Diazepam and Benzodiazepines

Diazepam (DZ) belongs to the group of 3000 and odd benzodiazepine (BZD) drugs. The use of most anxiolytic drugs is limited by several side effects, most notably their serious toxicity at high doses and their high liability to addiction. BZDs are very useful and comparatively safer drugs when used alone in the treatment of anxieties, sleep disorders, muscle pain, convulsions and alcohol withdrawal. They however, can be very toxic when used with other CNS depressants and produce physical dependence when used chronically (Sloan, 1994). They have a lower potential for addiction than many other drugs that were used earlier and are less likely to cause death or serious damage when taken in large overdoses. The various benzodiazepines differ primarily in their pharmacokinetics rather than in their clinical effects (Barchas et al, 1994).

The term benzodiazepine (BZD) refers to the portion of the structure (Fig. 1) composed of a benzene ring (A) fused to a 7-membered diazepine ring (B). However, since all the important BZDs contain a 5-aryl substituent ring (C) and a 1,4-diazepine ring, the term has come to mean the 5,aryl-1,4-benzodiazepines.
Various modifications in the structure of the ring systems have yielded compounds with similar activities. The effects of BZDs, virtually all, result from actions of these drugs on the CNS, the most prominent of these being sedation, hypnosis, decreased anxiety, muscle relaxation, anterograde amnesia, and anticonvulsant activity. Only two effects of these drugs appear to result from actions on peripheral tissues: Coronary vasodilation, seen after intravenous administration of therapeutic doses of certain BZDs, and neuromuscular blockade, seen only with very high doses (Gilman, 1996).

**BZDs**

<table>
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<tr>
<th>BZDs</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₂'</th>
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<td>-H</td>
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<td>-H</td>
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<td>-H</td>
<td>-NO₂</td>
<td>-Cl</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>-CH₂CH₂N(C₃H₇)₂</td>
<td>=O</td>
<td>-H</td>
<td>-Cl</td>
<td>-F</td>
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<tr>
<td>Nitrazepam</td>
<td>-H</td>
<td>=O</td>
<td>-OH</td>
<td>-Cl</td>
<td>-H</td>
</tr>
</tbody>
</table>

**Fig. 1:** The general structure of benzodiazepine (Gilman *et al.*, 1991).

BZDs are well absorbed after oral administration, and concentrations in plasma are usually maximal within 1 to 4 hours. After i.v. administration, they are redistributed in a manner typical of that for highly lipid-soluble agents. Central effects develop promptly but wane rapidly as the drug moves to other tissues. Diazepam is redistributed especially rapidly with a half life of
redistribution of about 1 hour in adults and 15-30 mins in children (Gilman, 1991). The extent of binding of BZDs to plasma proteins correlates with lipid solubility, ranging from approximately 99% for diazepam to about 85% for clonazepam.

The major metabolite of diazepam, N-desmethyldiazepam (Kaplan et al, 1973), is somewhat less active than the parent drug and may behave as a partial agonist. Both diazepam and N-desmethyldiazepam are slowly hydroxylated to other active metabolites, such as oxazepam. The half life of diazepam in plasma is between 1 and 2 days while that of N-desmethyldiazepam is about 60 hours. Less than 1% of the drug is recovered unchanged in the urine.

Clinical Effects

Acute overdose toxicity has been observed more often with short acting BZDs (midazolam and triazolam) and the intermediate acting flunitrazepam than with diazepam, lorazepam and nitrazepam (Meir et al, 1993). Death caused by BZDs alone in the absence of other significant toxicologic agents or pathology is uncommon, although BZDs alone can cause death in the absence of significant natural disease or advanced age (Drummer et al, 1993). Death has been reported after consumption of high doses of flunitrazepam, alprazolam, triazolam, temazepam and flurazepam.

Tolerance and Physical Dependence

Tolerance, physical dependence, and withdrawal are all biological phenomena. They are the natural consequences of drug use. They can be produced in experimental animals and in any human being who takes certain medications repeatedly and the drug is terminated abruptly rather than gradually. Studies on tolerance in animals often are cited to support the belief that disinhibitory effects of BZDs are separate from their sedative-ataxic effects. For example, tolerance to
the depressant effects on rewarded or neutral behaviour occurs after several days of treatment with BZDs; the disinhibitory effects of the drugs on punished behaviour are augmented initially and decline after 3 to 4 weeks (File and Andrews, 1993). If doses of BZDs are given for long periods and then abruptly withdrawn, severe withdrawal symptoms can occur (Woods et al., 1987). Moreover, diazepam has been suggested to operate both acutely and chronically through the same pathway (Lucas et al., 1997). Although most patients who chronically ingest benzodiazepines report that drowsiness wanes over a few days, tolerance to the impairment of some measures of psychomotor performance usually is not observed. The development of tolerance to the anxiolytic effects of BZDs is a subject of debate (Lader and File, 1987). Some studies suggest that drug tolerance observed due to repeated BZD exposure may be associated with the development of a subsensitivity to GABA in dorsal raphe and hippocampal neurons. In other areas such as the substantia nigra, such sub-sensitivity has not been found, (Hutchinson et al., 1996). Withdrawal signs in BZD-dependent rats comprise behavioural changes such as enhanced anxiety, reduced seizure threshold and alterations in vegetative functions and weight loss (Bonnafous et al., 1995). This hypersensitivity after chronic BZD treatment of rodents is considered a reliable and useful index of physical dependence on BZDs (Mizoguchi et al., 1993). It has been postulated that this withdrawal response may reflect CNS hyperexcitability as a result of an adaptive response to chronic treatment with BZDs, although the neurochemical basis of this phenomenon has not yet been clarified. Moreover, BZD withdrawal has been shown to reduce the ability to develop an adaptive response to stress (Martijena et al., 1996).

BZD receptors located in the CNS are involved in behavioural withdrawal syndrome, whereas BZD receptors located at the peripheral level are responsible for digestive withdrawal syndrome involving capsaicin sensitive neurons as shown
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by rats chronically treated with DZ (15 mg/kg/day; i.p.) for 7 days (Bonnafous and Bueno, 1994). Abrupt termination of the chronic diazepam treatment (3 weeks) showed withdrawal hyperactivity which was reversed by calcium channel blockers (Chugh et al, 1992) as $^{44}\text{Ca}^{2+}$ uptake was found to be increased during diazepam withdrawal (Andrews et al, 1992).

Experimental data obtained after prenatal application of BZDs indicate that these drugs can cause malformations, functional deficits, behavioural disturbances, neurological deficits and long lasting behavioural anomalies in animals and man (Schroeder et al, 1995). The prenatal toxicity of BZDs is probably due to their interaction with neurotransmitter systems. Post natal application of BZDs can bring about behavioural disturbances and neurological deficits in animals and man (Schroeder et al, 1995). A part of this pathology can be compensated by means of the functional and structural redundancy as well as the tolerance (Piazza and Mole, 1996).

Toxic Reactions and Side Effects

The expected side effects of CNS depressants of drowsiness and ataxia are extensions of the pharmacological actions of these drugs. With diazepam, antianxiety effects can be expected at blood concentrations of 300 to 400 ng/ml, while some sedative effects and psychomotor impairment begin at similar concentrations; gross CNS intoxication can be expected at concentrations over 900 to 1000 ng/ml (Morselli, 1977). A high dosage of parenteral administration can produce respiratory depression and hypotension (Martindale, 1993).

A few deaths have been reported at doses greater than 700 mg of diazepam or chlordiazepoxide. Menstrual irregularities have been noted, and women may fail to ovulate while taking benzodiazepines. Diazepam has been shown to have carcinogenic effects in experimental animals (Andersson et al, 1981; Giri and Banerjee, 1996) in the in vivo systems, which makes it difficult to draw conclusions
about the clastogenic potential of this drug; though highly contradictory results have been reported for diazepam especially overdosage with the benzodiazepines is frequent, but serious sequelae are rare unless other drugs or ethanol are also taken. The striking advantage of this group of drugs is their remarkable margin of safety. The question of teratogenic effects of BZDs or other toxic effects on the fetus is controversial (Laegreid et al, 1992). BZDs depress CNS function in the neonate and especially in the newborn, as is the case with diazepam which crosses the placenta rapidly and can depress the fetus (Errkola et al, 1973).

**Biotransformation of Diazepam**

The BZDs are metabolized extensively, particularly by several different microsomal enzyme systems in the liver. The basic metabolic pathway leading to diazepam elimination in humans is as follows:

![Biotransformation Diagram](source: Skinner and Thompson, 1992).

Because active metabolites are generated that are biotransformed more slowly than the parent compound, the duration of action of many BZDs bears little relationship to the half time of elimination of the drug that has been administered. For example, the half life of flurazepam in plasma is 2 to 3 hours, but that of a major active metabolite (N-desalkylflurazepam) is 50 hours or more (Rall, 1991). The major metabolite of diazepam, N-desmethyl Diazepam
is somewhat less active than the parent drug and may behave as a partial agonist. Both diazepam and N-desmethyldiazepam are slowly hydroxylated to other active metabolites, such as oxazepam. The half life of diazepam in plasma is between 1 and 2 days, while that of N-desmethyldiazepam is about 60 hours (Greenblat et al., 1981). Thus, due to long half lives and conversion to active metabolites with long durations of action, withdrawal or abstinence symptoms after prolonged use may not appear for a week or more after abrupt discontinuation of the drug and are likely to be mild (Lader, 1994; Rickels et al., 1988). Since most drawbacks with the use of BZDs arise from long term use, recommendations both for insomnia and for anxiety disorders have stressed the importance of short term use (upto 4 weeks for generalised anxiety and 1-2 weeks for acute insomnia) (Magrini, 1996).

