CHAPTER-III

Studies on Brain Mitochondria in Relation to Calcium and Membrane Changes Due to Diazepam
Introduction

Mitochondria are convenient test models for understanding any interrelation between oxidative stress and calcium functions being a major site of formation of active oxygen species, along with ready availability of transition cations (Mattson, 1998), and an organelle of importance in intracellular calcium homeostasis (Takeyama et al, 1993). Working with isolated mitochondria is also an ideal reductionist approach to answer the above question, uninterfered by many physiological factors influencing calcium influx and efflux and associated metabolic changes. Earlier studies in this laboratory, with freshly isolated rat liver mitochondria led to considerable new information on oxidative calcium deregulation through following TBARS formation, antioxidant profile, *in vitro* swelling and ultrastructural observations (Mehrotra *et al*, 1991,1993; Kakkar *et al*, 1992,1996).

Moreover, there are reports on the cerebral cortex, hippocampus and other regions of cerebrum and the brain stem being affected in acute and chronic diazepam treatment as well as during withdrawal (Lidow *et al*, 1998; Eghbali
et al, 1997; Toki et al, 1996; Andrews et al, 1992a), and the uncertainty about the actual functions and role of cerebellum (Herrup and Kuemerle, 1997). Therefore in this chapter the effects of diazepam administration on mitochondria of cerebrum and brain stem has been studied.

Some of the studies reported in the previous chapters of this dissertation also show the involvement of mitochondrial processes in diazepam mediated effects. In view of these, some of the questions regarding the central theme of this thesis were investigated using status of calcium in the organelle through fluorescent dye (Fura-2 AM) binding, any altered phospholipid turnover and following the swelling pattern of mitochondria. It may be pointed out that in an earlier study with brain tissue, an increased formation of lysolecithin was observed following phenobarbitone administration (Awasthi et al, 1991). Thus attempts have also been made to estimate the phospholipid turnover in diazepam treated rat brain, since diazepam does not cause gross CNS intoxication (Gilman, 1991;1995). Any change in structural and functional aspects of the organelle as a result of in situ generation of active oxygen species has also been studied in detail by transmission electron microscopy (TEM) alongwith assay of inner and outer membrane marker enzymes. A possible leakage of protein during ROS generation has been tested by SDS-Polyacrylamide gel electrophoresis of diazepam treated mitochondria.

Materials and Methods

The experimental details were normally same as in earlier chapters and only the details of the additional experiments are given here.

Diazepam Treatment

The diazepam treatment in the following studies was both in vivo and in vitro. For in vivo studies, the 3 mg/kg i.p. dose was administered an hour before sacrificing while for the in vitro experiments, the isolated mitochondria were subjected to
doses lower and higher i.e., 1 mg/kg and 9 mg/kg to confirm the protective as well as harmful effects of the drug. The details of drug administration are given with each experimental procedure described below.

Mitochondrial Swelling

The mitochondria were isolated as described earlier, unless specified otherwise according to the protocol for the experimental procedures, as specified:

Effect of diazepam on in vitro swelling of isolated mitochondria from brain regions and liver of rat was carried out according to the spectrophotometric method of Lehninger et al (1959) using Milton Roy Spectronic 1001 spectrophotometer. 2 mM CaCl$_2$ was used as swelling agent. Hypoxanthine (5x10$^{-3}$M) and xanthine oxidase (0.025 units) system was used for the generation of superoxide anion. To study the quenching effect, superoxide dismutase (SOD) (70 units) was used as scavenger of superoxide radical. The effect of diazepam on free radical generation and swelling of mitochondria was studied by preincubating for 2 mins. The swelling was recorded at 520 nm as decrease in the optical density at 1 minute interval for a period of 5 minutes at room temperature.

Incorporation of $^{14}$C-choline in Phospholipids

A group of four albino rats were given diazepam (3 mg/kg body weight, i.p) and 0.2 μci of $^{14}$C choline chloride was injected per animal. Another set of 4 animals were given normal saline (i.p.) and 0.2 μci of $^{14}$C choline chloride in the same manner. The animals were sacrificed after 1 hour of $^{14}$C choline injection, brain was immediately taken out and processed. All operations were carried out under cold conditions (0-4 °C). Mitochondria were prepared in 0.15 M KCl according to the method of Mustafa (1974).
Lipid Extraction and Separation

Lipid extraction of the mitochondria of CB and BS was done by the method of Folch et al (1957) as modified by Suzuki (1965) and lyophilised on Heto FD3 Drywinner lyophiliser. Separation of different classes of phospholipids on thin layer silica gel chromatography plates (E. Merck) was done by the method of Horrocks and Sun (1972) using chloroform:methanol:glacial acetic acid: water (65:25:4:1) as solvent system and compared with standards of lecithin and lysolecithin.

Measurement of Radioactivity

Radioactivity was counted in mitochondrial fraction of BS on Rack Beta counter (LKB, Sweden, Model 1215/1216). The composition of the scintillation cocktail was 52g Naphthalene, 2.25g 5-diphenyloxazole, 65 mg, 1,4 bis-(5-phenyloxazolyl) benzene, 250 ml dioxane, 150 ml methanol and 250 ml toluene. Standards of $^{14}$C choline chloride were treated similarly.

Measurement of radioactivity in lipid fraction

Radioactivity was measured in lipid fraction as well as in different groups of phospholipids of BS mitochondria by eluting the phospholipid spot from TLC plate in chloroform. Same area of unstained portion on silica plate was also eluted and used as control. The eluted phospholipid fractions were centrifuged, suspended in scintillation fluid and radioactivity determined.

