PART I: INVESTIGATION OF MECHANISM(S) OF Cu(II) AND Zn(II) NEUROTOXICITY

PART II: PRE-STEADY STATE KINETICS OF THE REDUCTION OF CYTOCHROME
b_{561} WITH ASCORBATE

A Dissertation by

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the requirements for the degree of
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PART I: INVESTIGATION OF MECHANISM(S) OF Cu(II) AND Zn(II) NEUROTOXICITY

PART II: PRE-STEADY STATE KINETICS OF THE REDUCTION OF CYTOCHROME $b_{561}$ WITH ASCORBATE

The following faculty members have examined the final copy of this dissertation for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy with a major in Chemistry.

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DEDICATION

To my loving Parents
ACKNOWLEDGMENTS

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ABSTRACT

The adverse effects of copper on the catecholaminergic nervous system have been well documented and primarily attributed to its redox related properties. To determine the significance of the redox properties of copper, a comprehensive study of the catecholaminergic toxicity of Cu(II) has been carried out using MN9D, PC12 and SH-SY5Y cells and compared the results with that of non-redox active Zn(II) and non-neuronal HepG2 cells. While Cu(II) is more toxic than Zn(II), membrane permeable metal chelators increase the toxicities of the both metals to catecholaminergic cells in comparison to HepG2 cells. The toxicities of the two metals are parallel to their uptakes and no protection from toxicity is observed with high concentrations of bathocuproine which stabilizes the redox active Cu(I). Thus, the redox properties of Cu(II) are not the primary cause of catecholaminergic toxicity. The increase of reactive oxygen species (ROS) and the apoptotic DNA fragmentation induced by both metals suggest that the apoptotic cell death is due to the excessive ROS production. Significance these findings with respect to intracellular calcium perturbation is discussed.

Part II of my research is focused on structural characterization of cyt b_{561}. The protein was isolated from bovine adrenal glands and pre-steady state kinetics of oxidized protein was carried out using stopped-flow absorption spectrophotometry. The reduction kinetics was complex and would be fitted into a linear combination of three exponential functions, providing further evidence for the presence of two heme b centers in the protein.
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<td>6-hydroxydopamine</td>
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<td>Aβ</td>
<td>Amyloid beta</td>
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<td>AD</td>
<td>Alzheimer’s Disease</td>
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<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
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<td>AMPA</td>
<td>(S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
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<td>Asc</td>
<td>Ascorbic Acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine -5'-Triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance Units</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BCP</td>
<td>Bathocuproine</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>Cyt b₅₆₁</td>
<td>Cytochrome b₅₆₁</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>DA Transporter</td>
</tr>
<tr>
<td>DβM</td>
<td>Dopamine β-Monooxygenase</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydroascorbate</td>
</tr>
</tbody>
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LIST OF ABBREVIATIONS (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent Metal Transporter 1</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3, 4- Dihydroxy Phenylacetic Acid</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3, 4- Dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>E</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction Coefficient</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron Paramagnetic Resonance</td>
</tr>
<tr>
<td>FALS</td>
<td>Familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutric Acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HPLC-EC</td>
<td>High Performance Liquid Chromatography – Electro Chemical</td>
</tr>
<tr>
<td>HS</td>
<td>Horse Serum</td>
</tr>
<tr>
<td>K_m</td>
<td>Michaelis Constant</td>
</tr>
<tr>
<td>KRB</td>
<td>Krebs-Ringer Buffer</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidase</td>
</tr>
<tr>
<td>MAO-B</td>
<td>Monoamine Oxidase B</td>
</tr>
<tr>
<td>mDβM</td>
<td>Membrane Bound Dopamine β-Monoxygenase</td>
</tr>
<tr>
<td>MD</td>
<td>Menkes Disease</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NCP</td>
<td>Neocuproine</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NET</td>
<td>NE Transporter</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>PAR</td>
<td>4-(2-pyridylazo)-resorcinol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PHM</td>
<td>Peptidyl glycine-α-Hydroxylating Monooxygenase</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein</td>
</tr>
<tr>
<td>PrPc</td>
<td>alpha-helix rich prion protein</td>
</tr>
<tr>
<td>PrPSc</td>
<td>beta-structure-rich insoluble conformer of prion protein</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>SDA</td>
<td>Semidehydroascorbate</td>
</tr>
<tr>
<td>sDJβM</td>
<td>Soluble Dopamine β-Monooxygenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper Zinc Superoxide Dismutase 1</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris-[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TRPM7</td>
<td>Transient Receptor Potential Melastatin 7</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>vis</td>
<td>Visible</td>
</tr>
<tr>
<td>WD</td>
<td>Wilson’s Disease</td>
</tr>
<tr>
<td>ZnT-1</td>
<td>Zinc Transporter 1</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Wavelength at which absorption is maximum</td>
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</table>
CHAPTER I
INTRODUCTION

1.1 Part I

The effects of copper on the functions of the central nervous system (CNS) are multiple, complex and not completely understood (1-3). Copper is required for normal synaptic transmission and high concentrations are continuously released from the nerve endings upon stimulation (4, 5). Copper is an essential cofactor for several important enzymes in the CNS, including dopamine-β-monooxygenase, peptidyl-α-amidating monooxygenase, Cu/Zn superoxide dismutase, cytochrome c oxidase, and lysine oxidase. On the other hand, the imbalance of copper homeostasis in the CNS is closely associated with the pathophysiology of neurodegenerative disorders including Parkinson’s, Alzheimer’s, Wilson’s, and Prion diseases, and amyotrophic lateral sclerosis (6). The age related accumulation of high levels of copper in the CNS has been attributed to the primary reason for its adverse effects (7). Although the vital proteins associated with neurodegenerative disorders including the prion protein (8), β-amyloid protein (9), α-synuclein (10), and S100B (11) possess a specific and high affinity for copper, neither the significance of the copper binding properties nor the physiological functions of these proteins are fully understood.

Copper is redox active and can cycle between Cu(II) state and Cu(I) state in the presence of intracellular reducing agents. Cu(II) reacts efficiently with O₂ to generate H₂O₂ and consequently related reactive oxygen species (ROS) by Fenton-type
reactions causing oxidative stress and cellular damage. The catecholaminergic nervous system is specifically more susceptible to copper induced oxidative stress. It was shown that catecholamines effectively interact with Cu(II) and reduce it to Cu(I) catalyzing the ROS production under physiological conditions (12, 13). Based on these and other evidence, high catecholaminergic toxicity of copper has been primarily attributed to its redox related properties. In addition, high levels of free copper interact with and alter the activity of many proteins under physiological conditions causing cellular malfunctions, since both Cu(I) and Cu(II) have high affinities towards cysteine and histidine residues of proteins. For example, early studies show that copper binds to intact mitochondria and inhibits the mitochondrial respiration by modifying the essential thiol residues (14). Similarly, Cu(II)-phenanthroline complex cross-links the subunits of gastric H⁺-K⁺-ATPase (15) and E. coli ATP synthase (16) through disulfide bond formation. The inhibition of Mg²⁺-ATPase (17) and Na⁺/K⁺-ATPase (18) by copper has also been reported. Recently, we have shown that low levels of free copper inhibits the vesicular H⁺-ATPase leading to the dissipation of intra-granular pH and catecholamine gradients (19). However, the relative contributions of these adverse effects of copper toward the catecholaminergic nervous system have not been fully understood.