The GABA<sub>a</sub>/Benzodiazepine Receptor Complex

GABA (y-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and has been implicated in the etiology of a number of clinically important disorders, including epilepsy (Gale, 1989). BZDs appear to potentiate the effects of GABA (i.e. they facilitate inhibitory GABA neurotransmission) and other inhibitory transmitters by binding to specific BZD receptor sites. The activity of BZDs may involve the following sites: spinal cord (muscle relaxation), brain stem (anticonvulsant properties), cerebellum (ataxia), limbic and cortical areas (emotional behaviour). Anxiolytic effects are distinct from non-specific consequences of CNS depression (i.e. sedation and motor impairment) (Drug: facts and comparisons, 1995). GABA is localized in nerve terminals from which it is released in a calcium-dependent manner by depolarizing stimuli. Following release, GABA can interact with two, possibly three, subtypes of GABA receptor, namely GABA<sub>a</sub>, GABA<sub>b</sub> and GABA<sub>c</sub>.
The GABA<sub>A</sub> receptor is a ligand-gated Cl<sup>-</sup> channel which consists of four subunits, α, β, γ and δ (Smith and Olsen, 1995). Activation of this receptor produces an increase in membrane Cl<sup>-</sup> conductance, and this effect can be allosterically modulated by benzodiazepines, barbiturates and neurosteroids (Malcangio and Bowery, 1996). Fig. 2 gives a cutaway view demonstrating targets for a variety of compounds that influence the receptor complex. Each drug interacts with a different site on the receptor complex (DeLorey and Olsen, 1994). The GABA-receptor is a macromolecular protein that contains specific binding sites at least for GABA, picrotoxin, barbiturates, BZDs and the anaesthetic steroids, and forms a chloride ion selective channel (Macdonald and Olsen, 1994) and its numerous molecular variants underlie the observed diversity in the properties of BZD sites (Wieland et al., 1992). Since neuronal tissue is a vulnerable site for free radical generation and receptor proteins along with membranes can be the attack sites, this aspect has also been reviewed in detail.

**Fig. 2:** Structural model of the GABA<sub>A</sub>/benzodiazepine receptor chloride (Cl<sup>-</sup>) ionophore complex. The cut-away view demonstrates targets for a variety of compounds that influence the receptor complex. No specific drug receptor location is implied (Smith and Olsen, 1995).
Other Aspects

The toxicokinetics (Ellenhorn, 1997; Roman and Krishnamurthy, 1993), biotransformation (Skinner and Thompson, 1992; Magrini, 1996), mechanism of action (Eghbali et al, 1997; Toki et al, 1996; Birnir et al, 1994), molecular pharmacology of benzodiazepine receptor (Sigel and Buhr, 1997, Kanner, 1996, Li et al, 1994) stimulatory effects (Narahashi, 1997; Voigt et al, 1998; Silva and Tomas, 1995) and other aspects related to diazepam (Zavala, 1997; Yamagishii and Kawaguchi, 1998) are well studied and reported. They are not discussed in detail in this review since the dissertation centres around diazepam and oxidative stress.

Free Radical Processes and Oxidative Stress

A free radical is any species capable of independent existence (for however brief a period) that contains one or more unpaired electrons (Halliwell and Gutteridge, 1989) which can react with other molecules by taking or giving one electron (Fulbert and Cals, 1992). These reactive oxygen species (ROS) may be produced in the cellular environment due to many metabolic activities. Diatomic molecular oxygen ($O_2^-$) readily reacts to form partially reduced species, which are generally short-lived and highly reactive and include the superoxide anion ($O_2^{-}$, a free radical), hydrogen peroxide ($H_2O_2$) and the most reactive species, the hydroxyl free radical (OH). Other non-oxygen reactive species also exist such as nitric oxide (NO$^-$), the quinone moiety of certain xenobiotics (an exogenous pharmacologically, toxicologically or endocrinologically acting substance), the neurotoxin MPTP and the herbicide paraquat (Acworth and Bailey, 1995). OH radical is a highly reactive species that can attack all biological molecules, usually setting off free radical chain reactions (Halliwell and Gutteridge, 1989;1995). If a reaction is thermodynamically feasible, its reaction rate depends primarily on the concentrations of the reacting partners. Thus, to evaluate effects of ROS on
biomolecules, their concentrations and sites of production have to be considered (Gotz et al., 1994). Such activated oxygen species are increasingly recognised to be the mediators of cell injury in many human diseases (Nicotera et al., 1992; Kakkar et al., 1993, 1995).

Brain is particularly vulnerable to oxidative stress due to the presence of a number of polyunsaturated fatty acids (PUFAs) and low antioxidant defences (Clemens and Panetta, 1995). Several different structures that represent interfaces between brain tissue and blood are able to produce superoxide anions during a NADPH-dependent redox cycling of various blood-borne molecules. As these free radicals can alter the functional properties of cell membranes, superoxide formation may promote damage to choroid plexus and cerebral microvessel cells, resulting in the disruption of some properties of the blood-brain and blood cerebro-spinal fluid barriers, possibly resulting in cerebral dysfunctions (Lagrange et al., 1994).

Superoxide radical ($O_2^-$) is much less reactive than OH (Panasenko et al., 1995), but a number of biological targets can be attacked by it. Though, because of charge restriction, it cannot cross the membranes easily, except RBC membranes but in the presence of transition metal ions, it can give rise to highly reactive OH radical due to Fenton type (from hydrogen peroxide) or Haber-Weiss (from the superoxide anion) reaction (Halliwell, 1992).

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^- \quad \text{(Fenton)}$$

$$O_2^- + \text{H}_2\text{O}_2 \rightarrow O_2 + \text{HO}^- + \text{HO}^- \quad \text{(Haber-Weiss)}$$

$\text{H}_2\text{O}_2$ which is not a free radical, has nevertheless, a high oxidant capacity, via this reaction. $\text{H}_2\text{O}_2$ is able to cross biologic membranes and is capable of inducing cellular damages by the Fenton reaction (Delattre and Bonnefont-Rousselot, 1998).

Interaction of $O_2^-$ with nitric oxide (NO), a free radical produced by several cell types, especially phagocytes and vascular endothelial cells, to give peroxynitrite
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has attracted much attention in recent years (Saran et al, 1990; Huie and Padmaja, 1993; Iadecola, 1997). Peroxyl (ROO) and alkoxyl (RO) free radicals are also synthesized, essentially from PUFAs, either in a direct or controlled way or in an indirect and uncontrolled way. In the first case, these free radicals result from the action of oxygenases (Cyclooxygenases, lipoxygenases) and lead to the eicosanoid pathway. In the second case, they result from the action of OH, leading to the formation of ROO. This constitutes the initiation phase of lipid peroxidation (Delattre and Bonnefont-Rousselot, 1998). It is now accepted that free radicals, especially active O₂ centered radicals: OH, alkoxyl and peroxyl radicals, attack lipids, carbohydrates, proteins and DNA to induce membrane damage, protein modification (Stadtman and Oliver, 1991; Shacter et al, 1994), enzyme inactivation (Oliver et al, 1990) and strand break (Halliwell and Aruoma, 1993) etc. which eventually cause a variety of pathological events including cancer and aging (Sies, 1991; Sohal and Dubey, 1994).

Methionyl residues in proteins are quite often the target of free radical attack (Vogt, 1995). O₂⁻ may also inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Zhang et al, 1990). On the whole, however, O₂⁻ and H₂O₂ have limited chemical reactivity. Interest has, therefore, focused on their ability to generate more reactive species, such as OH in vivo (Halliwell and Gutteridge, 1990; Minotti, 1993) and together with other radicals cause oxidative stress.

Oxidative stress occurs when the production of free radicals overwhelms the body’s natural antioxidant defence systems, resulting in oxidative damage of the cells (Sies, 1985), thereby harming that particular cell or tissue (Ryan and Aust, 1992). Unsaturated lipids (Sohal et al, 1994), proteins (Stadtman, 1992; Jain, 1998) and DNA (Ames et al, 1991; Mchugh and Knowland, 1997) are the components of the cell that are most sensitive to oxidative damage (Fig. 3). Oxidative cell injury is usually accompanied by perturbations of thiol homeostasis i.e., depletion of soluble thiols, mainly glutathione (GSH), and protein-bound thiols (Reed, 1990) [even though mitochondrial GSH pool is significantly more resistant to depletion than that of cytosol (Slater et al, 1995)], peroxidation of lipids, modification of
proteins, cleavage of carbohydrates and oxidation and cleavage of DNA (Molyneux and Davies, 1995), including down-regulation of mammalian mitochondrial RNAs (Crawford et al, 1997). As mitochondrial transcription is extremely sensitive to inhibition by peroxyl radicals, this inhibition occurs prior to detectable evidence of lipid peroxidation as measured by TBARS. Oxidative cell injury is also accompanied by 4-HNE accumulation and oxygen consumption (Kristal et al, 1994). Another intriguing aspect of reactive oxygen toxicity is the ability of superoxide anion as well as redoxcycling xenobiotics to release iron from ferritin, thereby promoting the generation of OH− radicals and iron-oxygen complexes (Reif, 1992).

