Chapter 4

Effect of Picrotoxin-induced Epileptic Condition and Antiepileptic Drug Treatment on Rat Liver Mitochondrial ATPase Kinetics Properties

Introduction

In Chapter 2 it was shown that PTX-induced chronic epileptic condition not only affects cerebral mitochondrial oxidative energy metabolism, but also a peripheral tissue and major metabolic organ i.e. liver. Drastic functional alterations in the peripheral system are reported to be associated with excessive ROS production and compromised antioxidant system in the chronic epileptic condition (1-3). Additionally, sporadic or spontaneous mutations in mitochondrial and/or nuclear DNA that encodes for essential components of mitochondria may lead to abnormalities in functioning of CNS and peripheral tissues that is associated with epileptic phenotype e.g. CPEO (chronic progressive external opthalmoplegia), LHON (Lerber's hereditary optic neuropathy), MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes) to name a few (4-6). The process of energy transduction i.e. oxidative phosphorylation is dependent on the membrane phospholipids as is the function of F_oF_1ATPase (7-9). In the light of above, it was of interest to find out the effect of PTX-induced epileptic condition and treatment with AEDs on the kinetic properties of F_oF_1ATPase in liver mitochondria. The results on these findings are summarized in this chapter.

Materials and Methods

Details of chemicals used are as described in Chapter 3.

The treatment with PTX and AEDs were as shown in Chapter 2 and 3. The animals were killed by decapitation after the end of treatment period (i.e. on the day 8th after AEDs treatment and day 21st after PTX or PTX-AEDs treatment). Isolation of mitochondria was essentially the same as described in Chapter 2.

Assay of ATPase

Liver mitochondrial ATPase activity was measured in the assay medium (total volume 0.4 ml) containing 50 mM Tris-HCl buffer pH 7.4, 75 mM KCl and 4 mM EDTA. The assays were performed in the absence and presence of $MgCl_2$ (6 mM) and 100 μM DNP, or a combination of thereof. After pre-incubating the liver mitochondrial protein (Ca. 1 mg) in the assay medium at 37 °C, the reaction was initiated by the addition of ATP at a final concentration of 5 mM. The reaction was carried out for 10 min and then terminated by the addition of 0.1 ml of 5% (w/v) SDS and the amount of liberated inorganic phosphorus was estimated by the method of Katewa and Katyare (2003) (10).

Substrate and temperature kinetics

The kinetics studies were carried out in the assay medium described above containing both Mg^{2+} and DNP. For substrate kinetics studies, the concentration of ATP was varied from 0.01 to 5 mM.

The temperature dependence of the enzyme activity was measured in the presence of fixed substrate concentration at 5 mM and the temperature was varied from 5 to 53 °C (4 °C steps).

Analysis of substrate kinetics data for determination of Km and Vmax was done by the Lineweaver-Burk and Eadie-Hofstee methods (11). The values of Km and Vmax obtained by both the methods were in close agreement and were averaged.

For the temperature kinetics data the determination of energies of activation for the high and low temperature ranges (E1 and E2, respectively) and phase transition temperature (Tt) were calculated form the Arrhenius plots (12).

Analyses of the data were carried out by employing Sigma Plot, version 5.0 (13), Microsoft Excel XP and Prism version 3.0.

Protein estimation was done by the method of Lowry et al. (1951) with BSA as the standard (14).

Results are given as mean ± SEM. Statistical evaluation of the data was performed using the Students' *t*-test.

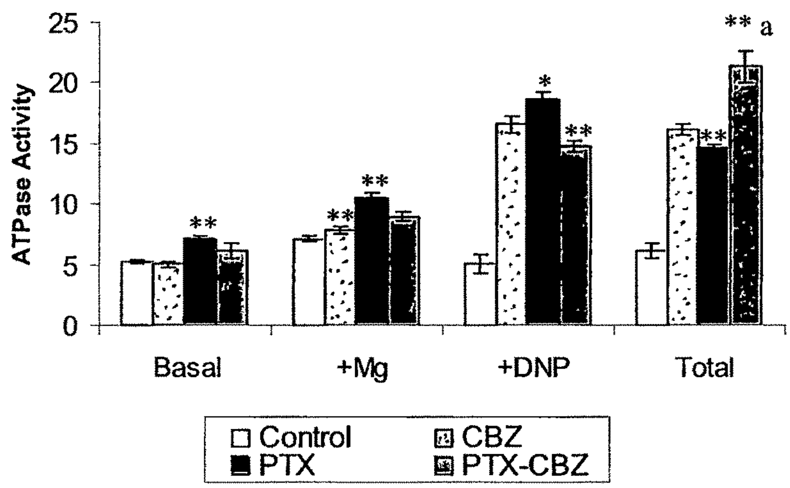
Results and Discussion

As shown in Fig 3 (Chapter 2) that PTX treatment caused elevation of basal and Mg^{2+} stimulated activities, therefore composite decrease in the total activity (+Mg^{2+} and +DNP) was observed.

CBZ, LTG and CLB treatment to the control animals resulted into 1.3 to 1.5 fold increase in the Mg-stimulated activity (Fig 1-3). LTG treatment showed 1.3-fold increase in DNP-stimulated ATPase activity (Fig 2). Despite of no significant alterations in the total activity, Mg^{2+} stimulated activity increased, indicating increased mitochondrial membrane fragility after AED treatment.

CBZ treatment to the epileptic animals resulted in 1.5 fold increase in total ATPase activity, while the basal and DNP-stimulated activities were restored back to normal (Fig 1). 1.5-fold increase in the total ATPase activity was observed in treatment with LTG to the epileptic animals (Fig 2). CLB treatment decreased DNP-stimulated and total ATPase activity by 14-16% with 1.7 to 1.9 folds elevation in basal and Mg-stimulated activities in the epileptic animals (Fig 3). In general, altered ATPase activity after PTX treatment was not restored after AED treatment to the epileptic animals.

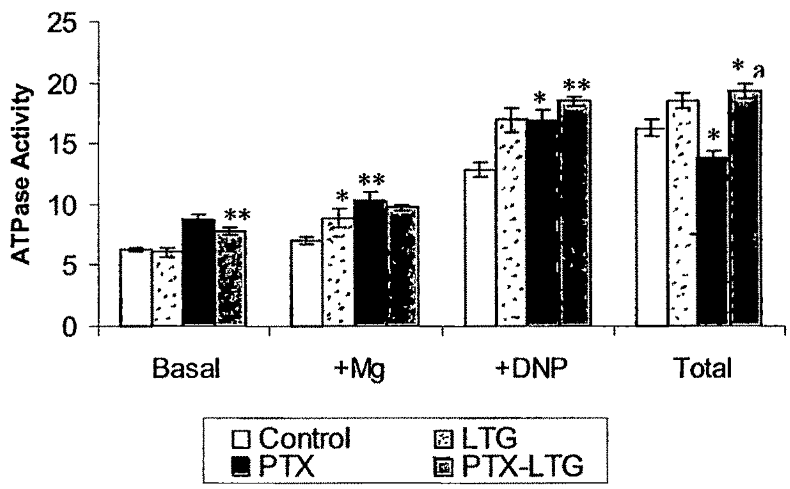
Figure 1. Effect of Carbamazepine (CBZ) treatment on liver mitochondrial ATPase activity in Control and PTX-induced epileptic condition



The experimental conditions are as described in text. The results are given as mean ± SEM of 8 independent observations. The ATPase activity is given in μmole P_i liberated /h/mg protein.