We have carried out a comprehensive study of the neurotoxicity of Cu(II) using catecholaminergic cells MN9D, PC12 and SH-SY5Y and compared the results with that of non-redox active metal Zn(II) and non-neuronal HepG2 cells. These studies show that while free Cu(II) is more toxic to catecholaminergic cells than Zn(II), in the presence of membrane permeable metal chelators both metals are comparably and highly toxic to catecholaminergic cells in comparison to HepG2 cells. In addition, while the toxicities of
two metals are parallel to their uptakes, the characteristics of the DA depletion induced by two metals were similar with minor differences. In addition, no protection was observed with high concentrations of BCP which is known to stabilize the redox active Cu (I). These and other findings suggest that the redox properties of Cu(II) may not be the primary cause of its catecholaminergic toxicity. Both VGCC blocker nitrendipine and extracellular calcium partially inhibit the uptake and protect catecholaminergic cells from the toxicity of both metals. The increased ROS and the apoptotic DNA fragmentation induced by both metals suggest that the apoptotic cell death is due to the excessive ROS production. We propose that the accumulation of the metals above the intracellular buffering capacity causes the membrane depolarization and opening of VGCC and other cation channels. Thus, the increased oxidative stress caused by the perturbation of intracellular calcium levels and ion gradients could be the major cause of the catecholaminergic toxicity of these metals.

1.2 Part II

Ascorbic acid (Asc) acts as a cofactor for enzymes in a variety of secretory vesicles including dopamine β-monooxygenase (DβM) in synaptic and adrenal medullary chromaffin vesicles and peptidylglycine α-amidating monooxygenase (PAM) in pituitary neuropeptide secretory vesicles (20). During DβM and PAM turnover, Asc is oxidized to semidehydro-ascorbic acid (SDA). Since Asc or SDA cannot pass through the vesicle membrane, the regeneration of SDA is facilitated by a membrane span protein, cytochrome b_{561} (cyt b_{561}) (21).
Bovine adrenal chromaffin granule cyt $b_{561}$ has been purified, sequenced, and shown to be a single polypeptide of 27,700 Da (22). Several other isoforms of cyt $b_{561}$ with similar functions have been identified from other sources such as macrophage lysosomes (23) and plants (24). Cyt $b_{561}$ is a highly hydrophobic hemoprotein and the chromaffin granule isoform has six spans across the membrane (25). In contrast to the early proposal that cyt $b_{561}$ contains only one $b$ type heme (22, 26, 27), recent studies suggest that it has two heme centers (28-30) with distinct characteristics.

Kinetics of the electron transport process mediated by cyt $b_{561}$ have been studied in chromaffin granule ghosts (21). Recently, stopped-flow analysis on Asc reduction kinetics of the oxidized cyt $b_{561}$ purified from bovine chromaffin vesicle membranes (31) and the purified protein expressed in insect and yeast cells (32) have been reported.

The specific objectives of the research were to study the pre-steady state kinetics of purified bovine chromaffin granule cyt $b_{561}$ and to analyze the results with respect to electron transport and the proposed structural model of the protein.
CHAPTER II
BACKGROUND AND SIGNIFICANCE

2.1 Part I

2.1.1 Copper

Copper is essential for the metabolic needs of all cells in the human body (33) and is required for infant growth (34, 35) brain development (36), host defense mechanisms (37), iron transport (38) and bone strength (39). Copper is the third most abundant trace metal in the body next to iron and zinc. The brain contains high amounts of copper (ranges from 3.6 to 6 μg/g) (40) compared to other organs in the body and it is second only to the liver (41). Copper is present in all major cell components including cytosol, nuclei and mitochondria. However, it is not uniformly distributed in the brain. The highest concentrations of copper (and also zinc) were found in the hippocampus (up to 15 μg/g) (42).

Numerous evidence suggests the involvement of copper in neurotransmission. While free copper concentration in the brain is very small, glutamatergic synapses in the cortex and hippocampus contain higher concentrations of copper, and during neuron depolarization high amount of copper is reported to release into synaptic cleft (43). Free copper modulates ligand- and voltage-gated ion channels (44-46) and may regulate neuronal excitability.

Copper serves as a cofactor of a number of key enzymes, including cytochrome c oxidase, dopamine-β-monoxygenase, Cu/Zn superoxide dismutase, lysine oxidase
and peptidyl-α-amidating monooxygenase (47). In mitochondria, Cytochrome c oxidase catalyzes the electron transfer process of the respiratory chain (48). Dopamine-β-monooxygenase is important in catecholamine synthesis as it converts dopamine to norepinephrine, which is an important neurotransmitter. Cu/Zn superoxide dismutase protects cells from highly reactive superoxide radicals generated in the cell by converting them to hydrogen peroxide. Mutation of Cu/Zn superoxide dismutase (SOD1) is known to cause the neurodegenerative disorder, familial amyotrophic lateral sclerosis (FALS) (49). Copper containing ceruloplasmin and tyrosinase are important in iron transport (50) and pigmentation respectively (51). Lysyl oxidase (collagen and elastin cross-linking) and peptidylglycine-α-amidating monooxygenase (polypeptide amidation) (52) are another two important copper containing enzymes.

Dietary copper is absorbed in the stomach and through the small intestine and stored in the liver. Hepatic copper is mostly bound to specific metal-binding proteins, primarily metallothioneins, or incorporated into several cuproenzymes. Metallothioneins are cysteine rich proteins that bind to heavy metals and play a critical role in copper and zinc homeostasis (53). Copper distributed from the liver to the blood stream is taken up by cellular copper transporter 1 (CTR1) (54) (which is the major pathway of copper entry), CTR2 (55) and divalent metal transporter 1 (DMT1). Ceruloplasmin, albumin, and transcuprein (56) are major copper-transporting proteins in the plasma. In the cytosol copper is incorporated with copper chaperones and delivered to copper-ATPases (ATP7A/ATP7B), superoxide dismutases, cytochrome c oxidase and secretory vesicles.
Copper transport through the body is tightly regulated to maintain appropriate levels. Altered copper homeostasis results in a number of health problems including neurodegenerative disorders. Copper deficiency may cause anemia, neutropenia (57) and impaired bones (58). Menkes (MD) and Wilson’s disease (WD) are two genetic disorders associated with the alteration of copper transporters. MD is caused by the mutation of copper transporting ATPase (ATP7A), which is expressed in most tissues other than the liver (59). Impaired copper efflux from enterocytes into blood in MD patients results in inadequate copper transport to the brain. Delayed development, mental retardation, seizures and poor body temperature control are some of the symptoms of MD. Mutation of ATP7B, which is predominantly expressed in the liver, results in WD. In WD patients copper accumulates in the liver and the brain (60, 61) due to inefficient copper transport from the liver to bile resulting in inefficient excretion from the body. Both liver and central nervous system are affected by WD.