![Diagram of Reactive Oxygen Species]

Fig. 3: Possible mechanism by which free radicals can damage cellular components (adapted from Kehrer, 1993)

The spectrum of pathological conditions in which the involvement of active oxygen species has been indicated grows broader and broader (Rodenas et
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al, 1995). ESR studies have demonstrated the formation of oxygen derived free radicals in the brain (Zini et al, 1992) and there is evidence of free radical induced lipid peroxidation following cerebral ischemia-reperfusion (Floyd, 1990).

Lipid peroxidation (LPO), is probably the most extensively studied of free radical related processes in biological context and the best known manifestations of oxidative cell injury (Masaki et al, 1989) as it is a process initiated and propagated by free radicals resulting in destruction of the unsaturated fatty acids of membrane phospholipids (Farber, 1994). Slices from brain regions provide a good model for LPO study (Fauconneau et al, 1994) as it was found in a study that after hyperbaric oxygen exposure, LPO was confined to the frontal cortex and hippocampus, while protein oxidation (in both cytoplasmic and membranous fractions) and increased GSSG was evident throughout the brain (Chavko and Harabin, 1996). LPO can be defined as the oxidative destruction of lipids and in most cases refers to fatty acid oxidation (Cheeseman, 1993). The significance of LPO as a damaging process is that (i) its most susceptible targets are the polyunsaturated fatty acids (PUFA) that are common in all cell membranes and (ii) once initiated, it is a self-propagating chain reaction.

Cell membranes represent a mixture of PUFA's, mostly linoleic, arachidonic and docosahexaenoic acids containing 2, 4 and 6 double bonds, respectively. Their respective primary fatty acyl radicals can rearrange to a number of different isomers yielding peroxide products from the fatty acids. Oxyradicals attack double bonds of unsaturated fatty acids in cell membranes in a process called membrane lipid peroxidation (MLP) (Fig. 4). 4-HNE might play a central role in MLP-induced alterations in plasma membrane and mitochondrial protein functions (Mattson, 1998). MLP can also impair mitochondrial function (Mark et al, 1995).

Halliwell and Chirico (1993) observed that "the detection and measurement of LPO is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and human disease", since:
Fig. 4: Pathway involved in induction of membrane lipid peroxidation (MLP) and mechanisms whereby MLP leads to alterations in ion homeostasis and energy metabolism (Mattson, 1998).

[Abbreviations: ER - endoplasmic reticulum; Aβ - amyloid β-peptide; COX - cyclooxygenases; LOX - lipoxygenases; CaM - Ca²⁺/calmodulin kinase; Depol - depolarization; LTs - leukotrienes; NOS - nitric oxide synthase; PGs - prostaglandins; PLA₂ - phospholipase A₂; THR - thromboxanes].

1. It is one of the most likely consequences of reactive free radical production in a cell since the PUFA substrates are so abundant.

2. It is a very destructive process, being a chain reaction in nature and attacking the membrane structures that are essential for normal cell metabolism and viability.

3. A vast amount of expertise has been developed to study LPO more so than has hitherto existed to study other processes.

The extreme damaging potential of LPO makes it one of the most important processes in free radical biochemistry. It is damaging directly through its effects...
on the cell membranes and indirectly by the production of aldehydes, the most biologically active are the hydroxy alkenals and the most important member of this class is 4-hydroxynonenal (4HNE) as it has potent biological activity (Schaur et al., 1991). Destruction of membranes leads to the inactivation of membrane-bound enzymes and the loss of decompartmentalisation that is essential for ordered cell metabolism. Clearly, uncontrolled LPO could represent a catastrophic occurrence for a cell. Of all the effects of LPO, possibly the ultimate lethal event is the loss of calcium homeostasis due to destruction of membranes and inactivation of ion pumps (Kakkar et al., 1995), and Ca\(^{2+}\) buffering capacity of mitochondria and endoplasmic reticulum plays an important role in it.

**Mitochondria and Oxidative Stress**

Mitochondria being the main locus of oxidative metabolism facilitate reactive oxygen species (ROS) generation. Under drastic conditions, the formation of ROS can be elevated and remain unquenched and possible mitochondrial damage may occur (McCabe and Pounds, 1997). Free radicals are formed during the mitochondrial electron transport (Paraidathathu et al., 1992) and the rate of \(O_2^-\) formation is proportional to mitochondrial oxygen utilization. Considerable amounts of \(O_2^-\) are produced when the electron flow is inhibited by antimycin or rotenone (Emster et al., 1992). Endogenous, or exogenous inhibitors of the mitochondrial electron transfer chain could cause a continuous chronic oxidative stress to mitochondria, finally leading to cell death. Thus, it seems reasonable to assume that a decrease in enzymic activity in the electron transfer chain, due to a decreased formation of enzymes or due to inhibitors, probably results in a chronic decrease in ATP levels and an increase in \(O_2^-\) formation. (Gotz et al., 1994). Mammalian mitochondria are highly sensitive targets of the cytotoxic effects of superoxide (\(O_2^-\)) and nitric oxide (NO\(^{\cdot}\)). In turn, when \(O_2^-\) and NO\(^{\cdot}\) are simultaneously produced, they rapidly react with each other to yield the highly
oxidizing peroxynitrite anion (ONOO⁻) which may also be toxic to mammalian mitochondria (Radi et al, 1994). Peroxynitrite is a highly toxic species capable of damaging protein, lipid and nucleic acid molecules (Iadecola, 1997). This assumes significance in the light of the fact that nitric oxide is a highly versatile molecule and acts as a retromessenger (Ignarro, 1996).

While mitochondria are an important cellular source of \( \text{O}_2^\cdot \) radicals (Radi et al, 1993), they also represent a preferred intracellular target of free radical attack. Increased mitochondrial free radical production is observed under diverse pathological conditions and is associated with impairment of mitochondrial structure and functions which results in mitochondrial swelling, inhibition of respiration, lipid peroxidation, depletion of mitochondrial antioxidants and dissipation of \( \text{Ca}^{2+} \) gradients (Zhang et al, 1990; Mehrotra et al, 1991, 1993; Kakkar et al, 1996).

Though the mitochondria are cellular organelles where the generation of ROS may be high, they are, however, effectively protected by their high capacities of antioxidative systems as enzymes and either water or lipid soluble low molecular weight antioxidants. These antioxidative defences can be effectively regenerated after or during an oxidative stress as long as the mitochondria are in an energized state (Augustin et al, 1997). The delicate balance between pro/antioxidative activities can be shifted towards oxidation, if prooxidants were added experimentally. After exhaustion of the antioxidative defences, damages of mitochondrial functions become expressed followed by membrane injuries along with the oxidative degradation of mitochondrial lipids and proteins leading finally to the total degradation of the mitochondria (Castilho et al, 1994).

**Mitochondria and \( \text{Ca}^{2+} \) Homeostasis**

Apart from the well established role in bioenergetics and specific metabolic reactions, mitochondria have been recently implicated in ion regulation and
cellular signal transduction (Miller, 1998). Several reports indicate that the inhibition of the mitochondrial respiratory chain may be caused by the uptake of calcium (Vlessis and Mela Riker, 1989). Mitochondria have been shown to accumulate calcium electrogenically, using the $H^+$ gradient for this purpose instead of producing ATP, even in the presence of ADP. Calcium overload of mitochondria may activate phospholipases or proteases, either of which may cause uncoupling and inhibition of respiration. Ca$^{2+}$ entry into the mitochondrial matrix occurs via a Ca$^{2+}$-permeable "uniporter" (presumably a channel) in the inner mitochondrial membrane (Nicholls and Akerman, 1982; Miller, 1991). Normally respiring mitochondria pump protons out of the mitochondrial matrix, resulting in a potential of about -150 mV to -200 mV across their inner membrane. Thus, when Ca$^{2+}$ increases in the cytoplasm, it can enter the matrix down a steep electrochemical gradient (Hajnoczky et al., 1995). However, recent reports have also started to show that the mitochondria are not always on the receiving end of cytoplasmic Ca$^{2+}$ signals and that they might be in a position to participate actively in them as well. One reason for this is that following a period of intense cellular activity Ca$^{2+}$ is accumulated by mitochondria, and this Ca$^{2+}$ has to leave again following cessation of the stimulus (Miller, 1991; Thayer and Miller, 1990). Until recently, the major route of Ca$^{2+}$ egress from the mitochondrial matrix had been considered to be a Na$^+$/Ca$^{2+}$ exchange system in the inner mitochondrial membrane that probably exchanges two Na$^+$ for each Ca$^{2+}$ (Miller, 1991). This route of Ca$^{2+}$ efflux is rather slow and easily saturated. Thus, if Ca$^{2+}$ influx is relatively fast and extensive, Ca$^{2+}$ will accumulate in the matrix. Accumulated Ca$^{2+}$ leaving the matrix will have the effect of buffering the cytoplasmic Ca$^{2+}$ concentration at relatively high levels until all the Ca$^{2+}$ has exited, thus increasing the length of the cytoplasmic Ca$^{2+}$ signal far beyond the cessation of the original stimulus. In effect, the mitochondria can act as a sort of Ca$^{2+}$ signalling memory storage device. The importance of this for synaptic function has been recently demonstrated by Tang and Zucker (1997).
Although the role of Na\(^+\)/Ca\(^{2+}\) exchange in mitochondrial function has been explored for many years, another route of Ca\(^{2+}\) release is presently causing a great deal of excitement. Indeed with the aid of this novel pathway mitochondria can behave as excitable organelles (Ichas et al., 1997).