*, p<0.01 and **, p<0.001 compared with control.
a, p<0.001 compared with PTX treated group.

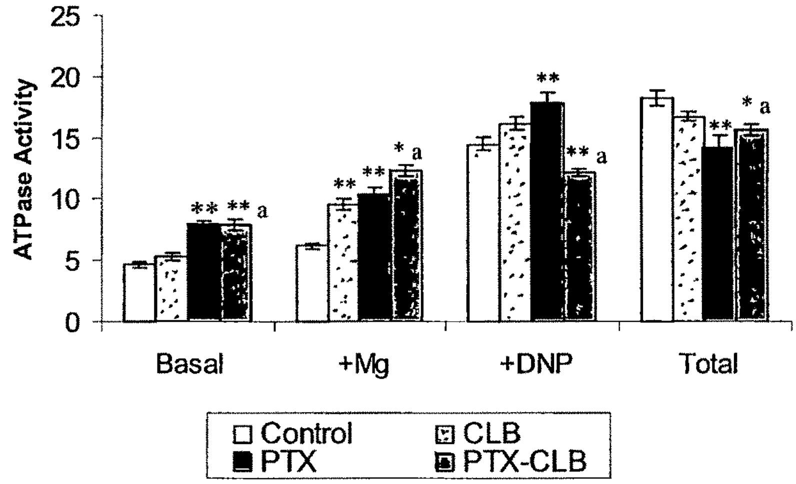
Figure 2. Effect of Lamotrigne (LTG) treatment on liver mitochondrial ATPase activity in Control and PTX-induced epileptic condition



The experimental conditions are as described in text. The results are given as mean ± SEM of 8 independent observations. The ATPase activity is given in μmole P_i liberated /h/mg protein.

*, p<0.01 and **, p<0.001 compared with control.
a, p<0.01 compared with PTX treated group.

Figure 3. Effect of Clobazam (CLB) treatment on liver mitochondrial ATPase activity in Control and PTX-induced epileptic condition



The experimental conditions are as described in text. The results are given as mean ± SEM of 8 independent observations. The ATPase activity is given in µmole P_i liberated /h/mg protein.

*, $p<0.05$ and **, $p<0.001$ compared with control.
a, $p<0.001$ compared with PTX treated group.

In the next series of experiments the substrate kinetics properties of mitochondrial ATPase was carried out. The results on effects of PTX-induced epileptic condition and effect of AED treatment on non-epileptic control and epileptic animals are shown in Fig 4 to 9. The substrate saturation curves for the control and treatment groups are shown in Fig 4 to 6. It is evident that typical substrate saturation patterns were obtained for the control and treatment groups.

From the Eadie-Hofstee plots (Fig 7 to 9) it is clear that in control group the liver mitochondrial ATPase activity could be resolved into three kinetic components. This is consistent with previously reported observations by us and other investigators (15, 16), However, with either PTX or AED treatment resulted in abolishment of one component (Fig 7-9). Thus only two components (I and II) were evident. PTX exposure resulted into 1.5 to 5.5 fold increase in Km and Vm for component II with the loss of component III (Table 1). AED treatment to the control animals in general resulted in increased Km (3.5-5.4 folds) and Vm (1.8 to 2.2 folds) for component I and II (Fig. 7-9 and Table 2-4).

CBZ treatment to the epileptic animals resulted in 1.4 to 5.6 fold elevation in Km and Vm of component I and II (Table 2). Similar trend was observed for LTG treatment to the epileptic animals (Table 3). CLB treatment in general showed increased Vm for component I and II by 1.2 to 1.8 folds in the epileptic animals (Table 4). Component III was not restored in all the AED treated groups (Fig 7-9).

Figure 4: Typical substrate saturation curves for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with CBZ and epileptic treated with CBZ (PTX-CBZ) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents [S]. Reaction velocity is in μmol Pi liberated/h/mg protein.