2.1.2 Zinc

Zinc is one of the most abundant trace metals in the body second to iron. Zinc can be found in many body tissues including liver, bone, skin, muscle and brain. There are more than 300 zinc containing metalloenzymes and zinc containing proteins which play catalytic, regulatory as well as structural roles (62). Zinc is not redox active unlike copper and it acts as a Lewis acid in enzyme catalytic sites. Some important zinc containing enzymes are carbonic anhydrase, carboxy peptidase, amino peptidase (catalyzes hydrolysis of peptides or proteins), alkaline phosphatase (increased level of serum indicates bone tumor, hepatitis and gall bladder disease), alcohol dehydrogenase and DNA and RNA polymerases. Zinc acts as a calcium antagonist and neuromodulator
in synaptic transmission. It is also crucial in insulin biosynthesis and exocytosis. In pancreatic β cells, a high concentration of zinc in the vesicles were reported to release into the extracellular medium with insulin during exocytosis and controlled insulin secretion (63).

In the regulation of gene expression zinc plays an important role. Transcription initiation is regulated by proteins that bind to DNA through their specific binding domains. Many eukaryotic DNA-binding proteins have a zinc finger motif that binds to the corresponding site on DNA that initiate transcription. In the zinc finger motif, about 30 amino acid residues are held together by a single Zn\(^{2+}\) ion. Zinc is coordinated through cysteine and histidine residues. Zinc fingers can also be found in nuclear hormone receptors that bind steroids such as estrogen (64). They are also involved in RNA and protein interactions.

Zinc is needed for brain development and zinc deficiency can cause neurological diseases, abnormal CNS development and alteration in behavior. Zinc deficiency can cause learning impairment and also growth impairment (65) and deficiency of zinc in the hippocampus has shown to increase susceptibility to seizures in epilepsy (66). It was reported that after ischemia or prolonged seizure, zinc can accumulate in hippocampal pyramidal neurons and induce synaptic failure, mitochondrial depolarization and cell death (67). A number of studies suggest the involvement of zinc in depression. While low levels of plasma zinc were observed in depressed patients (68), zinc deficiency in adult rats had shown depression-like behavior and their behavior had improved by the antidepressant drug fluoxetine (69). The role of zinc in depression is not clear. However,
zinc is an antagonist of the NMDA receptor and antidepress drugs were also shown to inhibit the NMDA receptor (70). Therefore, at least one of the antidepressant activities of zinc may be the inhibition of NMDA receptor activity.

Some other symptoms of zinc deficiency are diarrhea (71) nail dystrophy, dermatology problems such as dandruff, acne and diaper rash (72), decrease in immunity and decrease of growth and deregulation of neuronal proliferation and apoptosis (73). Burning mouth syndrome patients have shown zinc deficiency, indicating the possible role of zinc in the disease (74).

Zinc is absorbed in the upper small intestine and excretes through bile. A large percentage of zinc in the body is found in bones and muscles. In addition, zinc is stored in the liver, pancreas, kidney, parts of the eyes, skin, hair and white blood cells. In the plasma, zinc and copper concentrations are about 15 μM (75), and are bound to albumin and other proteins. Therefore, free zinc in the cells is in the nanomolar range. Brain is rich in zinc compared to other organs (100-150 μM) (76). Zinc in the brain is mostly present as a prosthetic group in various metalloproteins in neurons and glial cells. Metallothioneins bind intracellular zinc and play an important role in zinc homeostasis (77, 78). Some parts of the brain such as amygdala and hippocampus are rich in zinc which is about 10% of the total zinc in the brain. In these regions, zinc is stored with glutamate in synaptic vesicles called glutamnergic vesicles that contain high concentration of zinc (~1 mM). Therefore, a high amount of zinc (100 μM) is released to the synaptic cleft along with glutamate during neuronal activity (79, 80). The released zinc has been shown to act as a modulator in neurotransmission (81, 82). For example,
Minami et al. (83) showed that zinc modulates excitatory NMDA and inhibitory GABA receptors in amygdala, part of the brain that is responsible in the formation and storage of memories associated with emotional events. Zinc is also proposed to modulate glutamate receptors such as (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kinate receptors and voltage gated Ca$^{2+}$, K$^+$ and Na$^+$ channels (84).

2.1.3 Mechanism(s) of Cellular Metal Toxicity

2.1.3.1 Copper Toxicity

While copper is an essential trace metal which participates in diverse processes in the brain, elevated levels are toxic to the cells. It was shown that Cu(II) induced degradation of dopaminergic neurons in the nigrostriatal system of rats (85). The mechanism of copper toxicity to neuronal cells is not clear. However, the reduced form of copper [Cu(I)] can catalyze hydroxyl radical formation in the presence of hydrogen peroxide by Fenton-type reactions thus increasing oxidative stress. Copper was shown to increase ROS and decrease cellular mitochondrial activity of cells (86, 87). Mitochondria are important in multiple cellular functions including ATP production, regulating intracellular calcium, and inducing programmed cell death. Sheline and Choi (86) have reported that Cu(II) inhibits neuronal and hepatocellular mitochondrial dehydrogenase activities and reduces mitochondrial transmembrane potential. Cu(II) has been shown to decrease tyrosine hydroxylase (TH) mRNA expression, DA content and mitochondrial transmembrane potential of MES23.5 dopaminergic cells (87). Cu(II) was reported to bind efficiently to bovine heart mitochondria and decrease O$_2$ uptake and also inhibit oxidative phosphorylation (88, 89). Extragranular free copper is shown to inhibit the vesicular H$^+$/ATPase of chromaffin granule ghosts suggesting that direct
inhibition of vesicular H\(^+\)-ATPase by copper could also be a factor for copper neurotoxicity \((19)\).

Redox active copper has been reported to react with catecholamines to form harmful intermediates. Co-incubation of copper and DA was shown to decrease cell viability of rat substantia nigra neuronal cells \((13)\). It was suggested that the copper-dopamine complex decomposed inside the cell and Cu(II) was reduced to Cu(I) while dopamine oxidized to dopamine \(\sigma\)-quinone and finally to aminochrome. Aminochrome further reduced to leukoaminochrome \(\sigma\)-semiquinone radical by quinone reductase and then reacted with biomolecules leading to cell toxicity \((13)\). Paris \textit{et al.} \((90)\) have suggested the Cu-DA induced mitochondrial autophagy followed by caspase-independent apoptotic cell death. Interaction of copper with serotonin was also reported to form a colored pigment and increase of ROS and cell death in undifferentiated PC12 cells \((12)\).