When Ca\(^{2+}\) is added to a medium containing isolated mitochondria, it is rapidly accumulated. When a certain degree of loading is reached however, addition of further Ca\(^{2+}\) leads to rapid release of accumulated Ca\(^{2+}\), which is followed by reaccumulation. Thus, the addition of Ca\(^{2+}\) at this point produces an ‘all or none’ Ca\(^{2+}\) spike that is reminiscent of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) produced by ryanodine receptors (intracellular Ca\(^{2+}\) channels with structural and functional similarities to inositol triphosphate receptors which are phosphorylated by Ca\(^{2+}\)-calmodulin-dependent protein kinase II). Mitochondrial CICR might co-operate with inositol (1,4,5)-triphosphate [Ins(1,4,5)P\(_3\)] induced Ca\(^{2+}\) signals and help to amplify them and facilitate the propagation of Ins(1,4,5)P\(_3\)-dependent Ca\(^{2+}\) waves. The juxtaposition of some mitochondria to Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores in the endoplasmic reticulum might be the anatomical mechanism for such an interaction (Miller, 1991). The molecular mechanisms that allow mitochondria to behave in this way are probably through a channel in the inner mitochondrial membrane called the ‘mitochondrial permeability transition pore’ (MPT) (Fig. 5) (Bernardi and Petronelli, 1996; Zoratti and Szabo, 1995). The MPT is a voltage gated, cation permeable channel, whose opening is favoured by several factors including depolarization, intramatrix Ca\(^{2+}\) and oxidizing agents and whose closing is favored by protons (that is low matrix pH) and adenine nucleotides. Ichas et al. (1997) have provided experimental data for a possible model of mitochondrial functioning which suggests that Ca\(^{2+}\) enters the mitochondrion and protons are pumped out, increasing the matrix pH. High matrix pH facilitates MPT opening. This leads to a collapse of the mitochondrial proton gradient and membrane
potential, the outward movement of $\text{Ca}^{2+}$ through the channel and acidification of the matrix which leads to closing of the channel. The respiratory chain now restores the proton gradient, and so on. In this way, transient opening of the MPT produces the spike like CICR. The mitochondrion is a low $\text{Ca}^{2+}$ affinity structure therefore, it is mainly a long term buffer for cellular $\text{Ca}^{2+}$. In normal cells, mitochondria must be regarded more as organelles that control their own internal $\text{Ca}^{2+}$ than as organelles that play a major role in cytosolic $\text{Ca}^{2+}$ homeostasis. Though it is also a fact that mitochondria can store very large amounts of $\text{Ca}^{2+}$ in the matrix without essential variations in its ionic concentration due to the simultaneous accumulation of phosphate and to the precipitation of insoluble calcium phosphate deposits (Carafoli, 1988), the sequestration of large amounts of calcium by mitochondria is driven by membrane potential. Recent studies of this process have led to the proposal that mitochondria may be a major regulator of cytosolic concentrations of calcium under pathological conditions (Richter and Kass, 1991). Storage sites for calcium in mitochondrial membranes apparently can maintain extra mitochondrial calcium at levels only 5 to 10 times higher than the normal cytosolic levels. It appears that under normal physiological conditions these
sites are responsible solely for regulating intramitochondrial calcium homeostasis. Fig. 6 gives a diagrammatic representation of mitochondrial Ca^{2+} cycle (Reed, 1994). Depicted are the mechanisms by which the redox state of mitochondria [NAD(P)H/NAD(P)] can affect the uptake and efflux of Ca^{2+} ADP-ribosylation. Also indicated is the possibility of Ca^{2+} cycling and the site at which ruthenium red and La^{3+} block Ca^{2+} uptake.

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Fig. 6: Diagrammatic representation of mitochondrial Ca^{2+} cycle (Reed, 1994).
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The depletion of soluble and protein thiols and disruption of mitochondrial calcium homeostasis due to a variety of toxicants has been implied due to a decrease in the activity of calcium ATP-ase in plasma membranes secondary to oxidation of secondary thiols in the protein or by formation of covalent adducts (Reed, 1994).

**Mitochondria and Neurodegenerative Conditions**

One of the major ways in which mitochondria appear to participate in cellular pathology is through activation of MPT (Miller, 1998). As discussed earlier, when the channel opens transiently it can mediate normal signalling events.
However, opening of the MPT in the high conductance mode appears to be irreversible and has some profound consequences for cell function. For example, MPT activation of this type is associated with osmotic changes and mitochondrial swelling. The mitochondrial cristae straighten out and as the inner membrane is larger than the outer, this leads to rupturing of the outer membrane and release of a miasma of destructive molecules from the intermembrane space into the cytoplasm. Included in these are apoptosis inducing factor (AIF) and strangely, cytochrome C (Kroemer et al, 1997; Zamzami et al, 1997). The movement of both these molecules from the mitochondria into the cytoplasm appears to be an early event in the triggering of cell death in many instances.

The switching over of the reversible low conductance mode of MPT operation (associated with normal Ca\(^{2+}\) signaling) to the irreversible, high conductance state associated with cell death might be due to the propensity of the mitochondria to leak reactive oxygen species (ROS) (Bindokas et al, 1996). As discussed earlier, mitochondrial electron transport chain constantly leaks superoxide and hydroxyl free radicals, primarily from the ubiquinone site (Johnson et al, 1986). Among other things these ROS are powerful activators of the MPT (Chen et al, 1997). Thus, the MPT seems to play a central role in neuropathology and there is also a likelihood that drugs that inhibit it might be neuroprotective. There are several important clues provided by pharmacological studies of the MPT regarding its structure. It is thought that it might be a multiprotein complex that is located at contact sites where the inner and outer mitochondrial membranes come into close juxtaposition to one another (Zamzami et al, 1997). In particular, it is thought that the inner membrane adenine nucleotide translocator might be part of the complex. Drugs that bind to the translocator are also powerful regulators. In addition other molecules including the mitochondrial peripheral benzodiazepine binding protein and mitochondrial cyclophilin might also participate in the MPT complex.
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Mitochondrial dysfunction defects in energy metabolism and excitotoxicity might be involved in neurodegeneration in Alzheimer's disease. Effective buffering of cytosolic calcium is critical to neuronal survival because elevated cytosolic calcium would stimulate glutamate release, resulting in activation of N-methyl-D-Aspartate (NMDA) receptors, which in turn would result in massive Ca^{2+} influx and cell damage and death (Luo et al, 1997). A reduction of Ca^{2+} storage in mitochondria has been detected in old rats (Martinez-Serrano et al, 1992).

Mitochondria as Test Systems in Toxicology

The effects of many toxic agents on cells are mediated via damage to one or more of the subcellular compartments. Specific organelles may become damaged by toxic agents, when they perform a primary role in the metabolism of a particular toxicant, when a toxicant is stored intracellularly, or as a result of an inherent sensitivity of some essential biochemical pathways in the organelle to perturbation (Fowler et al, 1994). In terms of understanding the mechanisms of cellular toxicity, it is clear that evaluation of organelles as basic units of subcellular function may provide useful insights into the basis of toxicant action.

Mitochondria are essential organelles that play an important role in cell metabolism by mediating a number of metabolic functions (Fig. 7). Enzymes involved in energy production, carbohydrate metabolism, heme biosynthesis and the urea cycle are localized within specific subcompartments such as the outer and inner membranes and matrix.

In terms of the effects of toxicants on this organelle, it is important to understand the relationship between particular metabolic functions and the physical integrity of the mitochondrion as a structure, because frequently in vivo biochemical perturbations result directly from structural damage.
Mitochondrial protein synthesis studies are essential for determining whether changes in the specific activities of mitochondrial marker enzymes following *in vivo* chemical exposure are the result of a direct chemical enzyme interaction, a change in the synthesis of that enzyme, or both. Fowler *et al* (1994) found these studies of value for interpreting the results of morphological as well as other biochemical studies such as specific activities of the mitochondrial marker enzymes, monoamine oxidase, cytochrome oxidase and Mg\(^{2+}\) ATPase (Fowler and Woods, 1977; Fowler *et al*, 1979).