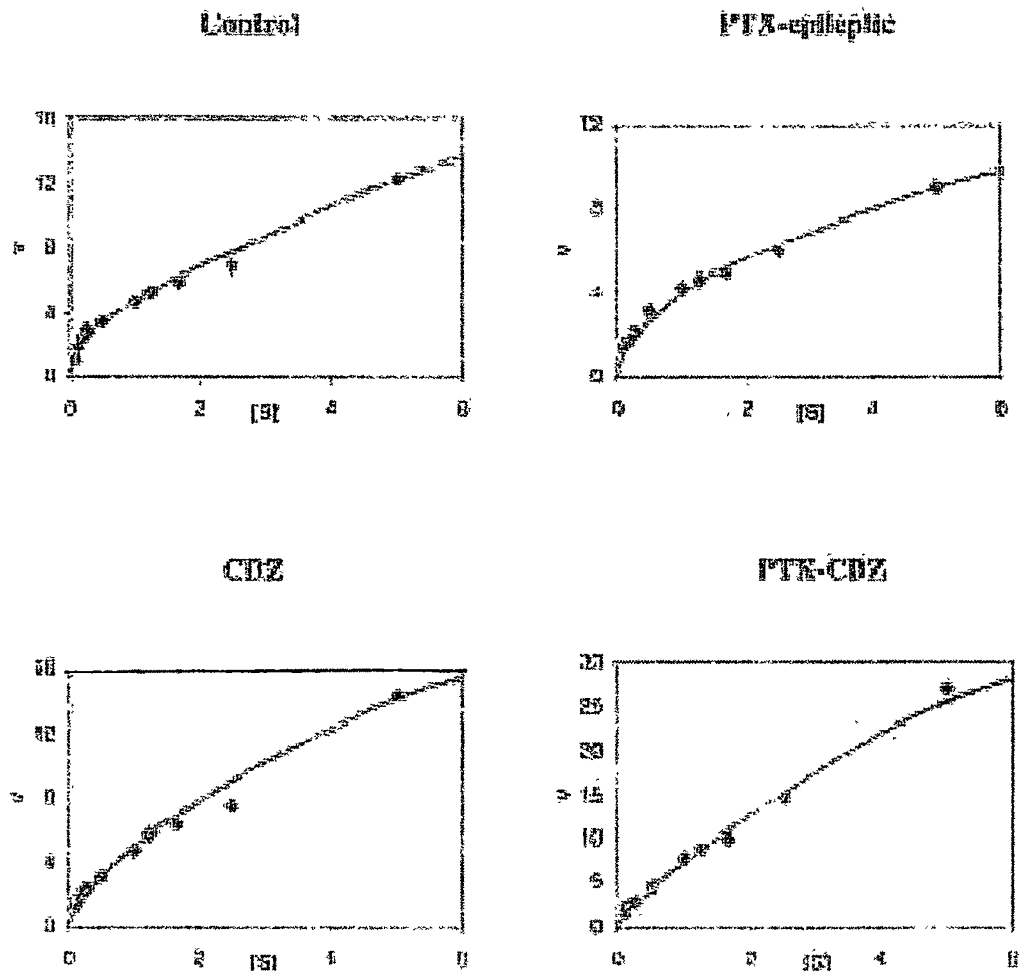


Figure 5: Typical substrate saturation curves for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with LTG and epileptic treated with LTG (PTX-LTG) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents [S]. Reaction velocity is in μmol Pi liberated/h/mg protein.

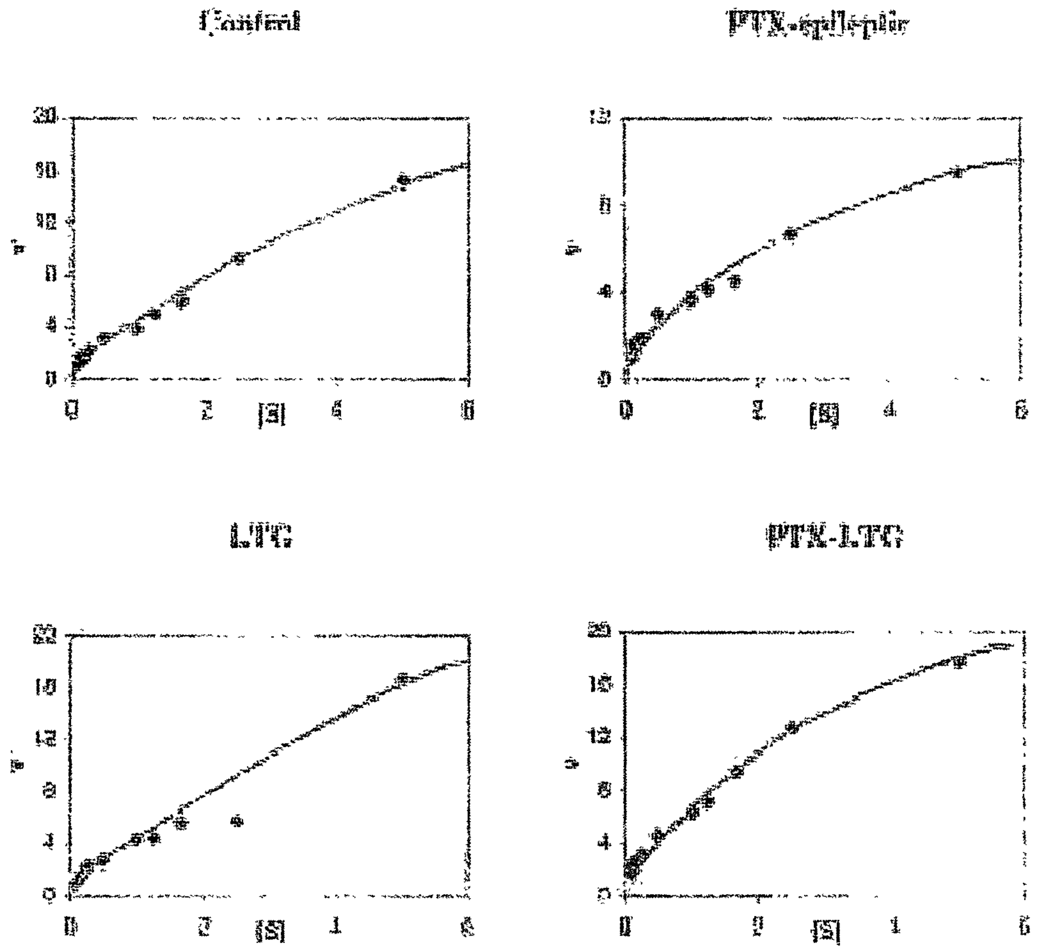


Figure 6: Typical substrate saturation curves for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with CLB and epileptic treated with CLB (PTX-CLB) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents [S]. Reaction velocity is in µmol Pi liberated/h/mg protein.