Calcium Content of Mitochondria from Brain Regions Following In vitro Concentration and Time Dependent Diazepam Treatment

The mitochondria were isolated in 0.32 M sucrose. Isolated mitochondrial suspension (~0.2 mg protein) was incubated with diazepam, diluted to 1 ml with incubation buffer and then 4 μM of Fura-2AM was added. These Fura-2 AM loaded mitochondria were then incubated at 37 °C for 20 mins. in a shaking water bath. This
was then diluted to 9 ml with incubation buffer and again incubated for another 25 mins. at 37 °C according to the method of Martin et al (1991). This diluted preparation was centrifuged at 17,800g for 5 mins. The pellet was suspended in 2 ml incubation buffer which contains all the ingredients of the assay buffer (described below) with the addition of 0.64 mM NaH$_2$PO$_4$. 0.5 ml of this suspension was further centrifuged at 12,000g for 2 mins. The mitochondrial pellet was then suspended in 2 ml assay buffer (containing 124 mM NaCl, 4 mM KCl, 0.66 mM K$_2$HPO$_4$, 15.2 mM NaHCO$_3$, 20 mM HEPES, 5.56 mM glucose, 1.2 mM MgCl$_2$ and 1 mM CaCl$_2$). The ratio of fura-2 AM fluorescence at excitation wavelengths of 340 and 380 nm with emission at 510 nm was determined at 32 °C using a Perkin-Elmer LS50 spectrofluorometer and associated software for fura-2 measurements. After obtaining a baseline reading, 20 μl of 0.1% SDS, 30 μl of 0.5 mM Tris and 20 μl of 1 mM EGTA (final concentration) were added to the cuvette to disrupt the mitochondria. This gave the F$_{\text{min}}$ (minimum ratio of fluorescence at 340:380). The F$_{\text{max}}$ (maximum fluorescence ratio) was determined after addition of 125 μl of 6 mM (final concentration) of CaCl$_2$.

For the dose-dependent assay, 0.088 μM, 0.176 μM and 0.7 μM diazepam for CB and 0.044 μM, 0.088 μM and 0.35 μM for BS was taken whereas for time dependent assay, 0.44 μM (for CB) and 0.29 μM (for BS) of diazepam was added for 0, 5, 10 and 15 mins before adding Fura 2 AM (in DMSO). The calcium concentration was calculated as described by Gryniewicz et al (1985) by the equation:

$$[\text{Ca}^{2+}] = \frac{K_d (F-F_{\text{min}}/F_{\text{max}}-F)}{R}$$

where $K_d$ is the dissociation constant for fura-2/Ca$^{2+}$ complex (224 nM).

$R = S_f/S_b$ calculated by the method of Jiang et al (1993) using EGTA-MOPS buffers. $S_f$ is the fluorescence intensity of the dye at 380 nm when $[\text{Ca}^{2+}] = 0$ and $S_b$ is that at $[\text{Ca}^{2+}]$ saturation (Ca$^{2+}$ saturated buffer contains 1 mM free Ca$^{2+}$).

This viscosity correction factor was used to correct for alteration of fura-2 due to the intracellular environment (Poenie, 1992).
Mitochondrial Calcium & Membrane Changes

F is the ratio of basal calcium concentration at 340 and 380 nm excitation and 510 nm emission.

\[ F_{\text{max}} \text{ and } F_{\text{min}} \text{ are the ratios obtained in Ca}^{2+} \text{ saturated and Ca}^{2+} \text{ free buffers respectively at the above wavelengths.} \]

Transmission Electron Microscopy

After pelletting by centrifugation in isotonic sucrose medium, (SEE) containing 150 mM sucrose, 10 mM HEPES (pH 7.4), 1 mg/ml BSA, 0.5 mM EDTA and 0.5 mM EGTA (Lee et al, 1993) the mitochondria from control and treated rats were washed with sodium cacodylate buffer and suspended in 2.5% glutaraldehyde (fixative agent) for an hour or two and washed twice with sodium cacodylate buffer. The material was fixed in osmium tetroxide, washed with the same buffer and dehydrated with acetone. For embedding, araldite 502 (resin), DDSSA and DMP (all Ladd, USA) were used and sections were cut using LKB ultratome. After treating with KOH solution and washing with distilled water, the sections were stained with uranyl acetate and examined on Phillips 400 transmission electron microscope. The entire procedure was carried out according to the methodology of Hayat (1981). The mitochondria subjected to in situ generation of \( \text{O}_2^- \) and preincubated with diazepam were centrifuged after the incubation period and fixed in the same manner.

Cytochrome c Oxidase Assay

Cytochrome C oxidase in the mitochondria was assayed by the method of Cooperstein and Lazarrow (1951). The assay system contained 1.0 ml of 0.2 M phosphate buffer, pH 7.4; 0.45 ml of \( 1 \times 10^{-4} \) M ferrocytochrome c solution (the cytochrome c solution was reduced with sodium dithionite), 1.5 ml \( \text{H}_2\text{O} \) and 0.5 ml of appropriately diluted mitochondrial suspension (the stock suspension was 2.5 to 5 fold diluted with 0.25 M sucrose). The decrease in O.D. at 550 nm was
followed at 30 sec. intervals for 2 minutes. The initial rate of the reaction in μmoles was calculated according to Yonetani (1961), using $19.1 \times 10^3 \text{cm}^{-1}$ molar extinction coefficient.

**Determination of Monoamine Oxidase Activity**

MAO was determined in mitochondria both *in vivo* and *in vitro* (3 mg/kg dose for 1 hour) by the method of Tabor *et al.* (1954), using benzylamine as substrate. O.D. at 250 nm due to benzaldehyde formation was converted to nmoles by using the molecular extinction coefficient of benzaldehyde ($1.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$).