The technique of following mitochondrial swelling and contraction is based on the increased optical density of mitochondria in a contracted state and decreased density in a swollen or orthodox configuration due to cation influx. This gives useful functional tests of mitochondrial membrane integrity following *in vivo* or *in vitro* exposure to a chemical agent (Matlib and Srere, 1976; Fowler *et al*, 1979; Mehrotra *et al*, 1993).
Free Radical Mechanisms of Neurotoxicity

One of the most exciting areas of contemporary research is the involvement of free radicals in neurodegenerative diseases (Fig. 8). Many literature reports and previous detailed studies from this laboratory (Awasthi et al., 1989; Kakkar et al., 1991, 1992; Viswanathan et al., 1995, 1996) indicate the involvement of oxidative stress in toxicity as well as several neurological conditions. The emphasis in this dissertation is on the response of mitochondria in such conditions. Although the brain is an extremely active organ-consuming >20% of total oxygen intake - it is probably the least protected from ROS. It has a high concentration of PUFAs, a large iron store, low metal binding capacity, low antioxidant capacity and is incapable of neuronal regeneration (Acworth and Bailey, 1995). Oxidative stress has been found to lead to an increase in the levels of protein carbonyls in brain (Oliver et al., 1990) and protein oxidation is particularly relevant to neurological dysfunction because nerve cells do not divide and proteins play critical roles in their function. Thus, protein oxidation and loss of activity could contribute to the degeneration of nervous tissue (Stadtman and Berlett, 1997). Whether protein oxidation is responsible for neurological death or not, it is correlated to the process, so it constitutes an excellent biomarker of oxidative stress in cells (Marnett, 1997). As one of the hallmarks of Alzheimer’s disease is the presence of senile plaques containing aggregates of β-amyloid protein which leads to oxygen radical generation playing a causative role in neurological toxicity (Sayre et al., 1993; Smith et al., 1995). However, Hensley et al. (1994) suggest that a range of radicals may mediate neurological pathology. Nitric oxide and its derivative, peroxynitrite have been shown recently (Eiserich et al., 1998) to play a role in stroke and inflammatory disorders.

Due to the fact that brain contains a large amount of unsaturated fatty acids and catecholamines which are the target molecules of free-radical induced peroxidation, such oxidation plays an important role in the pathogenesis of cerebral
diseases such as Parkinson's disease (Duthie et al, 1994) dementia, brain ischemia, trauma, epilepsy and schizophrenia etc (Mori, 1993; Simonian and Coyle, 1996).

Fig. 8: Intracellular pathways that contribute to oxidative stress in neurons (Simonian and Coyle, 1996).

[Abbreviations: PLA₂ - phospholipase A₂; PLs - phospholipids; AA- arachidonic acid; CaMK - calcium and calmodulin-dependent protein kinase; NOS - nitric oxide synthase; Arg- arginine; NO⁻ - nitric oxide].

Oxidative stress might represent a mechanism of neurotoxicity of a number of environmental neurotoxicants (Pinsky and Bose, 1988) which might be relevant in the etiology of certain neurodegenerative diseases. The finding that most neuronal
cell bodies do not contain appreciable amounts of GSH indicates that other mechanisms must exist in neurons for preventing oxidative damage to their cytoplasmic constituents (Philbert et al., 1991).

A "final common pathway" to characterize and unify the cellular responses to xenobiotic neurotoxic agents has been proposed to involve enhanced free radical mediated oxidative stress a membrane dysfunction and elevation in cytosolic calcium (Kakkar et al., 1995). Free radical generation by cytochrome P450, the xenobiotic drug metabolising enzyme system, may also contribute to oxidative stress within the brain (Cheeseman and Slater, 1993).

The limited advancement in the knowledge of the biochemical changes occurring in humans exposed to environmental and occupational toxicants is due to the complexity of the nervous system and its distinctive peculiarities together with problems associated with the determination of the precise target for neurotoxic action (Anger, 1990).

**Cellular Antioxidant Defences**

In addition to the continuous production of free radicals, our body possesses several defence systems that are constituted by enzymes and radical scavengers (Sies, 1991; Fulbert and Cals, 1992). These latter ones are easily oxidisable compounds, which are present either in the cytosol (e.g. ascorbic acid and glutathione) or in membranes (\(\alpha\)-tocopherol). Table 1 sums up these defence systems and their protection mechanisms.

The relative importance of these as protective agents depends on which ROS is generated, how it is generated and what target of damage is measured (Halliwell and Gutteridge, 1995). These defence systems, called "first line defence systems" are not totally efficient, since almost all cell components undergo free radical damages (Delattre and Bonnefont-Rousselot, 1998). Therefore, "second line defence systems" are involved. They are constituted of repair systems for biomolecules that have been damaged by radical attacks (Davies, 1991).
Table 1
Free radicals and defence systems.

<table>
<thead>
<tr>
<th>Free radicals or prooxidants</th>
<th>Defence systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_2^-$ (superoxide anion)</td>
<td>Superoxide dismutases (SOD)</td>
</tr>
<tr>
<td></td>
<td>Mn-SOD</td>
</tr>
<tr>
<td></td>
<td>Cu/Zn-SOD</td>
</tr>
<tr>
<td>$\cdot$OH (hydroxyl radical)</td>
<td>Vitamin C, Glutathione</td>
</tr>
<tr>
<td></td>
<td>Taurin, Uric acid</td>
</tr>
<tr>
<td>ROO$^-$ (peroxy radical)</td>
<td>Tocopherols</td>
</tr>
<tr>
<td></td>
<td>Ubiquinone</td>
</tr>
<tr>
<td>$^1$O$_2$ (singlet oxygen)</td>
<td>Carotenoids</td>
</tr>
<tr>
<td>$H_2O_2$ (hydrogen peroxide)</td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>Se glutathione peroxidase (GPx)</td>
</tr>
<tr>
<td></td>
<td>Glutathione-reductase (GR)</td>
</tr>
<tr>
<td>ROO (hydroperoxides)</td>
<td>Se glutathione peroxidase (GPx)</td>
</tr>
<tr>
<td></td>
<td>Glutathione-reductase (GR)</td>
</tr>
<tr>
<td>Transition metals (Fe$^{2+}$,Cu$^{2+}$)</td>
<td>Chelators</td>
</tr>
</tbody>
</table>

Vroegop et al (1995) showed in a cell culture system that up to a certain limit, the cells are able to control the damage with glutathione, catalase, SOD, or other antioxidant mechanisms. However, once a threshold of damage or rate of damage is exceeded, the cellular defences are overwhelmed and even a slight additional insult results in severe cellular injury. The results led to two important and related conclusions: (a) there is a specificity to the location of damage produced in cells by particular ROS. (b) it is unlikely that a single protective agent will be able to protect from all the toxic species that may be generated near cells. Thus, it is important that a number of antioxidant defence systems be present in the cell. Therefore, all cells have a battery of antioxidant enzymes which are necessary for the survival of the cell even in normal conditions and they act in a cooperative or synergistic way to ensure a global cell protection (Michiels et al, 1994).

Halliwell and Gutteridge (1995) define an antioxidant as “any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate”. This
definition emphasizes the importance of the source of stress and the target (oxidisable substrate) measured.

Variations in the levels of antioxidants leads to differential tissue responses to oxidative stress. It has been reported that the rat brain possesses a relatively weak antioxidant defence system (Benzi et al, 1989). In spite of its high $O_2$ consumption, the brain curiously contains less active SOD, catalase and peroxidases than other enzymes (Choi and Yu, 1995). Lopez-Torres et al (1993) showed that catalase depletion led to very strong tissue-specific and time-dependent reactive increases of SOD, GR, GSH, and ascorbate. These inductions can explain the very good tolerance to catalase depletion.

The induction of SOD, GR, GSH, and ascorbate can help to control $OH^-$ indirectly (due to $O_2^-$ scavenging by SOD or reduction of GSH back to GSSG by GR) or directly (GSH and ascorbate) even in the presence of high $H_2O_2$ content.

Antioxidant systems are known markers of aging and according to the free radical theory of aging, it would be logical that the activity of the antioxidant enzymes would be altered in aging cells (Sohal, 1993). Aejermaeleus et al (1997) in their study highlight the presence of unidentified antioxidants that are involved in the total peroxyl radical scavenging antioxidant capacity (TRAP) of human plasma. Induction of endogenous antioxidants has been observed at the gene level in bacteria (Storz et al, 1990), Neurospora (Munkres, 1990), and even in human cells (Rushmore et al, 1991) in response to $H_2O_2$ or $O_2^-$. 

Superoxide dismutase [SOD EC.1.15.1.1] keeps turning up as a possible critical factor in regulating the rate of aging and/or disease incidence (Warner, 1994). SOD activity was discovered by McCord and Fridovich in 1969.

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Three distinct isoenzymes have been described with the same kinetic properties: one containing iron in its active site found in prokaryotes, one with
manganese (Mn-SOD) in prokaryotes and eukaryotic mitochondria, and one with copper and zinc (Cu/Zn-SOD) in the cytoplasm of eukaryotic cells (Fridovich, 1985). This enzyme seems to be the first line of defence against oxygen derived free radicals and can be rapidly induced in some conditions when cells or organisms are exposed to an oxidative stress (Coursin et al, 1985). SOD is known to scavenge \( \text{O}_2^- \) \textit{in vitro}; however, the precise \textit{in vivo} role of this enzyme other than scavenging of \( \text{O}_2^- \) is unclear and has been the subject of much debate (Culotta et al, 1993).

Role of Cu/Zn-SOD has been studied in a number of pathological conditions and mutations in Cu/Zn-SOD gene has been shown to be associated with familial amyotrophic lateral sclerosis, a chronic neurodegenerative condition (Rosen et al, 1993).