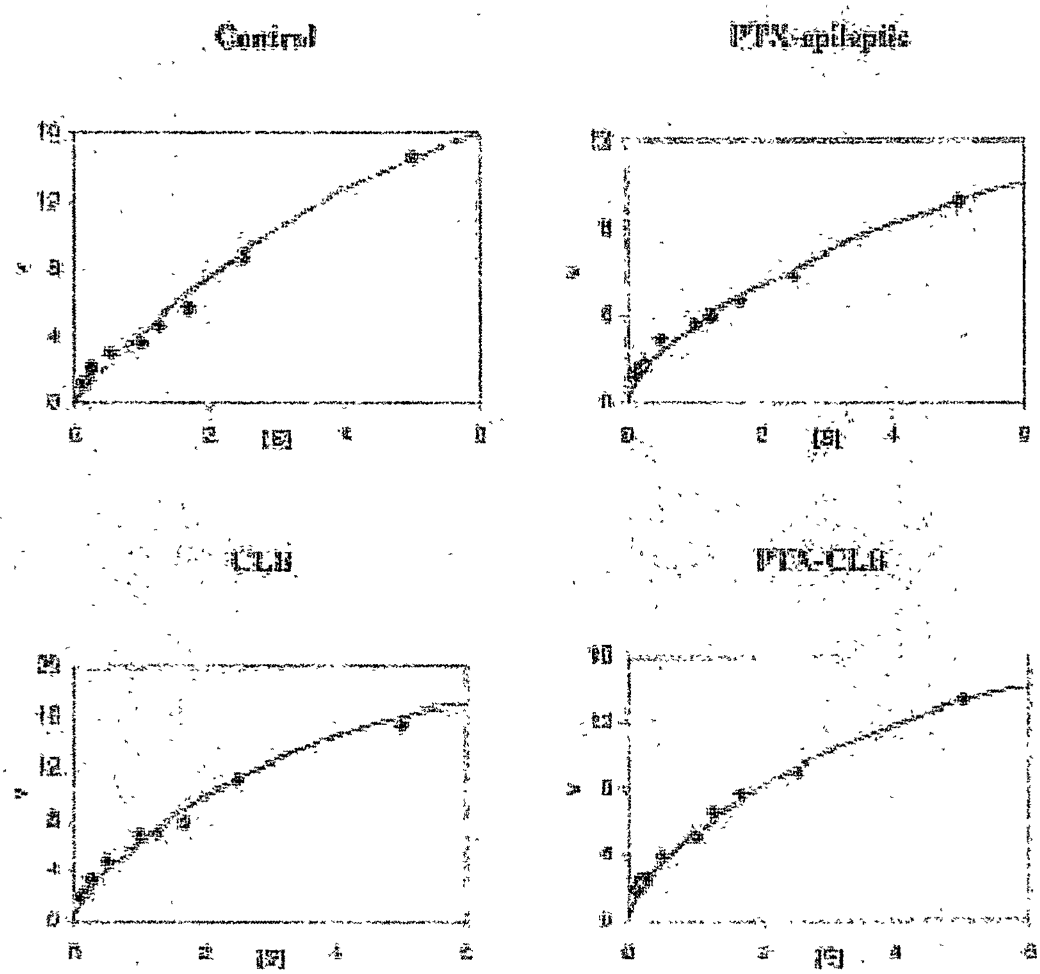


Figure 7. The respective Eadie-Hofstee plots for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with CBZ and epileptic treated with CBZ (PTX-CBZ) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents the *v*/[S] ratios. Reaction velocity is in µmol Pi liberated/h/mg protein. *v*/[S] is reaction velocity divided by the corresponding substrate concentration.

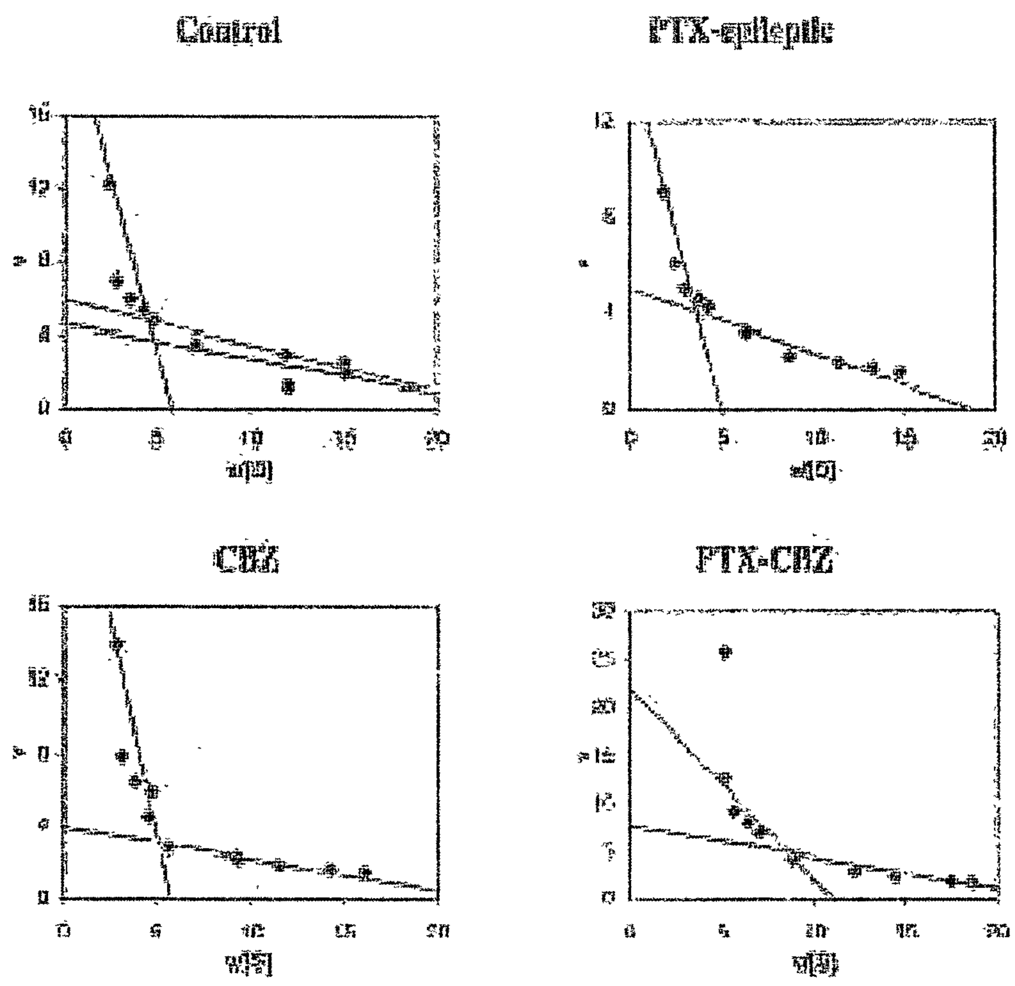


Figure 8. The respective Eadie-Hofstee plots for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with LTG and epileptic treated with LTG (PTX-LTG) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents the *v*/[S] ratios. Reaction velocity is in μmol Pi liberated/h/mg protein. *v*/[S] is reaction velocity divided by the corresponding substrate concentration.

