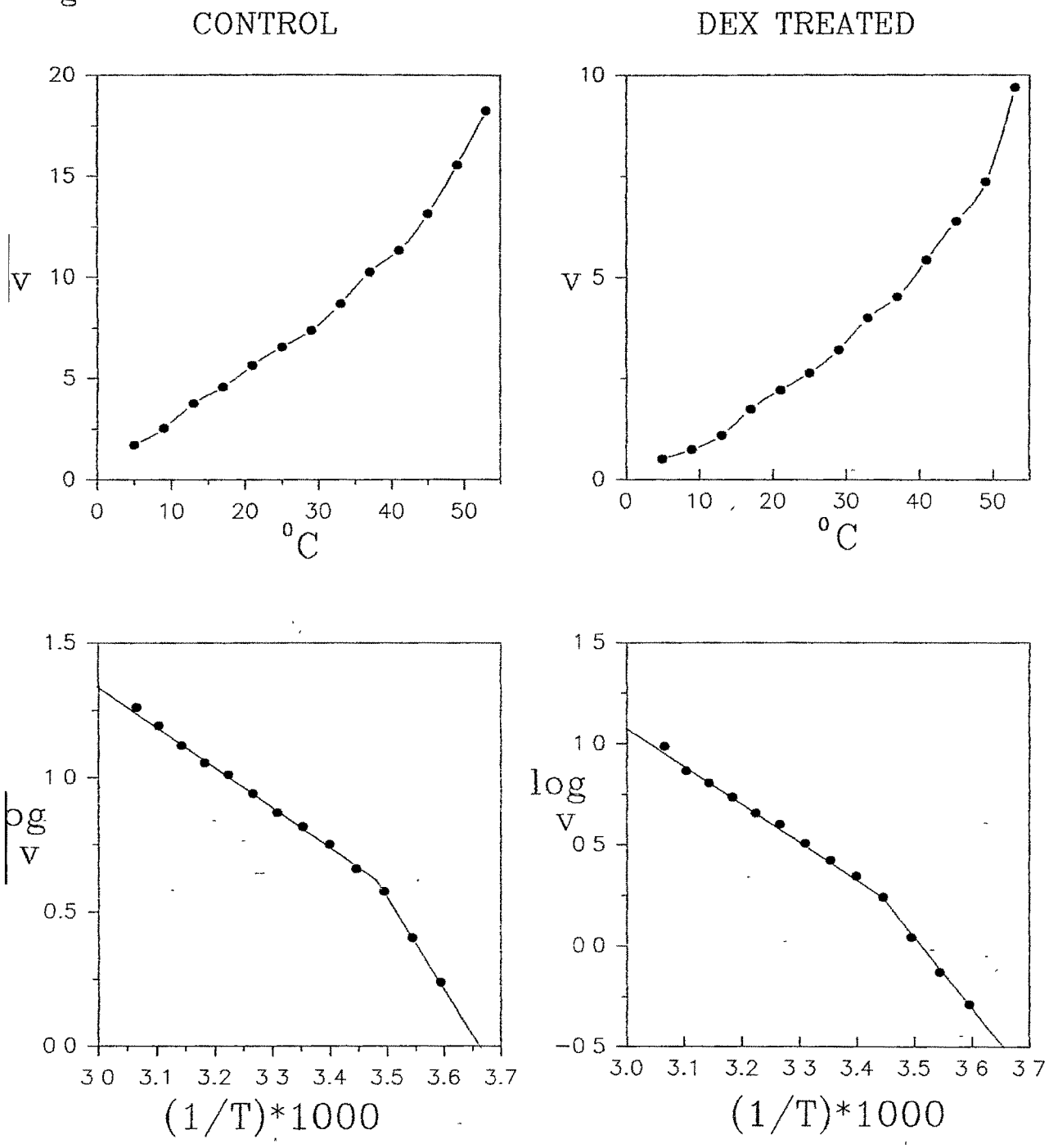
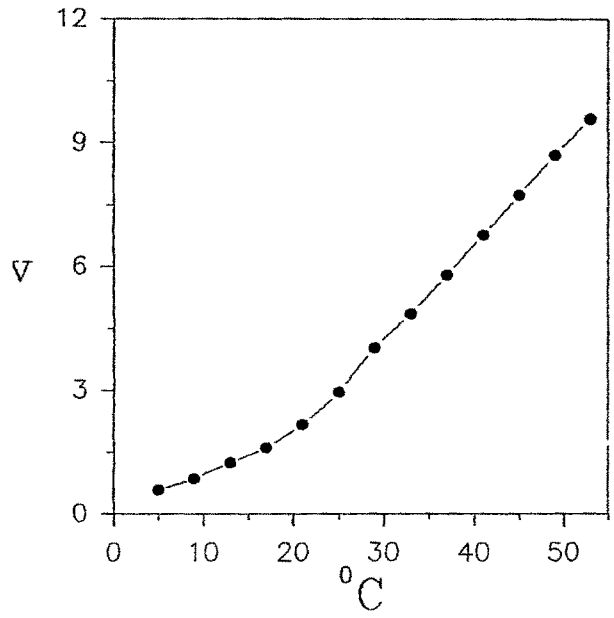
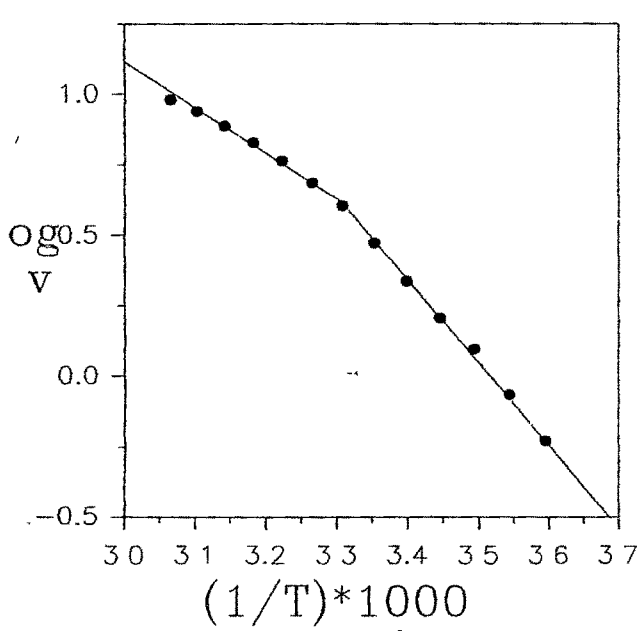