**Determination of Protein Leakage from Mitochondria by SDS-PAGE**

The sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of mitochondria was done after incubating freshly isolated CB and BS mitochondria (suspended in Tris-HCl buffer, pH 7.4) with xanthine (0.5 mM) and xanthine oxidase (0.056 units), that were either pretreated with diazepam (0.5 mg) or the same dose being given after the oxidative stress caused by xanthine-xanthine oxidase. The same schedules were also followed for 100 μM tBHP (another positive control for oxidative stress) in a 2 ml of final assay system. After 5 minutes of incubation under the above conditions, the mitochondria were centrifuged at 10,000 rpm for 5 mins and the supernatant was quickly decanted. The mitochondrial pellets were suspended in 1x running buffer (0.025M Tris-0.192M glycine, pH 8.3) containing 0.1% SDS and 1/4 volume of 5x sample buffer (1M Tris, pH 6.8, 10% SDS, 50% glycerol, 0.25 ml of β-mercaptoethanol, 0.1% Bromophenol blue). The supernatants were subjected to sucrose dialysis to remove inorganic salt interference and concentrate protein (if any). The residue after dialysis was also suspended in 1x running buffer and sample buffer was added as above. These samples were then stored as 30 μl aliquots at -20 °C. Before loading the samples, they were kept in a boiling water bath for 10 minutes.
SDS-polyacrylamide separating gel (12.5%) and 5% stacking gel were prepared and SDS-PAGE of both the mitochondrial as well as of their respective supernatants was carried out according to the method of Laemmli (1970) in 1x running buffer. The gel was polymerised using 6.25 ml of solution A (29.2% acrylamide + 0.8% N,N’ methylene bisacrylamide) 5.625 ml of 1 M Tris pH 8.8, 2.59 ml of H₂O, 15 ml of 10% SDS, 0.3 ml 50% glycerol, 7.5 µl TEMED and 75 µl of fresh 10% Ammonium persulphate (APS) fresh. After polymerization, it was layered with stacking gel using 0.5 ml of solution A, 0.5 ml of 1M Tris (pH 6.8), 40 µl SDS (10%), 2.93 ml H₂O, 3.125 µl TEMED and 31.25 µl APS (10%). 30 µl of sample was loaded after gel polymerisation using 1 mm comb and spacers and run at 24 mA constant current. The gel was stained with 0.17% coomassie brilliant blue R-250 (prepared in 50% methanol and 10% glacial acetic acid) and left overnight. This was then destained in methanol (10%) and glacial acetic acid (10%) until bands appeared.

Results

Effect of Diazepam on Swelling of Mitochondria *In vitro*

Effect of diazepam on *in vitro* swelling of isolated mitochondria of brain was studied and compared to its effect on swelling of liver mitochondria as diazepam is metabolised in liver. Isolated mitochondria from brain were subjected to calcium induced swelling (Fig. 24). As is evident from the figure, addition of 2mM Ca²⁺ to the assay system induced swelling of mitochondria which was reduced in the presence of 5 µg diazepam which was 30.76% lower than its control. Fig. 25 shows the effect of *in situ* generation of superoxide radical on rat brain mitochondria. In the absence of externally added calcium ions, superoxide radical generated due to the reaction of hypoxanthine and xanthine oxidase could itself cause swelling of mitochondria which was reduced by 21.4% in the presence of 70 units of superoxide dismutase. Diazepam in the presence of superoxide generating system could not show any effect on oxidative swelling of brain mitochondria.
**Fig. 24:** Effect of diazepam (5 µg) on CaCl₂ (2 mM) induced swelling of isolated rat brain mitochondria.
Fig. 25: Superoxide induced swelling of rat brain mitochondria. Effect of SOD and diazepam (5 μg) on $O_2^-$ induced swelling.
Since liver mitochondria possess peripheral benzodiazepine receptors and diazepam is metabolized in liver causing activation of biotransformation enzymes, its effect on isolated liver mitochondria was also monitored. In comparison to brain mitochondria, 2 mM CaCl\(_2\) could cause large amplitude swelling of rat liver mitochondria as is evident from Fig. 26. Preincubation with 5 \(\mu\)g of diazepam could reduce the swelling of mitochondria by 46% thus giving some protection to mitochondrial membrane impermeability against changes in calcium fluxes. Unlike rat brain where diazepam did not show any effect on oxidative swelling of mitochondria, in liver, a dose dependent protection against oxidative swelling of mitochondria was observed. Fig. 27 shows that 0.05 mg of diazepam reduced the oxidative swelling to 75% of that of corresponding control whereas 0.1 mg and 0.15 mg reduced it to 43.7% and 50% respectively of that of control. The data shows definite intervention of calcium and free radical induced swelling of liver mitochondria by diazepam, thus indicating towards its effects being mediated by ROS involving membrane integrity and calcium fluxes.

**Mitochondrial calcium ion changes**

Fig. 28 shows the changes in calcium ion concentration within the mitochondria of CB at different concentrations of diazepam *in vitro* for 5 minutes. Upto 0.17 \(\mu\)M diazepam, there were no changes in [Ca\(^{2+}\)], but at 0.7 \(\mu\)M of diazepam, there was a two fold increase in the [Ca\(^{2+}\)] in the CB whereas a different trend was observed in the BS mitochondria (Fig. 29). There was an uptake upto 0.088 \(\mu\)M of diazepam followed by release at 0.35 \(\mu\)M in BS. Fig. 30 gives an account of the time dependent changes in [Ca\(^{2+}\)] in the mitochondria of CB and BS. Cerebral tissue showed an increase initially for 5 minutes but there was a dramatic increase in the next five minutes followed by a sudden drop in the [Ca\(^{2+}\)] finally after 15 minutes of incubation with diazepam before adding the dye. However, BS showed a gradual uptake without release even after 15 minutes of incubation.
Fig. 26: Effect of diazepam (5 μg) on CaCl₂ (2 mM) induced *in vitro* swelling of rat liver mitochondria.
Fig. 27: Effect of different concentrations of diazepam on $O_2^-$-induced swelling of rat liver mitochondria.
Fig. 28: Changes in CB mitochondrial [Ca$^{2+}$] at different concentrations (µM) of diazepam (in vitro).
Fig. 29: Changes in BS mitochondrial [Ca$^{2+}$] at different concentrations of diazepam (in vitro).
Fig. 30: Time dependent changes in $[\text{Ca}^{2+}]$ in the mitochondria of CB and BS due to diazepam (0.44 $\mu$M for CB and 0.29 $\mu$M for BS) using Fura-2AM fluorescent dye.
with diazepam (0.29 μM). The concentration of diazepam was used according to the mitochondria present in the incubation system. Thus, Fig. 30 indicates towards greater calcium buffering capacity of BS mitochondria as compared to the CB where a sudden collapse of the mitochondrial membrane impermeability is indicated after 10 mins. On the other hand, CB mitochondria could hold 21 times more Ca^{2+}/mg protein as compared to the BS mitochondria but for a shorter duration of time.