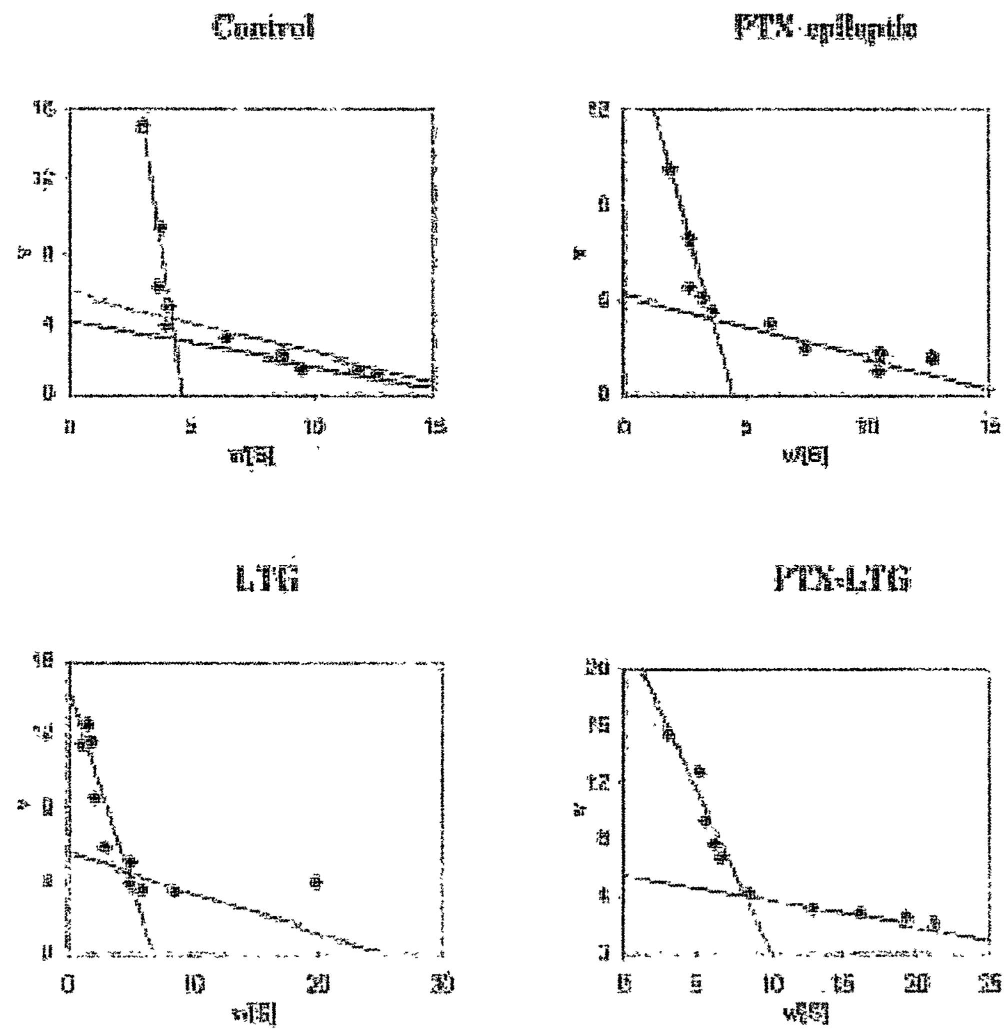


Figure 9. The respective Eadie-Hofstee plots for rat brain mitochondrial ATPase in control, PTX-epileptic, control treated with CLB and epileptic treated with CLB (PTX-CLB) animals. The experimental details are as given in the text. Concentration of ATP was in the range of 0.01 to 5 mM. The abscissa represents the reaction velocity *v*, while the ordinate represents the *v*/[S] ratios. Reaction velocity is in μmol Pi liberated/h/mg protein. *v*/[S] is reaction velocity divided by the corresponding substrate concentration.

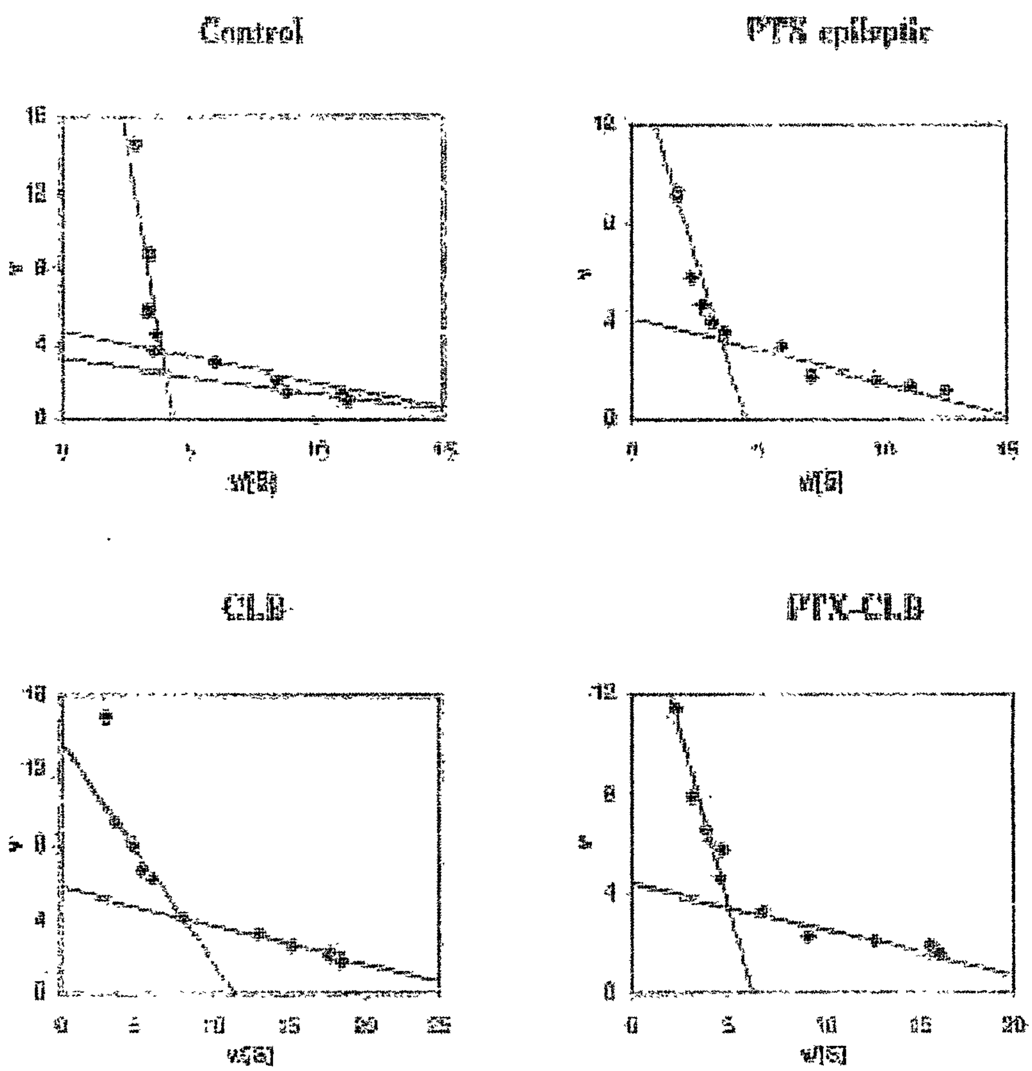


Table 1. Effect of PTX-induced seizures on substrate kinetics of rat liver mitochondrial ATPase

Animals	Component I		Component II		Component III	
	K_m	V_m	K_m	V_m	K_m	V_m
Control	0.25 ± 0.015	4.73 ± 0.33	0.42 ± 0.02	8.40 ± 0.41	3.23 ± 0.19	19.74 ± 1.16
PTX	0.21 ± 0.012	4.01 ± 0.22	2.34 ± 0.13*	12.61± 0.47*	--	--

Results are given as mean ± SEM of the number of observation indicated in the parentheses.
* p<0.001

Table 2. Effects of Carbamazepine (CBZ) treatment on temperature kinetics of rat brain mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Component I		Component II		Component III	
	K_m	V_m	K_m	V_m	K_m	V_m
Control	0.23 ± 0.014	4.97 ± 0.19	0.51 ± 0.03	7.35 ± 0.31	3.38 ± 0.22	18.90 ± 0.67
CBZ	0.25 ± 0.022	5.12 ± 0.21	2.19 ± 0.14**	14.04 ± 0.77**	--	--
PTX	0.24 ± 0.009	4.56 ± 0.14	2.63 ± 0.15**	12.64 ± 0.46**	--	--
PTX-CBZ	0.32±0.02*,a	7.53±0.37**,b	2.85±0.18**	21.08±0.88**,b	--	--

Results are given as mean ± SEM of 8 independent observations.
*, $p<0.01$ and **, $p<0.001$ compared with Control.
a, $p<0.01$ and b, $p<0.001$ compared with PTX treated group.