### 2.1.3.2 Zinc Toxicity

Similar to copper, zinc toxicity to neuronal cells has also been linked to oxidative stress and mitochondrial dysfunction. It has been reported that Zn\(^{2+}\) decreases ATP and GSH in differentiated PC12 cells preceding cell death \((91)\). Sensi \textit{et al.} \((92)\) have reported that Zn\(^{2+}\) induces rapid mitochondrial depolarization, and ROS production in cultured cortical neurons. Abe \textit{et al.} \((93)\) observed increased hydroxyl radical production and calcium influx in Zn\(^{2+}\) treated PC12 cells. They also showed that in Ca\(^{2+}\) free media or in the presence of the L-type Ca\(^{2+}\) channel antagonist, nifedipine, decreased ROS
production and partially attenuated Zn$^{2+}$ induced cell death. Their data indicate that Ca$^{2+}$ influx and ROS production are critical events in Zn$^{2+}$ cell toxicity (93).

It has been reported that zinc can be transported into the cells through multiple channels. Increase of zinc uptake through these channels and inhibition of their activity may increase cell toxicity. Zinc transport to the cells is believed to be through ZIP family proteins (94). However, numerous studies suggest that zinc can also enter into neuronal cells through VGCC (95, 96), transient receptor potential melastatin 7 (TRPM7) channels (97), NMDA receptor-gated channels (98), and Ca$^{2+}$-permeable AMPA/kinate channels (92). Activation of transient receptor potential melastatin 7 (TRPM7) channels by removing Ca$^{2+}$ and Mg$^{2+}$ had increased intracellular Zn$^{2+}$ and toxicity in mouse cortical neurons (97). In the same experiment, knockout of the expression of TRM7 channel by TRPM7-shRNA or blocking the function of the channel by Gd$^{3+}$ had reduced the zinc-mediated cell death. Depolarization of cortical neurons with KCl was shown to increase intracellular zinc entry through VGCC and it was completely inhibited by nonselective VGCC blockers, gadolinium (Gd$^{3+}$, 10 µM) and verapamil (100 µM) and partially blocked by Ca$^{2+}$ (1.8 mM), ω-conotoxin-GVIA (100 nM) and nimodipine (1 µM) (98). However nimodipine and mibefradil, L-type and T-type VGCC inhibitors respectively did not protect Zn$^{2+}$ induced toxicity in HT-22 murine hippocampal neuronal cells (99). Increased intracellular Zn$^{2+}$ content and cytotoxicity was observed in rat thymocytes treated with micromolar concentration of Zn$^{2+}$ in the presence of H$_2$O$_2$ suggesting that oxidative conditions could have increased Zn$^{2+}$ permeability to cells (100).
2.1.4 Oxidative Stress

Oxidative stress is a condition where there is an imbalance between the production of free radicals and anti-oxidant defense mechanism in the body. Free radicals are highly reactive chemical species which contains one or more unpaired electrons. Most common biological free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some of the reactive oxygen species are superoxide (O$_2^{-}$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH'). The main RNS is nitric oxide (NO'), in addition, peroxinitrite (ONOO'), nitrogen dioxide ('NO$_2$), dinitrogen trioxide (N$_2$O$_3$), and dinitrogen tetroxide (N$_2$O$_4$) can be formed. Tightly regulated ROS production modulates cellular signaling (redox signaling) (101) and some redox signaling dependent disorders are vascular smooth muscle proliferation, atherosclerosis and angiogenesis (102). ROS/RNS can chemically modify proteins, DNA and lipids and damage membranes. Severe oxidative stress can lead to apoptosis or even necrosis.

ROS originate mainly from mitochondria in the electron transfer chain. The O$_2^{-}$ forms in this process further breaks down to H$_2$O$_2$ and OH'. In addition, ROS is produced by activities of enzymes such as cytochrome P450, NADH oxidases and xanthine oxidase. ROS can be produced under certain conditions such as inflammation, smoking, UV radiation, toxins and aging. Redox active metals, copper and iron and organic compounds like quinones can also produce free radicals. For example, Fe$^{2+}$ ion produces ROS in Fenton reaction (equation 1). OH' radicals can also form in the cells by Haber-Weiss reaction (equation 2).
Scheme 1 - The Fenton reaction (equation 1) and Haber-Weiss reaction (equation 2)

Enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase, and antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamine E), uric acid, carotenoid and glutathione can react with ROS and protect cells from oxidative damage. SOD catalyzes the conversion of superoxide into oxygen and hydrogen peroxide (equation 3). While SOD is present in extracellular space, cytoplasm and in mitochondria, catalase is concentrated in peroxisomes and it reacts with hydrogen peroxide to produce water and oxygen (equation 4). Glutathione also reacts with peroxides and forms water in the presence of glutathione peroxidase.

Scheme 2 - Reaction catalyzed by superoxide dismutase (equation 3) and catalase (equation 4)

Increased oxidative stress is a common pathophysiology of neurodegenerative diseases such as Alzheimer’s disease (AD) (103). Increased oxidative stress is also reported to play an important role in a number of diseases such as heart failure,
atherosclerosis ischaemia–reperfusion, endothelial dysfunction, hypertension (102), rheumatoid arthritis (104) and diabetes (105, 106). However, Zhou et al. (107) suggests that oxidative stress may not be the sole deleterious factor in dopaminergic neuronal death in Parkinson’s disease (PD).

2.1.5 Mechanisms of Cell Death

All cells have a finite life span and the human body continuously eliminates old, unhealthy and unnecessary cells. When cells in multicellular organisms are badly damaged or infected, they are removed through a passive necrosis process or active apoptosis process. In addition, both necrosis and apoptosis are identified as irreversible and programmed processes. Necrosis is an inflammatory form of cell death where damaged cells swell and burst leading to the leakage of intracellular contents. Although apoptosis and necrosis are mediated by different pathways, the same insult can lead to either apoptosis or necrosis depending on the intensity of damage and type of cell involved.

Autophagy is another form of programmed cell death which is reversible and involves vesicular sequestration of cytoplasmic proteins and organelles (108). Usually nutrient starvation and infection can lead to autophagy. The common mechanism of autophagy involves the formation of a membrane around a targeted region of the cell separating the contents from the rest of the cytoplasm. These double-membrane vesicles (also called an autophagosome) then fuse with lysosomes which contain digestive enzymes to degrade proteins and intracellular organelles.
2.1.5.1 Apoptosis

Apoptosis is the most common form of cell death, in which a programmed sequence of events leads to the elimination of cells without causing inflammation and leaking cell substances. Apoptosis can initiate in response to the absence of a survival signal, or in the development process like embryonic development or due to stress, toxins, bacterial and viral infections etc.