**Ultrastructural Studies**

Studies were carried out in mitochondria subjected to oxidative stress and preincubated with diazepam to see its effect on structural integrity of mitochondria. Plate 2 shows mitochondria of the cerebral tissue after 1 hour of treatment with 3 mg/kg dose of diazepam. The mitochondria, though still intact, show a slight change in shape in terms of the outer membrane and the cristae are also not as well organised as in the control picture (Plate 1). Plate 3a shows the shape of CB mitochondria 18 hours after administration of the drug (3 mg/kg) and there is a clear revival of the shape although the cristae have become thread-like and elongated which is more clear at higher magnifications (Plate 3b). This may be due to the fact that BZD receptors are on the contact sites of outer and inner mitochondrial membrane and after the initial insult, the drug on binding to its receptors has provided a certain amount of protection, whereas at a higher dose (15 mg/kg) for 1 hour (Plates 4a and 4b) there seems to be nearly 80% degeneration of mitochondria. Preparation artifacts are also seen in the pictures. This is in conformity with the earlier Fura-2 binding studies indicating Ca^{2+} efflux from mitochondria at higher concentrations of diazepam leading to membrane changes. Plate 6 shows the mitochondria of BS after an hour of the 3 mg/kg dose of diazepam. The outer membrane is only slightly deshaped while the cristae show a certain amount of disorganisation in comparison to the untreated samples (Plates 5a and 5b) where the cristae are well organised which is different from the effect shown in CB. But an hour after the 15 mg/kg dose (Plate 7) the mitochondria of BS show highly swollen cristae and the outer envelope evaginates and disrupts.
Plate 1: CB mitochondria (untreated) at 18,500 x magnification.

Plate 2: CB mitochondria 1 hour after 3 mg/kg of diazepam (in vivo) at 18,500 x magnification.
Plate 3a: CB mitochondria 18 hours after 3 mg/kg of diazepam administration (in vivo) at 8,200 x magnification.

Plate 3b: CB mitochondria 18 hours after 3 mg/kg of diazepam administration (in vivo) at 18,500 x magnification.
Plate 4a: CB mitochondria 1 hour after 15 mg/kg of diazepam (in vivo) at 8,200 x magnification.

Plate 4b: CB mitochondria 1 hour after 15 mg/kg of diazepam (in vivo) at 18,500 x magnification.
Plate 5a: Mitochondria of BS (untreated) at 18,500 x magnification.

Plate 5b: Mitochondria of BS (untreated) at 49,000 x magnification.
Plate 6: BS mitochondria 1 hour after 3 mg/kg of diazepam (in vivo) at 18,500 x magnification.

Plate 7: BS mitochondria 1 hour after 15 mg/kg of diazepam administration (in vivo) at 49,000 x magnification.
Mitochondrial Calcium & Membrane Changes

Since the changes in BS due to diazepam administration are more pronounced, in vitro superoxide generation with xanthine and xanthine oxidase system was done and mitochondria were incubated with and without diazepam. Plate 8 shows disruption of inner membrane and cristae disorganisation along with swelling of mitochondria and breakage of membrane with loss of matrix material whereas with 0.5 mg diazepam (Plate 9a) added prior to in vitro oxidative insult, the mitochondria seem to be protected with only a slight disorganisation in the cristae, that too in only a few mitochondria which is well pronounced at higher magnifications (Plate 9b). Thus, in this region diazepam accords protection against oxidative stress to the outer membrane and the disruption of the inner structure is in keeping with the results of the single dose of 3 mg/kg for 1 hour in this region.

On comparison of CBL mitochondria from untreated rats (Plate 10) with that from rats subjected to single 3 mg/kg diazepam dose for 1 hour (Plates 11a and 11b) mitochondria are not as deformed as those observed for CB and BS and the cristae have also maintained their organisation though they are not absolutely without any effect. Whereas at 15 mg/kg dose, mitochondria from cerebellum even after 18 hours show highly swollen, blebbing cristae (Plates 12a and 12b) as is evident from these sections on a different plane.

Assay of Mitochondrial Membrane Integrity Using Marker Enzymes

To test the involvement of outer membrane of mitochondria during diazepam administration, the activity of the outer membrane marker enzyme, monoamine oxidase (MAO) was assayed by in vitro (Table 23) and in vivo (Table 24) administration of diazepam. At a dose of 1 mg/kg, 3 mg/kg and 9 mg/kg DZ, MAO activity in CB is slightly increased as compared to controls, and is nearly the same at all the doses while in BS, there is a significant decrease (p<0.001) at 1 mg/kg and 9 mg/kg dose. At 3 mg/kg, though it is slightly higher as compared to the other 2 doses, yet it is 28.38% lesser as compared to control. whereas due to in vivo
Plate 8: BS mitochondria treated with 0.5 mM xanthine + 0.25 U xanthine oxidase at 18,500 x magnification.

Plate 9a: Diazepam (0.5 mg) pretreated BS mitochondria subjected to in situ O₂ generation (0.5 mM xanthine + 0.25 U xanthine oxidase) at 18,500 x magnification.
Plate 9b: Diazepam (0.5 mg) pretreated BS mitochondria subjected to in situ $O_2$ generation (0.5 mM xanthine + 0.25 U xanthine oxidase) at 49,000 x magnification.

Plate 10: Untreated mitochondria of CBL at 8,200 x magnification.
**Plate 11a:** CBL mitochondria 1 hour after 3 mg/kg of diazepam administration (*in vivo*) at 8,200 x magnification.

**Plate 11b:** CBL mitochondria 1 hour after 3 mg/kg of diazepam administration (*in vivo*) at 18,500 x magnification.
Plate 12a: CBL mitochondria 18 hours after 15 mg/kg of diazepam administration (in vivo) at 30,000 x magnification.