Table 3. Effects of Lamotrigine (LTG) treatment on temperature kinetics of rat brain mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Component I		Component II		Component III	
	K_m	V_m	K_m	V_m	K_m	V_m
Control	0.26 ± 0.012	4.63 ± 0.24	0.47 ± 0.02	7.01 ± 0.34	3.61± 0.093	17.59 ± 0.84
LTG	0.29 ± 0.019	2.54 ± 0.16*	2.54 ± 0.16**	15.62 ± 1.09**	--	--
PTX	0.25 ± 0.016	4.47 ± 0.17	2.58 ± 0.14**	14.16 ± 0.49**	--	--
PTX-LTG	0.19±0.01**,b	6.72 ± 0.28**,b	1.98 ± 0.1**,a	23.72 ± 1.09**,b	--	--

Results are given as mean ± SEM of 8 independent observations.
*, $p<0.01$ and **, $p<0.001$ compared with Control.
a, $p<0.01$ and b, $p<0.001$ compared with PTX treated group.

Table 4. Effects of Clobazam (CLB) treatment on temperature kinetics of rat brain mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Component I		Component II		Component III	
	K_m	V_m	K_m	V_m	K_m	V_m
Control	0.25 ± 0.019	4.83 ± 0.21	0.48 ± 0.03	8.24 ± 0.56	3.70 ± 0.19	17.21 ± 0.49
CLB	0.26 ± 0.129	6.96 ± 0.46**	1.69 ± 0.09**	14.78 ± 0.91**	--	--
PTX	0.24 ± 0.012	4.36 ± 0.18	2.52 ± 0.14**	13.14 ± 0.48**	--	--
PTX-CLB	0.24±0.014*,b	5.61±0.23**,a	1.94±0.12**,a	14.95 ± 0.81**	--	--

Results are given as mean ± SEM of 8 independent observations.
*, $p<0.05$ and **, $p<0.001$ compared with Control.
a, $p<0.01$ and b, $p<0.001$ compared with PTX treated group.

In the temperature kinetics analysis, marginal decrease (19%) in E2 in liver was observed in PTX-treatment group (Table 5). When, the epileptic animals were treated with AEDs, a generalized decrease in E1 and E2 was seen for all the three AEDs (Table 6-8 and Fig 10-12). Tt was decreased by 5 °C after CBZ treatment to the epileptic animals. Similar trend of generalized decrease (10-35% decrease) was observed for all the three AEDs when these are given to the control animals. CBZ treatment elevated Tt by 5.1 °C (Table 6 and Fig 11).

Generation of free radicals and compromised oxidative energy metabolism in the epileptic condition could further aggravate in to peripheral system as well, especially in blood and RBCs (17, 18, 19). Increased risk of atherogenesis is proposed to be associated with the chronic epileptic condition (1). The situation further worsens when ascended ROS production remained unchecked because of compromised antioxidant system in the epileptic patients (2). Additionally, PTX is metabolized to picrotoxinin and picrotin (20-22). It may be possible that PTX by itself or its metabolites - picrotoxinin and picrotin - may affect the liver function. But this possibility needs to be confirmed by more direct experiments.

Since liver is the major metabolic and detoxification organ, accumulation of AEDs and its metabolites in hepatocytes is obvious. Diazepam and Phenobarbital treatment lead to structural and functional alterations in mitochondrial and other subcellular membrane in liver (23, 24).

Figure 10. Typical Arrhenius plots for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with CBZ and epileptic treated with CBZ (PTX-CBZ) animals. The experimental details are as given in the text. The ATPase activity was determined with 5 mM ATP. The abscissa represents reciprocal of absolute temperature $T \times 1000$. Reaction velocity is in μmol Pi liberated/h/mg protein and absolute temperature T in Kelvin.