Apoptosis is associated with specific cysteine proteases called caspases, several important genes such as Bcl-2, Bax and p53 and endonucleases. It can be characterized by morphological changes in the cells such as plasma membrane blebbing, loss of cell membrane asymmetry, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation. One of the hallmarks of apoptosis is DNA fragmentation into multiples of ~200 bp oligonucleosomal fragments (109). In human chromosome, histone & a DNA strand that includes approximately 180-200 bp are tightly combined to form one nucleosomal unit. In apoptosis activated nuclear endonucleases selectively cleaves DNA at a site located between nucleosomal units (110). Therefore the detection of DNA ladder in nuclear DNA cell extract by gel electrophoresis is a widely used procedure to reveal apoptosis.

2.1.6 Neurodegenerative Diseases and Metal Dyshomeostasis

Progressive neurological disorders of cognitive abnormalities characterized by the presence of excess misfolded proteins are collectively called neurodegenerative diseases. In these disorders, it has been observed that the proteins that are normally soluble have been converted into insoluble aggregates and deposited in the brain, skeletal and muscular tissues, heart and liver (111). In addition, loss of neurons and
oxidative stress are common pathologies of neurodegenerative disorders including Alzheimer's, Parkinson's (112), Wilson's, Prion disease and FALS. Numerous biochemical pathways are affected in neurodegenerative disorders. The molecular changes in nerve cells results in nerve cell degradation and ultimately nerve dysfunction and cell death.

Neurodegenerative diseases are chronic and the molecular mechanisms that give rise to pathological symptoms are not fully understood. However genetic susceptibility factors, environmental toxins and various other factors are appeared to be involved in some cases. Aging is a predominant factor of these diseases. A common key feature of neurodegenerative disorders and aging brain is increase in oxidative stress. Imbalance of copper and other essential trace metals in the brain is reported to increase oxidative stress (113). There is accumulating evidence linking metal dyshomeostasis and neurodegenerative disorders, including Parkinson's, Alzheimer's and Prion diseases, and amyotrophic lateral sclerosis (114, 115).

2.1.6.1 Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder, which is characterized by the accumulation of extracellular senile plaques (amyloid plaques) and intracellular neurofibrillary tangles and selective loss of neurons in the brain. AD is the most common cause of dementia in the elderly affecting more than 35 million people worldwide. Generally, AD is diagnosed in people over 65 years old and, early symptoms of AD is memory loss, such as difficulty remembering recently gained information. When the disease progresses, confusion, irritability, mood swings,
language breakdown and long–term memory loss can occur. Finally bodily functions are lost eventually leading to death.

AD was first described by a German psychiatrist and a neuropathologist, Alois Alzheimer who presented the case of "Frau Auguste D.", at a scientific meeting in 1906 and was named after him (116). In the autopsy of the patient, Dr. Alzheimer saw a significant shrinkage of the cortex of her brain and observed widespread fatty deposits in the small blood vessels, dead and dying brain cells, and abnormal deposits in and around cells, under the microscope.

Amyloid plaques in AD contain aggregated Aβ peptides that are produced from amyloid β precursor protein (APP) through the proteolytic cleavage of β- and γ-secretases (117). APP is a type 1 integral membrane glycoprotein and it has a large N-terminal domain, a single transmembrane domain and a short cytoplasmic tail. The Aβ sequence spans portions of the extracellular and transmembrane domains of APP. Aβ peptides consist of 39-43 amino acids and they tend to aggregate easily due to their hydrophobicity forming oligomers, protofibrils, fibrils and amyloid plaques. The formation of low molecular weight oligomers such as dimers and trimers of Aβ at the beginning of the process, shows the highest toxicity (118). Under normal conditions, Aβ is quickly removed by clearance mechanisms. However, deregulation of Aβ clearance from the brain and an increased rate of formation may increase the plaque formation.
Some of the important molecules in pathogenesis of AD such as Aβ, tau and apoE4 are given in Figure 1. Neurofibrillary tangles in AD brain are formed by aggregation of hyperphosphorylated tau protein. Tau is a microtubule-associated protein that is abundant in neurons. Genetic factors also contribute to small percentage of AD. Mutations of genes such as APP on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1, are some of them (120). Presenilin is the enzymatic centre of the γ-secretase complex. Inheritance of apolipoprotein E (APOE) ε4 gene which encodes the apoE4 lipid carrier is responsible for the development of AD at an early age (120). In addition, deposits of presynaptic protein α-synuclein are also found in AD brain.
The reason for neuronal loss, dysfunction and dementia in AD is not clear. Aβ and tau may disturb cellular function by impairing synapses between neurons that form and maintain microcircuits supporting learning, memory and other cognitive functions. Tau inhibits kinesin-dependent transport of peroxisomes, neurofilaments and Golgi-derived vesicles into neurites and transport of APP into axons and dendrites (121). Structural modification of tau such as hyperphosphorylation and aggregation may interfere with neuronal functions.

A notable characteristic of AD brain is altered metal ion concentrations (122). High levels of copper and zinc have been found in the neocortex and amyloid plaques of AD patients (123). A significant decrease in Cu, and significant increases in Zn and Fe were found in AD hippocampus and amygdala areas (124) and low plasma concentrations of copper were reported in AD patients (125, 126). While Danscher et al. (127) have reported high levels of zinc in hippocampus and amygdala of AD brain, some studies showed a decrease in zinc in AD brain (128, 129). Increased expression of divalent metal transporter 1 (DMT1) was also reported in AD brain (130).

Studies show that binding of copper, zinc and other biometals to APP (131) and Aβ (132-134) promotes protein aggregation and oxidative stress in the cells. Substoicheometric amounts of Cu(II) and Zn(II) were reported to accelerate kinetics of Aβ precipitation (135, 136) and cytotoxicity. Oxidative stress is a key pathogenisis of AD similar to other neurodegenerative disorders (137). Aβ-Cu\(^{2+}\) complex was shown to produce H\(_2\)O\(_2\) in the presence of ascorbic acid, dopamine, cholesterol and other biological reducing agents and mediate neurotoxicity (138). Sayre et al. (139) have also
reported that the incorporation of redox-active transition metals in neurofibrillary tangles and senile plaques can increase oxidative stress. Cu-APP complex was shown to induce oxidative stress and cell death in primary neuronal cultures (140).

2.1.6.2 Parkinson’s Disease

Parkinson’s disease (PD) is a progressive neurodegenerative disorder characterized by impaired motor function including tremor, muscle rigidity, slowness of movement and changes in speech. It was first described by the British physician James Parkinson in 1817 (141). Degradation of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of intracellular Lewy bodies (142) are key pathophysiology of PD. Lewy bodies mainly consist of fibrils made of α-synuclein (143) which is a small 14 kDa protein predominantly expressed in neurons of the central nervous system. One of the treatments to alleviate symptoms of PD is the administration of L-DOPA, precursor of dopamine (141).