Plate 12b: CBL mitochondria 18 hours after 15 mg/kg of diazepam administration (in vivo) at 60,000 x magnification.
diazepam administration, there is a significant decrease \((p<0.001)\) in the activity in both regions with a greater decrease in CB. This difference in the \textit{in vitro} and \textit{in vivo} treatments could be due to the difference in availability of substrate to the enzyme under both these conditions.

### Table 23

\textbf{Effect of diazepam at different concentrations \textit{(in vitro)} on the activity of monoamine oxidase in rat brain mitochondria.}

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Control (0.266\pm0.033)</th>
<th>1 mg/kg (0.362\pm0.020^a)</th>
<th>3 mg/kg (0.378\pm0.028^a)</th>
<th>9 mg/kg (0.353\pm0.035^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>(0.539\pm0.035)</td>
<td>(0.286\pm0.041^c)</td>
<td>(0.386\pm0.096^b)</td>
<td>(0.265\pm0.186^c)</td>
</tr>
</tbody>
</table>

Values are arithmetic mean \(\pm\)S.D. of six determinations in each case.
a:p<0.01; b:p<0.1; c:p<0.001.

### Table 24

\textbf{Effect of diazepam (3 mg/kg b.w. for 1 hour) on the activity of MAO in rat brain mitochondria \textit{in vivo}.}

<table>
<thead>
<tr>
<th>Brain Regions</th>
<th>Control (1.282\pm0.045)</th>
<th>Treated (0.930\pm0.033^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>(1.424\pm0.090)</td>
<td>(1.013\pm0.095^a)</td>
</tr>
</tbody>
</table>

Values are arithmetic mean \(\pm\)S.D. of six determinations in each case.
a:p<0.001.

The changes in the inner mitochondrial membrane (IMM) were also looked into by assaying the activity of the IMM enzyme, cytochrome \(c\) oxidase (Fig. 31). The \textit{in vivo} administration of diazepam (3 mg/kg for 1 hour) does not show any significant changes in the activity of the mitochondrial inner membrane marker enzyme, cytochrome \(c\) oxidase (Fig. 31) in CB while BS showed a 44% increase. In the earlier results also the response of BS mitochondria to diazepam administration has been more pronounced as compared to CB.
Fig. 31: Changes in cytochrome c oxidase activity 1 hr after 3 mg/kg diazepam administration \((in vivo)\)
Changes in Turnover of Mitochondrial Membrane Phospholipids

Since earlier studies from this laboratory showed significant conversion of lecithin to lysolecithin due to phenobarbitone administration, indicating changes in the membrane phospholipid turnover and functional status of membranes, studies were carried out to rule out such an effect due to diazepam.

The phospholipid profile of the three regions of brain in terms of total phospholipids was observed and compared to control animals (Table 25). A highly significant increase in the total phospholipid content was observed in cerebral tissue which was 2.35 fold higher in diazepam treated rats as compared to their corresponding controls. In CBL the increase in total phospholipid content was 62.3% more than the control whereas in brain stem it was 49.5% more than the corresponding control. The results indicate a higher turnover of phospholipids or phospholipid alteration in membranes of treated animals. Attempts were also made to see the changes in individual phospholipids qualitatively by doing the thin layer chromatography of the isolated lipids of brain regions of both control and experimental animals. Plate 13 shows the separation of individual phospholipids of cerebrum and BS region on TLC plate. Though a good separation of individual phospholipids was achieved no significant change was observed in the treated group as compared to controls as is evident from the colour intensity of the spots developed.

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Total phospholipid content (mg/g tissue wt. equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>20.931±3.215</td>
</tr>
<tr>
<td>CBL</td>
<td>24.407±1.947</td>
</tr>
<tr>
<td>BS</td>
<td>37.172±1.723</td>
</tr>
<tr>
<td>Diazepam (15 mg/kg; 1 hour)</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>49.285±1.883 (p&lt;0.001)</td>
</tr>
<tr>
<td>CBL</td>
<td>39.623±0.412 (p&lt;0.001)</td>
</tr>
<tr>
<td>BS</td>
<td>55.593±2.406 (p&lt;0.001)</td>
</tr>
</tbody>
</table>

The values are arithmetic mean ±S.D. of six determinations in each case.
Plate 13: Separation of individual phospholipids of CB and BS on TLC plate.
Table 26 gives an account of the incorporation of the $^{14}$C label and the subsequent counts/min in the lecithin (PC) and lysoleicithin (LPC) content of BS after separation on the TLC plate, 1 hour after the dose of 3 mg/kg. Since BS was found to be affected most by diazepam in the studies carried out so far, this region was chosen for further studies. Unlike phenobarbitone, the magnitude of conversion of phosphatidylcholine i.e., lecithin (PC) to lyso phosphatidylcholine i.e. lysoleicithin (LPC) due to diazepam is much less, PC is only slightly decreased in the treated animals whereas there is a non significant increase in the LPC content in the treated animals compared to their controls. The PC:LPC ratio in controls was 1.5 whereas it was 0.87 for the treated animals showing very slight activation of peroxidative processes.

Table 26

Effect of diazepam administration (3 mg/kg, 1 hr) on the incorporation of $^{14}$C choline in brain stem (BS) mitochondria.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>$^{14}$C-choline incorporation (cpm/mg fresh tissue weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Phosphatidyl choline (PC)</td>
<td>550±53</td>
</tr>
<tr>
<td>Lysophosphatidyl choline (LPC)</td>
<td>352±69</td>
</tr>
</tbody>
</table>

Plate 14 shows the thin layer chromatographic separation of different classes of phospholipids in the BS of diazepam treated (3 mg/kg for 1 hour) and untreated animals along with the incorporation of $^{14}$C-choline. Only PC and LPC were used as standards for identification and the plate shows no significant difference between the two phospholipids in the treated and control groups.