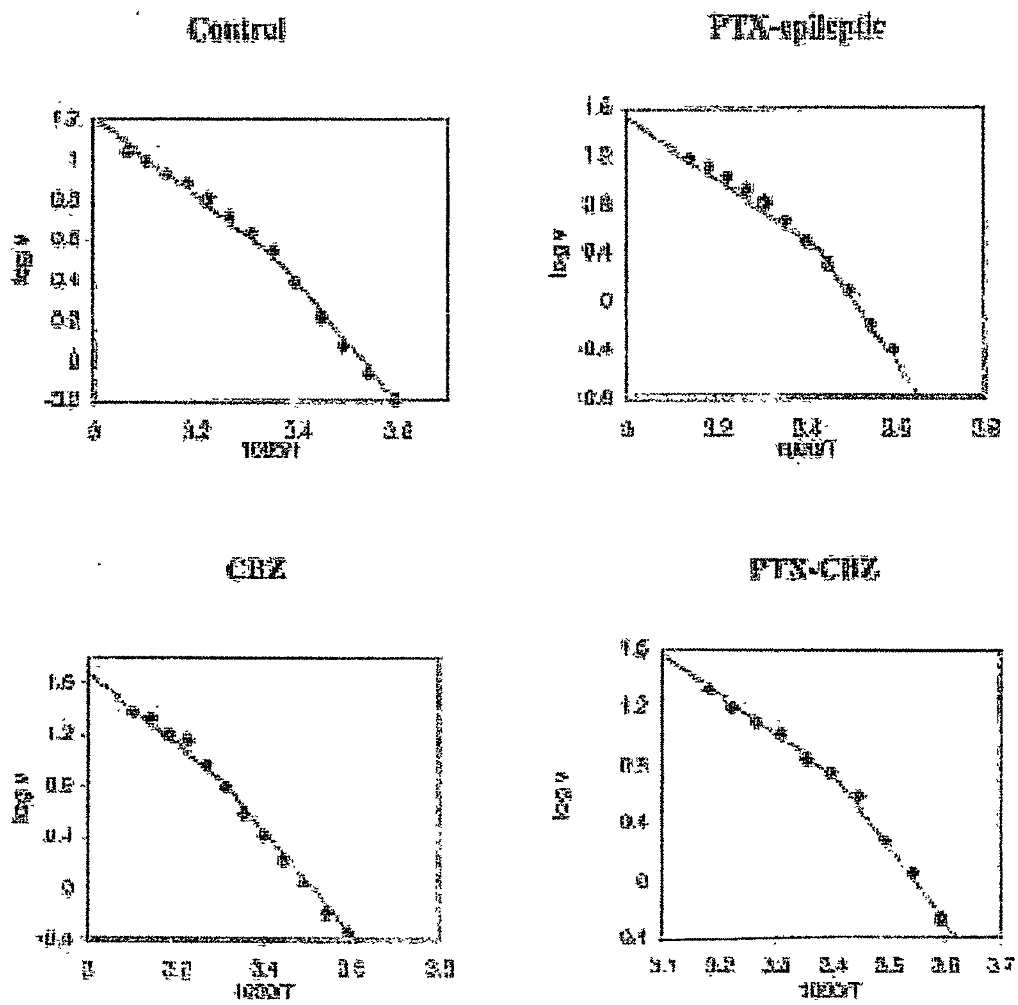


Figure 11. Typical Arrhenius plots for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with LTG and epileptic treated with LTG (PTX-LTG) animals. The experimental details are as given in the text. The ATPase activity was determined with 5 mM ATP. The abscissa represents reciprocal of absolute temperature $T \times 1000$. Reaction velocity is in μmol Pi liberated/h/mg protein and absolute temperature T in Kelvin.

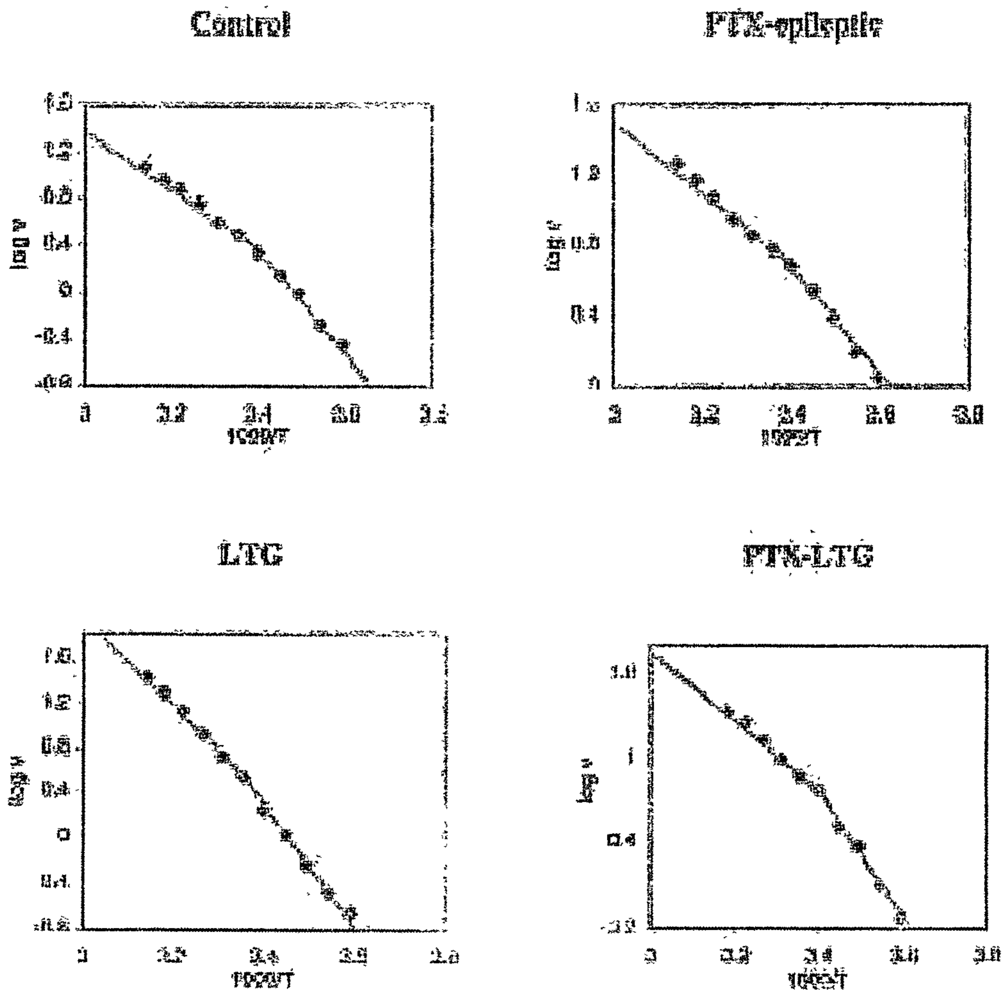


Figure 12. Typical Arrhenius plots for rat liver mitochondrial ATPase in control, PTX-epileptic, control treated with CLB and epileptic treated with CLB (PTX-CLB) animals. The experimental details are as given in the text. The ATPase activity was determined with 5 mM ATP. The abscissa represents reciprocal of absolute temperature $T \times 1000$. Reaction velocity is in µmol Pi liberated/h/mg protein and absolute temperature T in Kelvin.