The mechanism underlying the degradation of dopaminergic neurons in PD is still unclear. However, inflammation and oxidative stress are involved in disease progression. Evidence suggests that PD involves mitochondrial dysfunction in dopaminergic neurons (144). Dysfunction of mitochondrial complex I was first reported in the case of MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) developed PD (145). Inhibition of complex I can increase oxidative stress, reduce ATP formation and lead to neuronal death. Pesticides and herbicides such as rotenone and paraquat were shown to inhibit complex I and may also lead to aggregation of α-synuclein (146, 147). Oxidative stress may also promote α-synuclein aggregation in AD brain (148). α-synuclein is an unfolded protein and can change to an α-helical structure and then tend
to aggregate under conditions such as low pH (149), organic solvents, or in the presence of polyamines and certain metals (150). Copper (II) has been shown to be the most effective cation in accelerating the self-oligomerization of α-synuclein (10). Two copper binding sites were identified in α-synuclein and binding of Cu(II) to the highest affinity binding site was shown to increase the amyloid formation (151).

PD is caused by numerous factors including genetics, aging, environmental toxins such as pesticides (146), herbicides (147) and heavy metals. Increase in iron, zinc and aluminum content was observed in Parkinsonian substantia nigra. A recent study carried out by Willis et al. (152) has shown that PD incidence is greater in counties with high reported industrial release of copper or manganese in the United States indicating that environmental exposure to metals may be a risk factor for Parkinson’s disease in urban areas.

2.1.6.3 Prion Diseases (PrPD)

Prion diseases are a family of progressive neurodegenerative diseases commonly known as Transmissible Spongiform Encephalopathy (TSE’s). These include scrapie in sheep, bovine spongiform encephalopathy (BSE) or mad cow disease in cattle and several rare neuropathies such as Creutzfeld-Jacob disease, Fatal Familial insomnia, Gertsmann-Straussler Scheinker syndrome and Kuru in humans (153). Prion diseases affect the brain and other neural tissue and they are rapidly progressive, untreatable and always fatal.

Prion diseases are believed to be associated with prion protein (PrP), a membrane bound glycoprotein expressed by many cell types of all mammals and avian species and found at higher levels in neurons (154). PrP is monomeric in nature and
anchored to the cellular membrane through a glycosylphosphatidylinositol moiety (153, 155). Misfolding and accumulation of the abnormal isoform of the prion protein (PrPSc, scrapie isoform) is responsible for several devastating neurodegenerative diseases in human and animals. PrPc→PrPSc conversion which occurs as a posttranslational process without any detectable covalent modification to the protein likely represents a key molecular event in the pathogenesis of prion diseases (156, 157). PrPc is mostly α helical and monomeric while PrPSc is primarily β-sheet, multimeric and protease resistant. PrPsc induces the formation of stable amyloid aggregates that accumulates in infected tissue, causing cell death.

Even though PrPc is expressed in neurons and various cell types (157), its physiological role(s) is still not clear. Diverse activities and ligands have been described for PrPc. Some of the reported functions of PrPc are signaling, cell adhesion, anti-apoptotic, neuronal differentiation, T cell activation, SOD activity and copper homeostasis (157, 158). Fluorimetric analysis in b104 neuroblastoma cell model has revealed that PrPc mediates copper uptake by clathrin-dependant endocytosis (159).

Prion diseases are the best-understood family of diseases compared to other neurodegenerative diseases. TSE’s may be sporadic, inherited or infectious. Sporadic disease in which PrPc spontaneously misfolds into PrPSc is by far the most common form of disease. This accounts for nearly 85% of cases in humans. Inherited diseases typically arise from the mutations in the prp gene. Sporadic and infectious forms of TSC’s fall into the same category as Alzheimer’s and Parkinson’s disease in which the lethality is due to the abnormal accumulation of protein aggregates. The major feature which the prion diseases deviate from other such diseases is that they are infectious
The passage of PrPSc from infected animals to healthy animals is thought to cause symptoms of TSE.

Evidence suggests that transition metals such as copper binds to prion protein with high affinity (160) causing changes in its structure (161) and makes protein into more protease resistant form. Nishina et al. (162) have shown that while PrPsc is more than twenty fold sensitive to proteinase K digestion in the absence of divalent cations in low ionic strength buffers, addition of micromolar concentration of copper or zinc restore protease resistance. However it was reported that Cu(II) itself inhibits proteinase K activity (163). Changes in copper, iron, manganese and zinc levels were reported in the prion infected brains (111, 164). In the presence of redox active Cu(II) and Fe(II), PrP106-126 was shown to generate hydroxyl radicals (165). Interestingly, structures obtained by ab initio electronic structure calculations indicated that the binding of copper protects PrPc from conversion to the infectious form (166). Orem et al. (167) have also found that amplification of the conversion of PrPc to PrPSc in vitro was inhibited by Cu(II) and Zn(II). However, while it is evident that copper is involved in prion pathogenesis, molecular details of its role is not clearly understood yet.

2.1.6.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) also called Lou Gehrig's disease, is a rapidly progressive and always fatal neurological disease that attacks upper and lower motor neurons located in the mid-brain, brainstem and spinal cord. Degradation and loss of motor neurons in ALS ceases sending massages to the muscles. Inactive muscles
gradually weaken and finally the ability of the brain to start and control voluntary movement is lost. ALS affects approximately one in 200 000 people (6).

About 10% of ALS cases are inherited, while the others are believed to be sporadic cases. Of the familial cases about 20% result from a specific genetic defect that leads to mutations of copper zinc superoxide dismutase 1 (SOD1) (6, 49). Common pathology of FALS patients is aggregation of SOD1 in spinal cord (168, 169). SOD1 is found in eukaryotic species predominantly in the cytosol, nucleus, and mitochondrial intermembrane space and in the periplasmic space of bacteria. This enzyme acts as a powerful antioxidant that protects cells from free radical damage by dismutating the superoxide anion.

The detailed mechanism that causes ALS is not known. However, mutations of SOD1 was shown to accumulate free radicals that cause neuron degeneration and apoptosis (170). TDP-43 is a protein typically localized in the nucleus of healthy cells. However, pathophysiology of ALS indicates accumulated TDP-43 protein in the cytosol of affected nerve cells (171). While mechanism of pathological changes in this protein in ALS is not known, the absence of normal TDP-43 in the nucleus and the abnormal accumulation of the protein in the cytosol may affect cellular functions. Increase in glutamate levels were observed in the serum and spinal fluid of ALS patients indicating that glutamate may also play a role in ALS. Studies have demonstrated that neurons are toxic to high concentrations of glutamate.