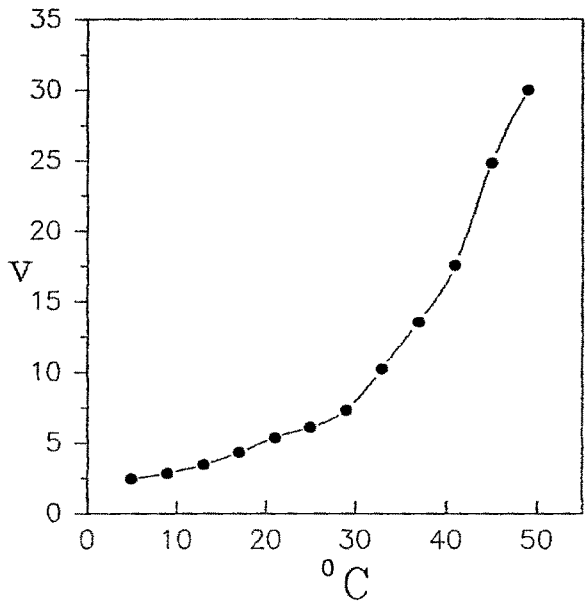
Fig.10.

CONTROL





DEX TREATED



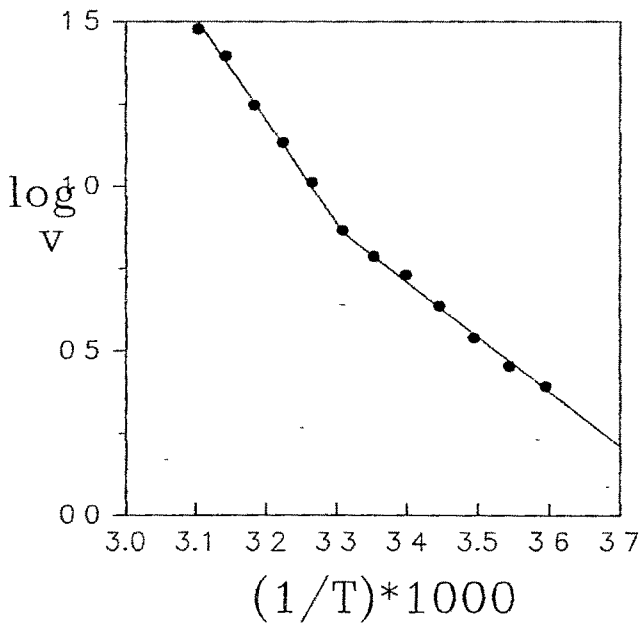
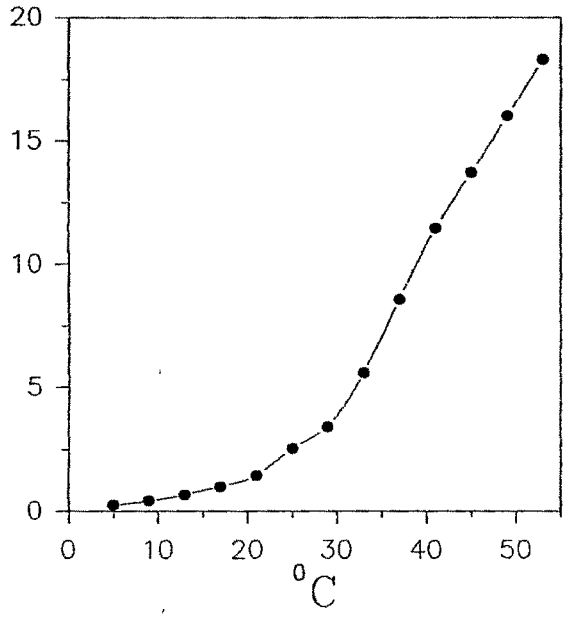