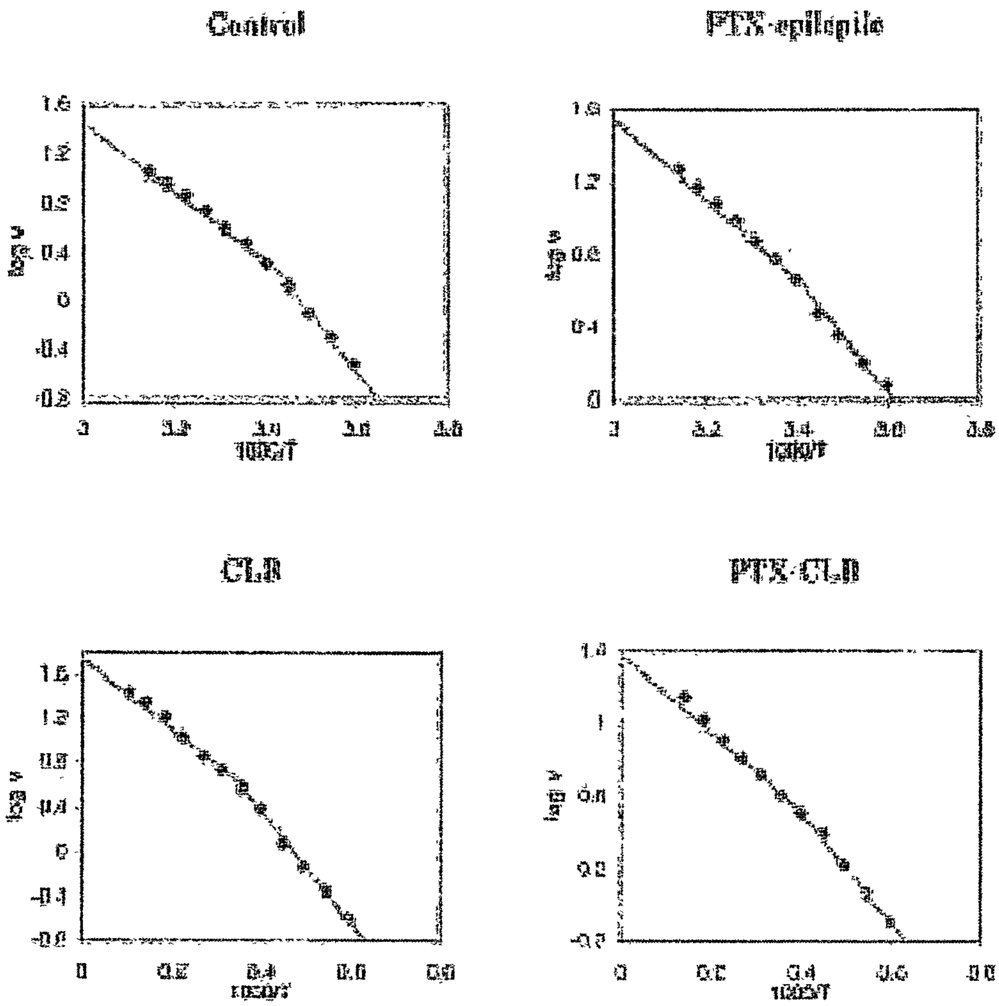


Table 5. Effect of PTX-induced seizures on temperature kinetics of rat liver mitochondrial ATPase

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt °C)
	E1	E2	
Control (8)	42.09 ± 1.12	64.29 ± 1.67	22.21 ± 0.31
PTX (8)	46.47 ± 1.87	52.12 ± 1.51*	22.57 ± 0.54

Results are given as mean ± SEM of the number of observation indicated in the parentheses.
**, $p<0.001$.

Table 6. Effects of Carbamazepine (CBZ) treatment on temperature kinetics of rat liver mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt °C)
	E1	E2	
Control (8)	43.49 ± 1.64	64.36 ± 1.75	22.95 ± 0.51
CBZ (8)	30.59 ± 0.66**	41.72 ± 1.85**	28.03 ± 0.72**
PTX (8)	44.37 ± 1.68	51.69 ± 1.57**	23.10 ± 0.66
PTX-CBZ (8)	34.26 ± 0.66**, a	45.30 ± 0.64**, a	19.65 ± 0.44**, a

Results are given as mean ± SEM of the number of observation indicated in the parentheses.
*, p<0.001 compared with Control.
a, p<0.001 compared with PTX treated group.

Table 7. Effects of Lamotrigine (LTG) treatment on temperature kinetics of rat liver mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt °C)
	E1	E2	
Control (8)	43.32 ± 1.79	60.88 ± 1.21	22.16 ± 0.77
LTG (8)	39.09 ± 0.82*	45.32 ± 1.63**	23.98 ± 0.80
PTX (8)	45.24 ± 0.91	50.58 ± 1.52**	22.59 ± 1.32
PTX-LTG (8)	30.09 ± 1.06**, b	44.68 ± 0.82**, a	21.28 ± 0.47

Results are given as mean ± SEM of the number of observation indicated in the parentheses.
*, $p<0.05$ and **, $p<0.001$ compared with Control.
a, $p<0.01$ and b, $p<0.001$ compared with PTX treated group.

Table 8. Effects of Clobazam (CLB) treatment on temperature kinetics of rat liver mitochondrial ATPase in control and PTX-induced epileptic condition

Animals	Energy of activation (KJ/mole)		Phase transition temperature (Tt °C)
	E1	E2	
Control (8)	44.08 ± 0.79	61.04 ± 1.56	23.80 ± 0.90
CLB (8)	32.44 ± 0.78**	40.75 ± 0.72**	24.46 ± 0.73
PTX (8)	44.81 ± 1.12	51.13 ± 1.60**	22.84 ± 0.72
PTX-CLB (8)	51.11 ± 0.85**, b	55.82 ± 0.63*, a	22.98 ± 0.85

Results are given as mean ± SEM of the number of observation indicated in the parentheses.
*, $p<0.01$ and **, $p<0.001$ compared with Control.
a, $p<0.02$ and b, $p<0.001$ compared with PTX treated group.

Valproic acid and its metabolites are reported to induce cytotoxicity by altering fatty acid metabolism, mitochondrial transmembrane potential and modulating antioxidant system in liver (25-27). On the other had neuroprotective action of topiramate is directly related to its inhibitory effect on the mitochondrial permeability transition pore (28). These reports supports evidence of possible oxidative stress and altered mitochondrial oxidative energy metabolism after AED treatment.

Taken together, chronic PTX treatment resulted in altered substrate kinetic properties of the liver mitochondrial ATPase. Temperature kinetics revealed only marginal change. However, AED treatment in general to the epileptic animals showed tissue specific differential alterations. AED treatment to the control animals exerts their own noxious effects by drastically altering the kinetic properties of mitochondrial ATPase. Significance of these changes could be related to changes in the mitochondrial lipid/phospholipid compositions, as ETC and F_oF_1ATPase are membrane bound systems. This possibility was checked in the subsequent chapters.

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Summary

Based on the earlier observations on mitochondrial respiratory chain dysfunction in the chronic epileptic condition (Chapter 2), studies were done to decode the effects of PTX-induced convulsions and treatment with AEDs on rat liver mitochondrial ATPase kinetics properties. Elevation of basal, Mg and DNP stimulated ATPase activities and decreased total activity indicates altered membrane integrity in the PTX-induced epileptic animals. CBZ, LTG and CLB treatment to the control animals resulted into 1.3 to 1.5 fold increase in the Mg-stimulated activity. CBZ and LTG treatment to the epileptic animals resulted in increased Km and Vm of component I and II. Opposite effect was seen with CLB treatment to the epileptic animals. AED treatment to the control animals resulted in increased Km (3.5-5.4 folds) and Vm (1.8 to 2.2 folds) for component I and II. In either PTX treatment or AED treatment, component III was abolished in all the groups. Temperature kinetics revealed only marginal change. Taken together, chronic PTX treatment resulted in altered substrate and temperature kinetic properties of the liver mitochondrial ATPase. However, AED treatment in general to the epileptic animals showed tissue specific differential alterations. AED treatment to the control animals exerts their own toxic effects by drastically altering the kinetic properties of mitochondrial ATPase.