Copper and zinc are essential cofactors of SOD1 that regulate its stability and activity and the imbalance of these metals has been implicated in the pathogenesis of
ALS (172). Accumulation of labile zinc has been observed in neurons and astrocytes in the spinal cords of G93A SOD-1 transgenic mice (173). G93A is one of the mutations of SOD1 that causes FALS. Treatment of SH-SY5Y cells with zinc has demonstrated decreased expression and aggregation of TDP-43 (171). Iron accumulation was also observed in the CNS of ALS and iron chelators have been shown to increase the life span of SOD1(G37R) transgenic mice (174). Smith and Lee (175) have suggested that zinc may be important in the initiation and development of ALS because it has been reported that mutation of SOD1 has altered zinc binding. In addition, the deletion of metallothioneins has increased disease progression of mutant SOD1 mice. Moderate supplement of dietary zinc has shown to increase survival of ALS mutant G93A SOD transgenic mice (176).

2.1.7 Chelation Therapy for Neurodegenerative Diseases

Use of metal chelators for the treatment of neurodegenerative diseases have been widely discussed in recent years (177-179). Some of the molecular targets for drug development for AD are Aβ and tau. For example, 49 percent decrease in Aβ deposition has been shown reported in AD transgenic mice (APP2576) brain after giving membrane permeable metal chelator clioquinol (CQ) orally for 9 weeks (180). Treatment of AD patients with CQ has been shown to decrease Tau protein and slight improvement of their symptoms (181). CQ has a high affinity to copper and zinc. Therefore, the decrease of Aβ and tau depositions has been proposed due to the extraction of copper and zinc in Aβ deposits by CQ resulting in dissolution of the aggregates (180). A significant reduction (85-90 percent) of Aβ secretion was reported in APP overexpressing Chinese hamster ovary cells when they were treated with CQ.
and Cu²⁺ or Zn²⁺ (182). The mechanism of reduced Aβ secretion and the degradation was proposed due to metal-dependent up-regulation of matrix metalloproteases (MMPs) (182). An increase in MMPs activities has been shown to reduce Aβ accumulation in vitro (183, 184). Further studies have shown that diverse metal ligands including CQ, 8-hydroxyquinoline, neocuproine and 1,10-phenanthroline along with copper and zinc could be used to decrease Aβ by elevating intracellular metal concentration followed by up-regulating metalloprotease activities (185). Structures of some of the membrane permeable metal chelators are given in Fig. 2. In addition, copper-bis(thiosemicarbazonoto) complexes treated APP/PS1 transgenic AD mice have shown significant inhibition of GSK3beta (glycogen synthase kinase 3β) activity and decrease of Aβ and phosphorylated tau (186). Rapid decrease of in soluble interstitial Aβ and improved cognitive performance have been observed in AD transgenic mice after treating with 8-hydroxy quinoline analog PBT2 orally (187). Treatment of copper chelator, D-(-)-penicillamine (D-PEN) has shown decreased copper levels in brain and blood and delayed onset of prion disease in mice (188).

Even though numerous evidence suggests the use of membrane permeable metal ligands as potential therapeutic agents for treatment of neurodegenerative disorders, increase in copper bio-availability by lipophilic metal chelators may increase cellular toxicity. For example, treatment of rat cortical astrocytes with substoichiometric levels of neocuproine (0.03-10 μM) and copper (1-10 μM) has increased free radicals, decreased mitochondrial membrane potential and depleted glutathione (GSH) and ATP. They have also stimulated activation of caspase-3 and degradation of PARP (poly-ADP-ribose polymerase) with time (189). Therefore, the use of membrane permeable metal
chelators to increase intracellular metal content to decrease protein aggregation in neurodegenerative disorders may not be effective.

![Structures of membrane permeable metal chelators](image)

Figure 2 - Structures of membrane permeable metal chelators

### 2.1.8 Calcium Channels

Ca$^{2+}$ regulation pathways play an important role in the survival of neurons. It is implicated that Ca$^{2+}$-deregulation is associated with aging brain and diseases related to cognitive dysfunction such as AD (190, 191). Age-related increase of calcium channels have been observed in rat brain (192). In addition, calcium channel blockers have been shown to regulate neuronal survival (193). As extracellular Cu(II) and Zn(II) may also perturb cellular calcium, it is beneficial to give an overview of function of calcium channels and calcium channel blockers.
Calcium is one of the most important nutrients; besides providing skeletal structure for bones and teeth, it acts as a second messenger and is involved in many cellular functions. Calcium aids contraction and relaxation of muscles and blood vessels, blood clotting, conduction of nerve impulses, glycogen break down, oxidative metabolism as well as the regulation of body fluids including hormones and enzymes. Elevated cytosol Ca\(^{2+}\) triggers exocytosis and apoptosis. Calcium release from the ER and cytochrome c release from the mitochondria coordinates apoptosis (194). Therefore intracellular and intercellular calcium transport is strictly regulated by specific receptors and transporters. Calcium transport to the cells is regulated by specific ion channels that can be either voltage-gated or ligand-gated. Active transporter Ca\(^{2+}\)-ATPase mediates calcium transport to cytosol across the cell membrane.

2.1.8.1 Voltage Gated Calcium Channels

Under resting conditions membrane potential is in the range of -50 mV to -70 mV (48). The transmembrane electric potential is maintained by electrogenic Na\(^{+}\)K\(^{+}\) ATPase. It creates charge imbalance across the membrane by pumping out 3Na\(^{+}\) for every 2K\(^{+}\) pumped in. Therefore, inside of the cell is negatively charged relative to the outside and under these conditions the membrane is said to be polarized. As shown in Figure 3, influx of positively charged ions such as Na\(^{+}\) or efflux of negatively charged ions such as Cl\(^{-}\) increases the membrane potential and depolarizes the cell membrane.
Figure 3 - Schematic of a cell membrane. Na+ and K+ ionic gradient across the cell membrane is primarily maintained by active transport system in the plasma membrane which involves the enzyme Na\(^+\)K\(^+\) ATPase.

Voltage-gated calcium channels (VGCC) are ion pores present in the plasma membrane that are activated by changes in transmembrane voltage. While calcium ion concentration in the extracellular space is \(~1.5\) mM the cytosolic calcium ion concentration is very low \((~100\) nM) \(\text{(195)}\). This large concentration gradient is maintained by active transport of calcium across the plasma membrane and endoplasmic reticulum at the expense of ATP by Ca\(^{2+}\)-ATPase. Plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger channel (NCX) is also a major contributor in maintaining the Ca\(^{2+}\)
gradient which transports three Na\(^+\) ions out down the electrochemical gradient across the membrane in exchange for the countertransport of one Ca\(^{2+}\) into the cytosol.

Since extracellular calcium ion concentration is higher than that in the cytosol, opening of VGCCs will allow spontaneous calcium ion influx. Increase of intracellular calcium can depolarize the cell membrane and thereby activate other voltage gated ion channels. Other than VGCCs there are voltage-gated K\(^+\) and Na\(^+\) channels. These three channels are important in neuronal signaling. They carry an electrical impulse (action potential) along the axon from the cell body.