Mn and Fe-SODs possess the same enzymatic activity as the Cu/Zn-SOD, yet they are clearly different being larger in size (40,000 MW) and lacking the unusual stability of the Cu/Zn-SODs. All Mn and Fe dismutases show a high degree of homology but the copper-zinc enzyme appears to be evolutionarily unrelated (Harris and Steinman, 1977). Both the Mn and Fe-SODs are present in the matrix space of mitochondria (Britton and Fridovich, 1977).

Binding of Mn-SOD to DNA demonstrated by Steinman and Weinstein (1993) has important implications that SODs may have biological "activities" other than superoxide dismutation. They also suggest that localization near targets of oxyradical stress may enhance the effectiveness of SODs in preventing oxyradical damage to those targets. Mn-SOD has been reported to be more effective than Fe-SOD in preventing oxyradical damage to DNA \textit{in vivo} (Hopkin et al, 1992). Mn-SOD but not Cu/Zn-SOD has been found to be susceptible to modification by methyl mercury (MMC) in mouse brain and the resulting alteration in structure appears to cause a loss of enzyme activities (Shinyashiki et al, 1996). The catalytic properties of
human Mn-SOD are qualitatively similar to those reported for Mn-SOD of Thermus thermophilus (Bull et al, 1991). MMC poisoning has been reported to contribute to the induction of oxidative stress in mouse brain following suppression of antioxidant activity, including that of SOD and glutathione, together with disturbances in the mitochondrial electron transfer chain (Yee and Choi, 1994).

Multiple missense mutations in the cytosolic Cu/Zn-SOD (SOD-1) have been identified in a subset of families with familial amyotrophic lateral sclerosis (FALS) (Rosen et al, 1993). Reduced SOD activity has been reported in red blood cells, lymphoblastoid cell lines and brain tissue from many individuals with these mutations (Robberecht et al, 1994). However, several lines of evidence now suggest that motor neuron degeneration in at least some of the FALS patients with SOD-1 mutations results from a gain of function rather than from a loss of SOD-1 activity alone (Simonian and Coyle, 1996; Brown, 1995). During action of oxidants on SOD, its structure is strongly changed and new molecular forms appear which possess the catalytic activity. -SH groups of SOD are easily oxidized during action of oxidants but its oxidation does not accompany the loss of functional activity. Thus, in contrast to oxidation of NH-groups, oxidation of -SH groups have the positive effect because -SH groups actively react with oxidants and play the role of “false” target for oxidants (Sharonov and Churilova, 1993). Human recombinant Mn-SOD was shown to be superior to Cu/Zn-SOD in efficacy in preclinical models. This property can be attributed to its longer biological half-life (Nimrod et al, 1994).

SOD activity appears as the best adapted cellular parameter for early detection of free radical toxicity as observed in Hep 3B cells exposed to lindane, a known inducer of free radical production (Descampiaux et al, 1996). In a variety of mammalian cells and tissues, Mn-SOD is a highly inducible enzyme responding to a variety of stimuli, including \( \text{H}_2\text{O}_2 \) in some cell types. In contrast, Cu/Zn-SOD
appears to be constitutively expressed at higher levels and is considerably less inducible than Mn SOD in mammals (Visner et al, 1990). White and Nguyen (1993) demonstrated that the expression of Mn-SOD in vivo was not affected by overexpression of the Cu/Zn-SOD and, therefore, the two enzymes are probably regulated independently. Diminution or elimination of SOD will increase sensitivity of cells to oxygen and to compounds which can divert univalent e− flow to oxygen; however, elevation of SOD above wild type levels should therefore, not be expected to provide complete protection against overproduction of O2− (Fridovich, 1993).

SOD also exerts part of its antioxidant protection by inhibiting the formation of the cytotoxic ONOO−. The dismutation product of O2−, H2O2, is less reactive than ONOO− and can be metabolized by specific enzymatic systems.

**Catalase [CAT; EC 1.11.1.6]** is one of the oldest known antioxidant enzyme. It catalyzes the reaction:

\[
2\text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

Most aerobic cells contain this enzyme. In animals, CAT is present in all major body organs. The brain, heart and skeletal muscles contain only low amounts of catalase, although the activity does vary between muscles and even among different regions of the same muscle (Marklund et al., 1982). It is especially concentrated in liver and erythrocytes. At the subcellular level, CAT is found mostly in peroxisomes (80%) and cytosol (20%) (Zaman and Pardini, 1996).

**Glutathione peroxidase [GPx; EC 1.11.1.9]** decomposes H2O2 to H2O by the following reaction,

\[
\text{H}_2\text{O}_2 + \text{RH} \longrightarrow 2\text{H}_2\text{O} + \text{R}
\]

at the expense of reducing equivalents from GSH. GPx is located in both the cytosolic and mitochondrial compartments. Once GSH is oxidized to GSSG, it is reduced back by the action of NADPH-dependent glutathione reductase. Sixty to seventy five percent of this enzyme activity is found in the cytoplasm of eukaryotic cells and 25-40% in the mitochondria (Zakowski et al., 1978). Intracellular
detoxification of H$_2$O$_2$ by the GSH-GPx cycle plays a critical role in resistance against oxidative stress produced by xenobiotics (Doroshow, 1995). GPx needs reduced glutathione (GSH) to detoxify peroxides. A high concentration of reduced GSH (0.5-10x10$^{-3}$M) is maintained within the cell (Kosower and Kosower, 1983).

**Glutathione reductase** [GR; EC.1.6.4.2] reduces oxidized GSSG back to GSH by the reaction shown in Fig. 9.

\[ \text{Glutathione peroxidase} \quad \text{ROOH} \xrightarrow{\text{ROH} + \text{H}_2\text{O}} \]

\[ \text{Glutathione reductase} \quad \text{GSH} \xrightarrow{\text{GSSG}} \]

\[ \text{NADP}^+ \xrightarrow{\text{NADPH}} \]

\[ \text{Dehydrogenase} \quad \text{G6P} \xrightarrow{\text{6-phospho-gluconic acid}} \]

*Fig. 9: Glutathione reductase (GR) sequential attack on toxic oxygen products* (Zaman and Pardini, 1996).

GR utilizes NADPH to reduce GSSG (Deshpande *et al*, 1996). NADPH is regenerated from the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH) in the hexose monophosphate shunt (Halliwell and Gutteridge, 1989). The Se-independent form of GPx is glutathione-S-transferase (GST), which has relatively low activity towards lipid hydroperoxides and none at all towards H$_2$O$_2$. Therefore, GST, GR and G6PDH are secondary antioxidant enzymes that help in the detoxification of active oxygen species by decreasing lipid peroxides or by maintaining the activity of the primary antioxidant enzymes (Sugiyama, 1994).
Glucose-6-Phosphate dehydrogenase \([G6PDH; \text{EC.1.1.1.49}]\) is an important enzyme in the intracellular synthesis of NADPH. Thus, a decreased ability to generate NADPH gives rise to low RBC NADPH level (Tsai et al, 1996). G6PDH deficient RBCs are more susceptible to radical induced cell damage than normal RBCs.

Non-enzymatic Antioxidants

Small molecular antioxidants include non-protein -SH (NP-SH) such as GSH, N-acetyl cysteine and thiols, vitamins C, A and E, metallothionein and other metal chelating proteins (Hochstein and Atallah, 1988). While the major source of antioxidant capacity within cells lies with GSH and ascorbate, these water-soluble compounds cannot directly act to protect membranes from oxidative stress. The presence of a sufficient amount of lipid soluble vitamins such as \(\alpha\)-tocopherol and \(\beta\)-carotene is essential. Thus, the dietary intake of lipid soluble vitamins is a critical element in the maintenance of antioxidant defence processes (LeBel and Bondy, 1991).

Glutathione (GSH) a tripeptide composed of L-glutamate, L-cysteine and glycine is considered to be the most prevalent and important intracellular non-protein thiol/sulfhydryl compound in mammalian cells (Wu et al, 1994). The nucleophile GSH conjugates the electrophilic xenobiotics (R) in the following type of reaction:
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\[ R + GSH \rightarrow R-S-G + H^+ \]
catalysed by glutathione-S-transferases which are present in high amounts in liver and cytosol and in lower amounts in other tissues. If the potentially toxic xenobiotics were not conjugated to GSH, they would be free to combine covalently with DNA, RNA or cell protein and could thus lead to serious cell damage. GSH is therefore, an important defence mechanism against certain drugs and carcinogens (Murray, 1996); it occurs at higher levels in astrocytes than in neurons (Lowndes et al, 1994; Thorburne and Juurlinke, 1996).

A crucial role for GSH is as free radical scavenger, particularly effective against the OH radical. There are no known enzymatic defences against this species of radical. The ability of GSH to non-enzymatically scavenge both singlet oxygen and OH (Coyle and Puttfarcken, 1993) provides a first line of antioxidant defence. The ratio of GSSG/GSH serves as a sensitive index of oxidative stress (Toborek and Hennig, 1994). Fig. 10 shows relationship of reduced GSH to oxidized (GSSG) glutathione (Bains and Shaw, 1997).