Test For Any Leakage of Proteins From Mitochondria During Oxidative Stress and Diazepam Treatment (In vitro)

The SDS-PAGE of the mitochondrial pellets of CB and BS as well as their supernatants after being subjected to in vitro oxidative stress (xanthine-xanthine
Plate 14: Separation of mitochondrial phospholipids of BS of $^{14}$C choline + diazepam and only $^{14}$C-choline treated rats.
oxidase system and t-BHP) both in the presence (before and after the stress) and absence of diazepam (0.05 mg) was done in 12.5% polyacrylamide gel. Plate 15 shows the SDS-PAGE of CB mitochondria. As is evident from the picture in lane 2 which shows the protein bands after the xanthine-xanthine oxidase stress in the absence of diazepam, certain protein bands are found missing as compared to control (lane 1) whereas in lane 3 (diazepam given after the stress) and lane 7 (diazepam given before the stress), intensity of certain protein bands has reduced but the missing and faint protein bands could not be detected in the electrograms of the supernatants (Plate 16). Plate 17 shows the SDS-PAGE of BS mitochondria. Lane 2 (xanthine + xanthine oxidase) shows faint bands whereas in lane 3 (diazepam before xanthine-xanthine oxidase), though all the bands compared to control are present but they are not as sharp. Lane 4 (only diazepam) also shows faint bands which further confirms slight membrane changes observed in TEM pictures due to diazepam whereas lane 7 (xanthine-xanthine oxidase before diazepam) shows very faint bands. Lanes 5, 6 and 8 of both CB and BS which have t-BHP (100 μM) both in presence and absence of diazepam (details in pictures) do not show any changes. As in CB, the supernatants of BS (Plate 18) also do not show any bands, thus ruling out any detectable protein leakage from the mitochondria.

**Discussion**

Since oxidative stress is central to cell damage process in many neurodegenerative disorders, the present data suggest a central role of mitochondrial response to active oxygen species in these processes. A growing body of literature (Coyle and Puttfarcken, 1993) suggests that mitochondrial dysfunction plays a pivotal role in the neurodegenerative process in a panoply of disorders ranging from Parkinson’s (deBelleruche et al, 1996) and Huntington’s diseases to hereditary encephalomyopathies to Alzheimer’s (Gsell et al, 1995) disease. The data do not suggest any serious irreversible deleterious effects due to
Plate 15: SDS-PAGE of CB mitochondria
1. Control; 2. Xanthine-xanthine oxidase; 3. Xanthine-xanthine oxidase + 0.05 mg diazepam; 4. 0.05 mg diazepam; 5. 100 μM t-BHP; 6. 0.05 mg diazepam + 100 μM t-BHP; 7. 0.05 mg diazepam + xanthine-xanthine oxidase; 8. 100 μM t-BHP + 0.05 mg diazepam

Plate 16: SDS-PAGE of CB mitochondrial supernatants (Lane 1-8 are same as described for plate 15)
Plate 17: SDS-PAGE of BS mitochondria
1. Control; 2. xanthine-xanthine oxidase; 3. 0.05 mg diazepam + xanthine - xanthine oxidase; 4. 0.05 mg diazepam; 5. 100 μM t-BHP; 6. 0.05 mg diazepam + 100 μM t-BHP; 7. xanthine-xanthine oxidase + 0.05 mg diazepam; 8. 100 μM t-BHP + 0.05 mg diazepam

Plate 18: SDS-PAGE of BS mitochondrial supernatants (Lanes 1-8 are same as described for plate 17).
diazepam even though there are reports of genetic toxicity due to benzodiazepines (Giri and Banerjee, 1996) as well as malformations, functional deficits and long lasting behavioural anomalies due to prenatal BZD application (Karkos, 1991). Other experimental data also suggest that prenatal and/or postnatal administration of BZDs at dose levels below those associated with overt signs of neurotoxicity produces both short and long-term alterations in rats (De Salvia et al, 1990). However, the regional differences in the response and reversibility of the immediate response of diazepam are salient features of the present finding.

The results show a difference in the degree of swelling in brain and liver mitochondria which could be due to difference in availability of the drug within the cells' micro-environment, localization of enzyme systems and the cellular distribution of antioxidant defense mechanisms. The calcium induced swelling in brain (Fig. 24) and liver (Fig. 26) was significantly reduced (30% in brain and 46% in liver) due to DZ administration. During the superoxide-induced oxidative swelling of mitochondria, diazepam did not affect brain mitochondria significantly whereas liver mitochondria were accorded protection against oxidative swelling in a dose-dependent manner. Swelling is far less in brain mitochondria as compared to that of liver.

Since this effect of mitochondrial swelling has earlier been shown by Mehrotra et al (1991) to be reduced in liver by externally added SOD, the same was tested for brain mitochondria which too showed a fair amount of reduction in O2^- mediated swelling (Fig. 25). The reason for a greater reduction in O2^- mediated swelling due to diazepam in liver could be due to the presence of much higher amounts of catalase in the liver, which in contrast, is very low in brain (Jackson et al, 1990; Simonian and Coyle, 1996) thereby providing lesser protection due to diazepam in this organ. This means that OH formation is more in liver which will be more directly involved in membrane changes.
Diazepam, like anaesthetics, is known to stabilize receptors (Krueger and Papadopoulos, 1992). The TEM picture (Plates 9a and 9b) also shows that O$_2^-$ mediated disruption of cristae was less in mitochondria treated with diazepam prior to in vitro oxidative stress. The data on mitochondrial swelling also shows higher level of swelling in liver (Fig. 24) due to externally added calcium compared to that in brain (Fig. 26). This may be due to a difference between brain and liver mitochondria in uptake of externally added calcium and also due to quantitative variations in aerobic metabolism.