Fig.11.

CONTROL



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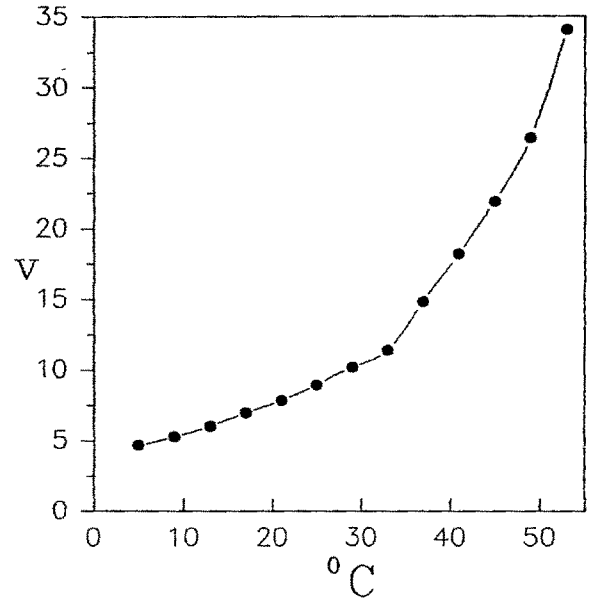

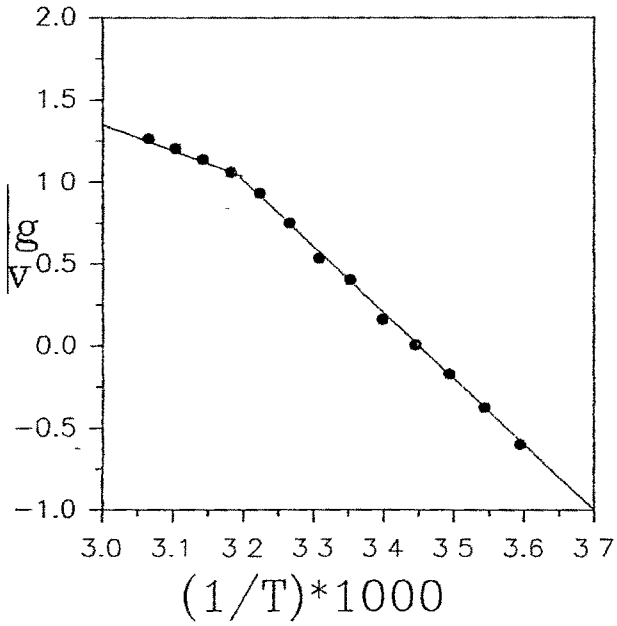

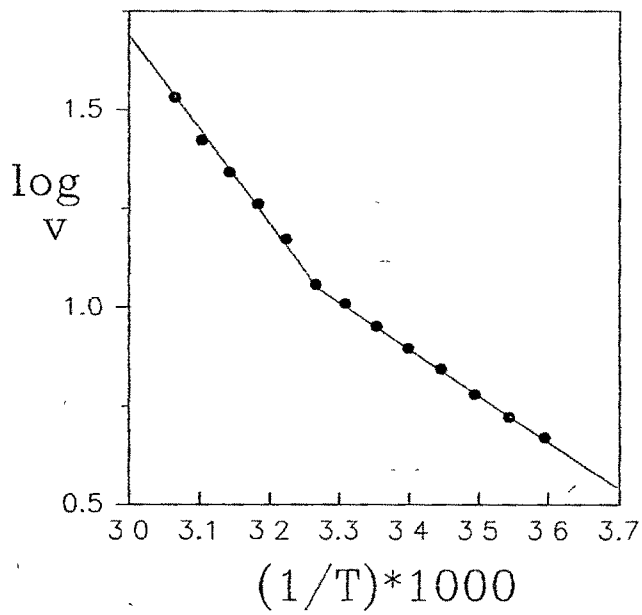

know that the typical biphasic Arrhenius plot were seen for all the age groups form control animals In dexamethasone treated rats the pattern was comparable with control up to 4th week, the biphasic pattern was reverse in 5 week group and adults (Table 3)