![Oxidation-reduction pathway](image)

**Fig. 10:** Oxidation-reduction pathway: relationship of reduced GSH to oxidized (GSSG) glutathione.

Thiol oxidation has been related to disturbances of Ca\(^{2+}\) homeostasis by several hepatotoxins eg., acetaminophen (Moore et al 1985) etc. The underlying mechanisms were supposed to be inactivation of Ca\(^{2+}\)-translocating ATPases as well as inhibition of mitochondrial Ca\(^{2+}\) sequestration (Kass et al, 1992), either by
modification of specific protein thiols or by an imbalance of the mitochondrial glutathione redox system. Nahagawa et al. (1992) showed that protein thiol oxidation starts early in cellular injury.

In addition to such antioxidative actions, GSH is involved in a number of other essential tasks including DNA synthesis and repair, protein synthesis, amino acid transport, enhancement of immune function, and enzyme activation (Lomaestro and Malone, 1995). Roles specific to the nervous system appear to include actions as a redox modulator of some inotropic receptor currents (Ogita et al., 1995) and as a potential neurotransmitter (Pasqualotto et al., 1997).

Due to such multiple roles in normal tissues, there is a considerable potential for alterations in GSH to be causally associated with disease and aging (Bains and Shaw, 1997). The traditional role of GSH is that of free radical scavenger. Oxidative stress arising from free radical formation can affect the ratio of GSH/GSSG of the cell as GSH is depleted to combat such radicals. A strong evidence of GSH depletion causing nerve cell death comes from cell culture studies by Li et al. (1997). They showed that a decrease in GSH triggers the activation of neuronal 12-lipoxygenase (12-LOx) which leads to the production of peroxides, the influx of Ca²⁺ and ultimately cell death. Peuchen et al. (1997) concluded that astrocytes in the CNS counteract oxidative stress due to their higher content of GSH as compared to neurons and alterations in GSH status may be deleterious to normal neuronal function. Fig. 11 shows a proposed GSH depletion model for neurodegenerative disorders (Bains and Shaw, 1997).

Approximately 90% of total cellular glutathione is localized in the cytosolic fraction, the rest being compartmentalized within mitochondria (Reed, 1990). The mitochondrial pool of GSH is likely to be involved in maintaining intra mitochondrial protein thiols in a reduced state which are essential for a number of functions, a key one being the regulation of selective membrane permeability to Ca²⁺. A clear example of the relationship between glutathione status, oxidative
Fig. 11: Proposed GSH-depletion model for neurodegenerative disorders (Bains and Shaw, 1997).

stress, mitochondrial damage and neuronal dysfunction/death due to excess free Ca\(^{2+}\) is shown by the effects of excessive production of H\(_2\)O\(_2\) within mitochondria leading to depletion of mitochondrial GSH, in turn causing the oxidation of protein thiols and impairment of mitochondrial function. This relationship may have relevant implications in terms of the degeneration of dopaminergic neurons. Monoamine oxidase acts on monoamines including dopamine producing H\(_2\)O\(_2\) within mitochondria which may lead to a decrease in mitochondrial GSH (Sandri et al, 1990). Glutathione has a neuroprotective role in NO mediated mitochondrial damage (Bolanos et al, 1996).

Thus, it is clear that the loss of GSH may cause mitochondrial damage (Heales et al, 1995) and it is likely that the converse situation is also true. As
GSH synthesis requires ATP, a deficiency of energy supplied by mitochondria is likely to affect the cellular turnover of GSH (Mithofer et al., 1992). These reports lend further support to the hypothesis that the oxidative stress and neuronal damage observed in the substantia nigra of patients with Parkinson's disease could be caused by a mitochondrial defect in GSH (Bains and Shaw, 1997). Changes in GSH status due to decreased synthesis or increased degradation could lead to a failure to combat free radical formation. Similarly, increased GSH degradation could increase the concentration of free glutamate (Coyle and Puttfarcken, 1993). Further, in human erythrocytes, ascorbate regeneration from dehydroascorbate is largely GSH-dependent, and occurs through either enzymatic or non-enzymatic reactions not involving the monoascorbyl free radical (May et al., 1996).

β-carotene has been identified as a sacrificial inhibitor of the propagation stage of lipid peroxidation (LPO) as it is a quencher of singlet oxygen. Thus, it prevents formation of hydroperoxide and interferes with LPO, but at higher oxygen pressures, β-carotene may become prooxidant (Burton and Ingold, 1984). A number of studies have shown the beneficial effects of β-carotene and other carotenoids in disease conditions. Lutein and zeaxanthine have been shown to be critical in age related macular degeneration (Mares-Perlman et al., 1995). Serum levels of total carotenoids, α-carotene and β-carotene have been shown to be lower in some cancer patients than in controls (Batieha et al., 1993; Helzlsouer et al., 1996; Negri et al., 1996). In a recent study, diet rich in β-carotene was shown to be well absorbed in blood and skin and gave protection against sunburn by UV rays (Stahl et al., 1998). β-carotene also protects against LDL oxidation at high doses (Lin et al., 1998).

Vitamin C is hydrophilic in nature and is primarily found in the cytosol, acts directly as an antioxidant and is an essential entity for the proper functioning of the various cellular defence mechanisms. It also appears to be involved in the bio-
transformation of xenobiotics and influences the activity of several oxidizing and hydroxylating enzymes (Davies et al., 1991). Its antioxidant reactions use its ability to donate a single electron to the free-radical species. The products of such reactions are the quenched reactive species and the less reactive ascorbyl free radical, which can be either reduced back to ascorbic acid or oxidized to form dehydroascorbic acid (Bendich et al., 1986).

\[
\begin{align*}
(a) & \quad \text{ascorbate} + \text{Fe}^{2+}\text{-complex} \rightarrow \text{Fe}^{2+}\text{-complex} + \text{semidehydro ascorbate} \\
(b) & \quad 2\text{-semidehydroascorbate} \rightarrow \text{ascorbate} + \text{dehydroascorbate} \\
(c) & \quad \text{dehydroascorbate} + 2\text{GSH} \rightarrow \text{GSSG} + \text{ascorbate}
\end{align*}
\]

\[\text{dehydroascorbate reductase} \rightarrow \text{complex reaction} \rightarrow \text{oxalic acid} + \text{L-Threonic acid}\]

Astrocytes take up vitamin C (dehydroascorbic acid) through plasma membrane transporters, reduce it to ascorbate and then release ascorbate to the extracellular fluid, where it may contribute to antioxidant defence of neurons (Wilson, 1997).

Ascorbate reacts rapidly with both superoxide and peroxyl radicals and even more rapidly with hydroxyl radicals (Halliwell and Gutteridge, 1989). It also scavenges singlet oxygen and reduces thyl radicals and protects tissues against LPO (Chakraborty et al., 1994). It plays a vital role in maintaining balance between oxidative products and the various cellular antioxidant defence mechanisms. It is required as a cofactor for some enzymes such as proline hydroxylase, dopamine β-hydroxylase etc. At low concentration it can act as a prooxidant in the presence of \(\text{H}_2\text{O}_2\). Human eye lens has low SOD activity but high content of ascorbic acid. Ascorbic acid also effectively protects low-density lipoproteins (LDL) against \(\text{Cu}^{2+}\)-induced oxidation. It can regenerate α-tocopherol from its oxidized form at
the water-lipid interface \textit{in vitro}. However, whether such a mechanism is operative \textit{in vivo} remains controversial (Mathiesen \textit{et al}, 1996).

\textbf{\textalpha;-tocopherol} (Vitamin E) is the principal component of the defence mechanism against free-radical mediated cellular injuries. In fact, it is the only natural physiological lipid-soluble antioxidant that can inhibit lipid peroxidation in cell membranes (Kappus, 1991). Evidence includes such \textit{in vitro} observations as its direct reactions with and quenching of superoxide and peroxyl radical and singlet oxygen:

\begin{align*}
\text{Vit.}E-\text{OH} + \text{ROO}^- & \rightarrow \text{Vit.} E-\text{O}^- + \text{ROOH} \quad (\text{H}^+ \text{ transfer}) \\
\text{Vit.}E-\text{OH} + \text{ROO}^- & \rightarrow \text{Vit.} E-\text{OH}^+ + \text{ROO}^-(e^-\text{transfer}) \\
\text{Vit.}E-\text{OH}^+ + \text{H}_2\text{O} & \rightarrow \text{Vit.} E-\text{O}^- + \text{H}_2\text{O}^+ \quad (\text{deprotonation})
\end{align*}

It is now widely recognized that \textalpha;-tocopherol is located primarily in the membrane portion of the cell and is a part of the cells defence against oxygen centered radicals. \textalpha;-tocopherol can function as a molecular "channel" via which the free radicals can leave the hydrocarbon zone of the membrane. Almost all enzymes that are affected by vitamin E status either are membrane bound or are concerned with the GSH-Px system (Catigani, 1980). Vitamin E, therefore, is unique in its more specific localization in membranes and the tenacity with which it remains in most tissues. High doses of \textalpha;-tocopherol have been tried as a therapeutic supplement in many neurodegenerative disorders (Jama \textit{et al}, 1996; Ebadi \textit{et al}, 1996; Drachman and Leber, 1997; Dorevitch \textit{et al}, 1997). As well as providing protection against risk to heart disease (Li \textit{et al}, 1998). Dietary vitamin E has been shown to increase the global antioxidant capacity of the heart and improve heart redox status (Rojas \textit{et al}, 1996). Topical application of vitamin E has been shown to bolster the skin's antioxidant status and protect against UV induced damage (Lopez-Torres \textit{et al}, 1998).