Further evidence for diazepam influencing calcium transport across mitochondria came from Fura-binding studies (Figs. 28-30). Diazepam shows an increasing trend in Ca$^{2+}$-sequestration in the mitochondria of CB with increasing doses of diazepam whereas, in BS, the trend was different which is in agreement with the differential response in other parameters as well due to regional heterogeneity in the brain (Ravindranath et al, 1994). BS mitochondria showed a marginal decrease as compared to the untreated samples at 0.044 µM diazepam, but at 0.088 µM, there was nearly a 2 fold increase in intramitochondrial Ca$^{2+}$ sequestration which is in agreement with experiments on synaptosomes from rat brain, which show that doses of diazepam ranging from 0.1 to 100 µM significantly enhanced Ca$^{2+}$ levels measured with Fura-2, compared to control incubations without the drug (Martin et al, 1991a). But at a higher dose of diazepam (0.35 µM), there was an apparent leakage of Ca$^{2+}$ from mitochondria showing Ca$^{2+}$ saturation at this dose which could be due to the calcium paradox (Duncan et al, 1992). This shows a slight differential response between synaptosomes and mitochondria. Moreover, lower doses of diazepam are capable of causing larger variations as well as showing greater Ca$^{2+}$ sequestering capacity in BS mitochondria as compared to CB which further justifies the differential response between the two regions shown in the preceding chapters. The above results could be due to the fact that isolated mitochondria have a large capacity to accumulate Ca$^{2+}$ and buffer the extra
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mitochondrial \([\text{Ca}^{2+}]\) at around 1 \(\mu\text{M}\) if their calcium content exceeds 10 nmol/mg protein (Lukacs and Kapus, 1987). In addition, oxidative stress has also been shown to promote \(\text{Ca}^{2+}\) release from mitochondria (Sandri et al, 1990). In a recent study mitochondrial permeability transition was compared in apoptosis and necrosis. Loss of mitochondrial membrane integrity was shown to precede caspase and nuclease activation in most models of apoptosis (Hirsch et al, 1998).

Since BS mitochondria have a higher calcium holding capacity than those of CB, as shown by the intramitochondrial \(\text{Ca}^{2+}\) concentration of the control samples. Even after longer incubation time (15 mins), BS mitochondria still hold \(\text{Ca}^{2+}\) without any leakage whereas CB shows a dramatic rise in \([\text{Ca}^{2+}]\) at 10 mins with subsequent leakage at 15 mins (Fig. 30) which is due to breakdown of membrane potential on massive accumulation of \(\text{Ca}^{2+}\) (Nicotera et al, 1992a). The concentration of diazepam used for BS is lesser than that for CB considering a lower protein content in the former. The above observations can be justified with the observations by Mendelson et al (1984) that pharmacologically relevant concentrations of benzodiazepines (\(\leq 1 \mu\text{M}\)) increase \(^{45}\text{Ca}^{2+}\) uptake into synaptosomes and also that diazepam (1 \(\mu\text{M}\)) significantly increased the uptake of \(^{45}\text{Ca}^{2+}\) to a crude synaptosomal fraction (P2) prepared from rat cerebral cortex and depolarised with 55 mM K\(^{+}\). Thus the pharmacologic action of BZDs may be mediated through effects on a calcium channel. In fact the capacitative calcium entry wherein release of calcium from intracellular organelles including mitochondria, secondarily signals to the plasma membrane, opening plasma membrane calcium channels, has attracted the attention of basic scientists as a potential target of therapeutic intervention.

The activity of mitochondrial outer membrane marker enzyme, monoamine oxidase (MAO) shows non-significant increases in CB with in vitro doses of 1 mg/kg, 3 mg/kg and 9 mg/kg diazepam (Table 23). This is in agreement with the electron micrographs of this region which show only slightly deshaped outer membrane with 3 mg/kg dose (Plate 2) and a greater but not complete degenera-
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The MAO activity in BS is decreased as compared to controls. This could be due to the disruption of outer envelope as shown by TEM picture of this region at 15 mg/kg (Plate 7). The TEM pictures clearly indicate marked damage by active oxygen species, confirming an earlier finding (Mehrotra et al., 1991). Also, structural collapse could be possible due to deranged calcium as disruption of mitochondrial membrane integrity has been implied to be the decisive factor in cell death process (Hirsch et al., 1998). The in vivo diazepam administration caused a decrease in MAO activity in both the regions (Table 24). This is supported by the observations of Nishikouri (1995) that the brain concentrations of monoamines in 1 day old rats exposed to 5 mg/kg s.c. diazepam were increased in comparison to the saline group which shows the effect of diazepam on the mitochondrial outer membrane which also possess BZD receptors, whereas in a different study (Gol’dina and Gankina, 1988), tranquilisers having a BZD structure were found to be inactive with respect to MAO of the brain during a comparative study of anti MAO activity of psychotropic agents of different classes.

Diazepam affects both the outer and the inner mitochondrial membranes due to the presence of BZD receptors at the contact sites of outer and inner membranes which typically cofractionate with inner mitochondrial membrane markers (Krueger and Papadopoulos, 1992). This could be the reason for the highly swollen cristae of BS at 15 mg/kg (Plate 7) and a slight disorganisation of cristae at 3 mg/kg (Plate 6) and also the protective effect of diazepam not being complete after in vitro oxidative stress, especially in terms of disorganized cristae, though only in few mitochondria (Plates 9a and 9b), yet the inner membrane in this region is not completely disrupted. The protective effect of diazepam has been observed in a study by Clement and Broxup (1993) where diazepam given 10 mins before
soman a neurotoxicant prevented the occurrence of soman induced convulsions and neuropathology (i.e., degenerative changes were not seen then). An increase (44%) in the activity of mitochondrial inner membrane marker enzyme, cytochrome oxidase of BS region was observed (Fig. 29), which could be due to greater availability of the substrate to the enzyme whereas the cristae of CB show degeneration even at 3 mg/kg diazepam (Plate 2) and even after revival (18 hrs after the 3 mg/kg dose), though the outer shape is regained, yet the cristae are thread like and elongated (Plates 4a and 4b). This is confirmed by a 32.7% decrease in the activity of cytochrome c oxidase in this region, thereby showing a differential response to diazepam in the two regions. Since CBL mitochondria do not show very deleterious effects even at 15 mg/kg (Plate 11a & 11b and 12a & 12b), other confirmatory parameters were not studied in this region.