Discussion

The dependence of F_o F_1 ATPase on acidic phospholipids especially PS, PI and DPG is well recognized (13,14) The age dependent and dexamethasone induced changes in the mitochondrial lipid / phospholipid composition are already detailed in the Chapter 6 In the light of the observed changes in the substrate and temperature dependent functions, and dexamethasone induce alteration in these parameters, an attempt was made to correlate the observed changes with the lipid make up by regression analysis The outcome is briefly summarized in Table 4

Thus across different age groups including the control and dexamethasone treated animals the total ATPase activity measured in the presence of Mg^{2+} +DNP did not correlate with either PS or PI content nor was any correlation seen with a combined PS+PI content However the activity correlated positively with SPM and no correlation with PE was observed The activity showed strong negative correlation with PC and with PC+SPM Likewise a strong positive correlation was seen with SPM/PE and SPM/PC ratio, PC/PE showed a moderate negative correlation (Table 4) It may hence be suggested the PS and PI although essential for enzyme activity may not be involved in modulation On the other hand the major phospholipid component PC is a strong negative modulation, SPM counter balances this effect

Table 3 Effect of dexamethasone treatment on Arrhenius kinetics properties of rat brain mitochondrial ATPase

Group	Treatment	(Energy of activation, KJ/mole) E_1	E_2	Phase transition temperature Tt (°C), Tt_1
2 week	Control	24.53±0 27	47 99±4 03	30 56±2 38
	Dex	19 68±2 43	82 90±10 62 [c]	21 53±4 98
3 week	Control	24 95±2 49	62 77±4 22	28 62±1 96
	Dex	48 01±4 84 [c]	90 42±7 41 [c]	27 00±2 13
4 week	Control	27 03±1 68	63 74±3 41	14 19±0 80
	Dex	36 14±1 44 [b]	88 30±6 26 [a]	13 35±0 47
5 week	Control	30 27±2 76	55 65±3 15	27 94±1 45
	Dex	24 91±2 03 [c]	69 17±4 23 [c]	28 08±2 33
Adult	Control	34 90±2 72	68 28±3 59	40 46±2 32
	Dex	13 67±1 27 [c]	57 47±2 29 [c]	32 71±0 35 [a]

The results are given as mean ± SEM of the 8 independent observations indicated in the parentheses

[a] $p<0.01$, [b] $p<0.002$ and [c] $p<0.001$ compared with the corresponding control

Table 4 Regression analysis of ATPase parameters with lipid parameter

Parameter	Regression coefficient (r)
ATPase activity	
PC	-0 7698
SPM	0 7521
PC+SPM	-0 6827
SPM/PE	0 7541
SPM/PC	0 7978
PC/PE	-0 6490
Energy of activation E_1	
SPM/PE	0 7023
SPM/PC	0 7228
Energy of activation E_2	
PI/BPL	-0 6696

BPL, basicc phospholipid, DPG, Diphosphotidylglycerol, PC, Phosphatidylcholine, PE, Phosphatidylethanolamine, SPM, Sphingomyelin

The regression analysis was extended to evaluate the modulatory role of phospholipid on energies of activation and phase transition temperature As can be seen in Table 4 energy of activation E_1 showed a positive correlation with total acidic phospholipids and a negative correlation with basic phospholipids Interestingly SPM/PE and SPM/PC showed a strong positive correlation. E_2 correlates negatively with PI/BPL ratio Tt showed no correlation with any of the lipid parameters The last observation is not really surprising since Tt is a reflection of membrane fatty acid unsaturation index Althogh the changes in ($\Delta 5$ $\Delta 6$ desaturase and in fatty acid synthase in liver microsomes after dexamethasone treatment have been reported (15) Information on the parameter in the brain is lacking Analysis of fatty acid composition could perhaps shed light on this aspect. However, in the present study the aspect had not been explored

Summary

Dexamethasone treatment resulted in age specific changes in ATPase activity In the control group normal substrate saturation pattern was noted for 2 week and 4 week groups, other groups showed sigmoidal saturation curve Dexamethasone treatment abolished allostericity in 5 week and adult groups In 2 and 4 week control groups ATPase activity resolved in two components differing with respect to the Km and Vmax After dexamethasone treatment in the adult group ATPase activity resolved in three components The typical biphasic Arrhenius plots were seen in all the age groups for control animals, the pattern was comparable up to 4^{th} week after dexamethasone treatment, the 5 week and adult showed a reverse pattern The energies of activation were generally high except for E_2 in 5 week The value of Tt decreased by 8-9^0C in 2 week and adult animals after dexamethasone treatment These changes in ATPase activity and energies of activation were found to be correlating with changes in lipid ratios

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