All these non-enzymatic lipophilic antioxidants act synergistically with
glutathione (GSH).

**Prooxidative Processes and Ca^{2+} Deregulation in Cell Death**

All cells contain elaborate systems for the spatial and temporal regulation of calcium ions, diverse calcium receptors and biochemical response systems that are regulated by these changes in intracellular calcium. Toxicants that perturb the mobilization or homeostasis of Ca^{2+} will place the regulation of these processes outside the normal range of physiological control. It has been documented by several investigators that local anaesthetics displace calcium from calcium binding sites and alter the functioning of different calcium regulating systems. Grant and Acosta Jr (1994) report that local anaesthetics have adverse effects on mitochondrial function and interact with cytoskeletal elements by elevating [Ca^{2+}]_{i} (intracellular calcium ion concentration) before cytotoxicity occurs and disruptions in calcium homeostasis may contribute to their toxicity. A disturbance of calcium homeostasis is thought to play a role in the cytotoxicity of a number of compounds (Nicotera et al, 1992a).

A series of reports in the recent past have brought in the concept of a relation between oxidative membrane damage and Ca^{2+} function (Fig. 12) (Kakkar et al, 1995). Masaki et al (1989) suggest that mitochondrial damage is the biochemical basis of the non-peroxidative mechanisms of oxidative cell injury. Endoplasmic reticulum Ca^{2+} release/depletion during cell injury may trigger a signalling cascade that causes extracellular Ca^{2+} influx followed by Cl⁻ influx, cell swelling and ultimately cell death/lysis (Waters et al, 1997). A disturbance in mitochondrial Ca^{2+} handling is essential for the generation of oxidative stress induced by Ca^{2+} deprivation (Reed, 1994). Data from several laboratories indicate that disruption in the homeostasis of Ca^{2+} and/cell thiols plays a major role in the pathogenesis of cell injury associated with oxidative stress caused by several toxic agents or pathologic states such as ischemia/reperfusion or xenobiotic poisoning (Castilho et
al, 1995; Reed, 1990). A possible participation of ROS and protein thiols has been suggested in the mechanism of mitochondrial inner membrane permeabilization by calcium (Valle et al, 1993).

In this regard, evidence has accumulated that the homeostasis of Ca$^{2+}$ and of thiols in mitochondria are closely linked either directly or through the pyridine nucleotides; an imbalance in one could affect the status of the other and diminish cell viability (Zweifach and Lewis, 1995).

The role of Ca$^{2+}$ as an intracellular regulator of many physiological processes is well established. Excitable tissues show extensive depolarization mediated calcium entry in the cell. Neurotransmitter and neuroendocrine release processes depend upon this rapid flux of Ca$^{2+}$ (Bondy, 1989). Calcium acts as a ubiquitous powerful trigger of conformational change in many proteins to initiate functions as diverse as muscle contraction, secretion and neurotransmission (Clapham, 1995). In excitable cells like neurons, voltage activated calcium selective channels located

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**Fig. 12**: Oxidative stress mediated calcium deregulation in toxicity (Kakkar et al, 1995).
in the plasma membrane provide an additional, faster route for raising intracellular calcium (Putney, 1990). Under steady state conditions, the cytosolic free calcium concentration is generally maintained at approximately $10^{-7}$ M (Nicotera et al., 1992). Similar to other neuronal types, it can be assumed that several mechanisms are responsible for maintenance of low internal $[\text{Ca}^{2+}]$ and for its restoration after transient elevation i.e., cytoplasmic $\text{Ca}^{2+}$-binding proteins (Zhou and Neher, 1993), $\text{Na}^+/\text{Ca}^{2+}$ exchange (Rasmussen et al., 1990), $\text{Ca}^{2+}$ pump in the surface membrane (Benham et al., 1992), a $\text{Ca}^{2+}$ pump of internal $\text{Ca}^{2+}$ stores (Kostyuk et al., 1989) and mitochondrial sequestration (Thayer and Miller, 1990). All these mechanisms of $\text{Ca}^{2+}$ clearance were found in rat hippocampal neurons, but their capacity clearly differed. $\text{Ca}^{2+}$ pumps in the surface membrane and in internal stores play the dominating role in slow $\text{Ca}^{2+}$ buffering whereas $\text{Na}^+/\text{Ca}^{2+}$ exchange and mitochondria are of marginal importance (Mironov, 1995).

The original idea that calcium might enter cells through a capacitative mechanism was first introduced by Putney (1986). Calcium entry in this hypothesis, was regulated by the state of filling of the calcium stores which prevent entry when they are charged up but immediately begin to promote entry as soon as stored calcium is discharged. This capacitative entry mechanism is present in many cells and has properties which are very similar from one cell to the next (Berridge, 1995). Hoth and Penner (1992) coined the term calcium-release-activated current ($I_{\text{CRAC}}$) to refer to the current flowing through these capacitative calcium entry channels. Perhaps the single most important property of capacitative $\text{Ca}^{2+}$ entry is its sensitivity to $\text{Ca}^{2+}$ (Zweifach and Lewis, 1995). $I_{\text{CRAC}}$ is expressed in many different cell types. It is potentiated by extracellular calcium but inactivated by intracellular calcium (Zweifach and Lewis, 1993; Missiaen et al., 1994). $I_{\text{CRAC}}$ is not the only type of channel bringing $\text{Ca}^{2+}$ into cells that is triggered by store depletion: such channels are known generally as storeoperated channels (Clapham, 1995a). Sustained increase in intracellular $\text{Ca}^{2+}$ concentration can lead to the
activation of degradative enzymes like proteinases, phospholipases and endonucleases, ultimately leading to breakdown of macromolecules (Davies and Chipman, 1994). The peroxidation of arachidonic acid, the major hydrolysis product of \( \text{PLA}_2 \) which is activated in presence of increased cytosolic \( \text{[Ca}^{2+}\text{]} \) and/or its metabolite interferes with \( \text{GABA}_A \)-receptor function leading to a decrease in \( \text{Cl}^- \) uptake (Schwartz et al, 1992). Rather than a sustained increase in cytosolic \( \text{[Ca}^{2+}\text{]} \), it is the localized cycling of \( \text{Ca}^{2+} \) across the plasma membrane that is the critically important \( \text{Ca}^{2+} \) messenger during the sustained phase of cellular responses mediated via surface receptors linked to the hydrolysis of phosphatidyl inositol-4,5-diphosphate (\( \text{PIP}_2 \)) which hydrolyses to give inositol 1,4,5-triphosphate (\( \text{IP}_3 \)) and diacyl glycerol (DAG). \( \text{IP}_3 \) acts to release \( \text{Ca}^{2+} \) from an intracellular pool, thereby causing a transient rise in cytosolic \( \text{[Ca}^{2+}\text{]} \) (Rasmussen, 1990).

The major pathway for entry of \( \text{Ca}^{2+} \) into excitable cells such as muscle, neural and secretory cells is through \( \text{Ca}^{2+} \)-permeable channels which are activated by depolarization of the cell (Varadi et al, 1995). These voltage-operated \( \text{Ca}^{2+} \) channels (VOCC) trigger neurotransmitter and hormone release, muscle contraction, and many other cellular functions (McCabe Jr. and Pounds, 1997). Beyond manipulation of the calcium channels by pharmacologic agents and toxins, VOCCs are sensitive to many toxic metals (Busselberg, 1995). Table 2 gives an account of the \( \text{Ca}^{2+} \)-transporting systems of cell membranes.

### Table 2

<table>
<thead>
<tr>
<th>Transporting mode</th>
<th>Membrane</th>
<th>( \text{Ca}^{2+} ) affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPases</td>
<td>Plasma membrane</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Endo(sarco)plasmic reticulum</td>
<td></td>
</tr>
<tr>
<td>Exchangers [( \text{Na}^+/\text{Ca}^{2+}) ]</td>
<td>Plasma membrane</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Inner mitochondrial membrane</td>
<td></td>
</tr>
<tr>
<td>Channels</td>
<td>Plasma membrane</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>Endo(sarco)plasmic reticulum ?</td>
<td></td>
</tr>
<tr>
<td>Electrophoretic</td>
<td>Inner mitochondrial uniporters membrane</td>
<td>Low</td>
</tr>
</tbody>
</table>

Carafoli (1988).
In the recent past a number of reports indicate a critical role of intracellular Ca$^{2+}$ concentration in neurodegenerative disorders (Verhratsky and Toescu, 1997). Any involvement of Ca$^{2+}$ fluxes in the pharmacological action of neuroactive drugs is of interest.

**Concluding Remarks**

The foregoing information indicate that the response of mitochondria towards generation and scavenging of active oxygen species in different regions of brain and the ensuing modulation of calcium fluxes and functions could assume significance in understanding the biological effects of benzodiazepines. Therefore studies were conducted in this dissertation towards answering the above possibility and the results are presented in this thesis.