Membrane phospholipids play an important role during any free radical mediated mechanism, therefore, the conversion, if any, of lecithin (PC) to lysolecithin (PC), observed earlier for phenobarbitone (Awasthi et al, 1991) was measured after diazepam administration. The results (Table 26) show that diazepam reduces the incorporation of $^{14}$C choline into the PC fraction with a slight increase in the LPC:PC ratio in the treated animals compared to their respective controls. There is only a marginal decrease in the PC:LPC ratio in the treated animals indicated by the rate of $^{14}$C incorporation into PC which decreases due to diazepam and PC gets converted to LPC presumably by the action of PLA$_2$. As shown in Table 25 in vivo administration of diazepam in high doses (15 mg/kg) caused a dramatic increase in total phospholipid content in all the three regions of treated rats. This shows that the amount of phospholipids including inositol (which has not been tested in this study) may have increased in response to diazepam. Since this dose causes drastic changes as evident from TEM studies 3 mg/kg diazepam (which is the more commonly used therapeutic dose) was used to see any subtle changes in PC:LPC ratio. As compared to control animals, the decrease
in PC:LPC ratio from untreated to treated animals was 1.5 to 0.87. However, this response is much less as compared to phenobarbitone, because diazepam is not a strong promoter of oxidative stress in brain as observed earlier in this thesis. The structural effect on membranes are also of a lower magnitude. Repeated exposure, unlike the above effects on single exposure, reduces LPO and accords protection to membrane. This could be due to the fact that unlike single exposure, repeated exposure could be causing metabolic adaptation to defend against the stress. It has also been indicated earlier (Goswami et al, 1991) that diazepam treatment has some protective ability from anoxia-induced imbalance in rat brain glutamatergic and GABAergic functions.

Finally, to confirm the protection of mitochondrial membranes by diazepam observed thus far, SDS-PAGE of mitochondria subjected to oxidative stress (both in presence and absence of diazepam) and their supernatants was done to rule out any possibility of protein leakage from the mitochondria. As shown in Plate 15, lane 2 of CB and BS (Plate 17) mitochondria (only xanthine-xanthine oxidase) shows either some missing protein bands (CB) or faint bands (BS) which confirms membrane changes due to oxidative stress whereas lane 3 of CB and lane 7 of BS (both having diazepam after oxidative stress) do not show very sharp bands, even though all the bands compared to control (lane 1) are present. This shows that a certain amount of protection from oxidative stress is provided by diazepam which is not complete as diazepam is given after incubating the system for 5 mins in the presence of xanthine-xanthine oxidase. Lane 4 (only 0.05 mg diazepam) of BS shows slightly fainter bands compared to control. This could be due to membrane changes, but not damage in the presence of diazepam in this region as shown in the TEM picture (Plates 9a and 9b). Lane 7 of CB and lane 3 of BS also show faint bands but no missing proteins and this could be due to presence of diazepam before oxidative stress, thus confirming a protecting ability of diazepam. 100 μM tBHP did not cause enough oxidative stress to lead to changes in membrane.
Therefore, lanes 5, 6 and 8 show clear and sharp bands (Plates 15 and 17). A point of interest that has emerged is that though diazepam + radical generated samples show either faint or missing bands, yet these are not detectable in their respective supernatants. This shows that though diazepam and xanthine-xanthine oxidase do cause protein changes (Slater et al, 1995), yet the membrane impermeability is not lost. It is known that membrane proteins in the mitochondria frequently form specific interactions in order to build a multifunctional protein complex. The correct and efficient assembly of these membrane complexes is essential for mitochondrial function (Rep et al, 1996). It has also been reported in alveolar type II cells that catalase activity was not changed after slight oxidative stress but decreased in severe oxidative stress (Gonzalez-Flecha et al, 1996) which further confirms all the earlier results of slightly decreased enzyme activities, in presence of oxidative stress along with the SDS-PAGE results of a decrease only in coomassie-blue stain intensity which could be due to protein carbonylation (Delattre and Bonnefont-Rousselot, 1998; Starke-Reed and Oliver, 1989; Dean et al, 1997).

In contrast, as shown in lanes 5, 6 and 8 (Plates 15 and 17) which show mitochondria exposed to 100 μM t-BHP both in the presence and absence of diazepam, there is no decrease in the intensity of coomassie blue staining and all the bands are clearly sharp as compared to control. This could be due to the fact that in t-BHP exposed mitochondria both transhydrogenation and transmembrane proton gradient appear to be important in NADPH regeneration and consequent GSSG reduction (Liu and Kehrer, 1996), thereby preventing any protein modification.

On the basis of data from phospholipid levels and turnover, function related ultrastructural changes, Ca fluxes and protein profile, a role for mitochondria in oxidative deregulation of Ca functions and a regional differential response of it, in diazepam effects are indicated.
Summary

In order to see whether the modulation of prooxidative processes due to DZ are related to altered calcium dynamics, several lines of in vivo and in vitro studies were conducted using CB and BS mitochondria. Brain and liver mitochondria showed difference in degree of superoxide induced oxidative swelling. Brain mitochondria showed 30% reduction in Ca\(^{2+}\) induced swelling in the presence of diazepam. Swelling was far less in brain mitochondria as compared to liver mitochondria. The transmission electron micrographs (TEM) show protection to mitochondrial membranes of cerebrum and brain stem incubated with diazepam before oxidative stress (in vitro), though diazepam alone does cause membrane alterations at higher doses compared to the untreated samples. Diazepam enhanced Ca\(^{2+}\) sequestration in mitochondria at different doses and time intervals and the pattern of sequestration was different in cerebrum and brain stem. It is evident from the data that brain stem mitochondria show greater capacity for holding calcium and that too for longer duration as compared to cerebrum mitochondria. The mitochondrial membrane marker enzymes, MAO and cytochrome c oxidase show only slight differences in activity due to diazepam treatment (in vivo and in vitro). There was a tendency of increase in lysolecithin/lecithin ratio as seen from \(^{14}\)C choline incorporation, yet the polyacrylamide gel electrophoresis showed no detectable leakage of proteins from mitochondria, thereby confirming that diazepam does not cause gross damage to mitochondrial membranes and the membrane impermeability is not lost. In the presence of diazepam, protection against oxidative stress to the membrane structure and functions along with the proteins was seen in these studies. Diazepam also enhanced the capacity of mitochondria for holding Ca\(^{2+}\) which is very critical in any toxic insult and ultimate cell death.