Voltage gated calcium channels are present in many cell types and regulate intracellular processes such as cell contraction, gene transcription, synaptic plasticity, modulate neuronal firing, memory, vision, hearing, control of mood and hormone secretion (196). Most of the receptor proteins are anchored to other regulatory proteins such as protein kinases and phosphatases, to target a specific site or process. Change in intracellular [Ca\(^{2+}\)] is detected by Ca\(^{2+}\) binding proteins that regulate Ca\(^{2+}\) dependent enzymes.

### 2.1.8.1.1 Classification of Voltage-Gated Calcium Channels

VGCCs were initially grouped as high voltage-active (HVA) and low voltage active (LVA) channels according to their current properties. While HVA channels are activated only when the membrane potential increase to -30 mV and their current is large and long lasting, LVA channels are activated around -70 mV and their current is small and inactivates quickly (196-198). VGCCs were further classified into L, P/Q, N, R and T types based on their pharmacological properties and amino acid sequence.
Figure 4 – Classification of voltage-gated calcium channels

L-type calcium channels are present in skeletal muscles, heart smooth muscles, neuroendocrine and endocrine tissues, retina and neurons. P, Q and N-type calcium channels are present in neurons. T-type calcium channels are present in cardiac tissues in addition to neurons.

It has been reported that Ca\textsubscript{v}1.3 channels serve as pacemaker channels in adrenal chromaffin cells (199). Pacemaking is required to maintain basal dopaminergic concentration in CNS. The basal dopamine release from the store (vesicles) in response to an increase of intracellular calcium through L-VGCC is important for proper functioning of neurons.
2.1.8.2 Ligand-Gated Calcium Channels

Some calcium channels are activated by various ligands as given in Table 1. In these channels, binding of extracellular or intracellular small molecules forces an allosteric transition in the protein which opens or closes the channel.

**TABLE 1 - SOME IMPORTANT LIGAND-GATED CALCIUM CHANNELS AND THEIR FUNCTIONS**

<table>
<thead>
<tr>
<th>Type</th>
<th>Gated by</th>
<th>Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP₃ receptor</td>
<td>IP₃</td>
<td>ER/SR</td>
<td>Release Ca²⁺ from ER/SR in response to IP₃</td>
</tr>
<tr>
<td>Ryanodine receptor (200)</td>
<td>Dihydropyridine</td>
<td>ER/SR</td>
<td>Calcium-induced calcium release in myocytes</td>
</tr>
<tr>
<td></td>
<td>receptors in T-tubules</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and increased</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>intracellular calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Store-operated</td>
<td>Indirectly by ER</td>
<td>Plasma membrane</td>
<td>Restore Ca²⁺ in ER</td>
</tr>
<tr>
<td>channels (201)</td>
<td>depletion of calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Acetylcholine</td>
<td>Plasma membrane</td>
<td>Increase cytosolic Ca²⁺ and regulate heart beat, muscle contraction</td>
</tr>
<tr>
<td>receptor channels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cGMP gated ion</td>
<td>cGMP</td>
<td>Rods and Cone</td>
<td>Increase of intracellular calcium making neuron depolarization</td>
</tr>
<tr>
<td>channels</td>
<td></td>
<td>cells in retina</td>
<td></td>
</tr>
</tbody>
</table>

2.1.8.3 Calcium Channel Blockers

L-type VGCCs are important in cardiovascular function. Blockers of L-VGCCs have cardiodepressive and vasodilating properties, therefore they have been commonly used as drugs to treat hypertension, cardiac arrhythmias and vasospastic disorders. This will increase blood flow leading to a decrease in blood pressure.
Three representatives of the common L-VGCC inhibitors are given in Figure 5. Nifedipine and nitrendipine belong to the dihydropiridine class of calcium channel antagonists (202). Verapamil is a phenylalkylamine class L-type calcium channel blocker. Nitrendipine binds tightly to the inactivated state of cardiac Ca\textsuperscript{2+} channels with an apparent dissociation constant (K\textsubscript{d}) of 0.36 nM and weakly to the normal resting state with K\textsubscript{d} of 700 nM (203). Using whole-cell-patch-clamped frog ventricular myocytes the equilibrium dissociation constant of nifedipine to the Ca\textsuperscript{2+} channels was determined to be 77 nM for the resting state and 0.17 nM for the inactivated state (204).

![Figure 5 - Structures of VGCC blockers](image)

2.1.9 Catecholaminergic Cell Lines as Neuronal Models

2.1.9.1 Cells Culture

The history of tissue culture runs back into the beginning of the 19\textsuperscript{th} century. However animal cell culture became popular in the 1950s when the first human cell line was established from a cervical carcinoma (205). Cell culture refers to a culture derived from dispersed cells originated from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation (205). Cells that are directly from the source that is from a tissue or tumor are called primary culture. Except tumor cells,
most of the primary cell cultures have limited life. Primary culture can be sub-cultured. After the first subculture or passage, the primary culture becomes a cell line.

Mammalian cells can be grown in suspension or adherent cultures. They are grown and maintained typically at 37° C and 5% CO₂. Culture conditions such as growth media vary widely for each cell type. For example pH, glucose concentration, growth factors and other nutrients can be varied in the growth media depending on the cell line. Once cells are confluent, that is when all the available growth area is utilized and cells make close contact with one another, they need to sub-cultured. The cell culture media is changed every 2-3 days until the cells are confluent.

2.1.9.2 PC12 Cells

PC12 cells are derived from pheochromocytoma of the rat adrenal medulla. This cell line is one of the most widely studied cell lines, well characterized and a useful neuronal model. PC12 cells synthesize and store catecholamine neurotransmitters, dopamine and norepinephrine but do not synthesize epinephrine (206). The catecholamine synthesis pathway is shown in scheme 3. PC12 cells also synthesize, store and release acetylcholine (207). These cells can be differentiated by culturing with Nerve Growth Factor (208).

Three distinct types of ion channels in PC12 cells have been identified and characterized i.e. voltage-dependent Na\(^+\) channels, voltage-dependent Ca\(^{2+}\) channels, and acetylcholine-activated channels permeable to both ions (209). While veratridine and scorpion venom open voltage-dependent Na\(^+\) channels, 5 µM tetrodotoxin inhibits more than 98% of \(^{22}\)Na\(^+\) uptake. Membrane depolarization due to influx of Na\(^+\) or by 50 mM KCl opens voltage-dependent Ca\(^{2+}\) channels. \(^{45}\)Ca\(^{2+}\) uptake is blocked by 1 mM Mn\(^{2+}\), \(^{[3]}\)Hnitrendipine and other calcium channel blockers. \(^{[3]}\)Hnitrendipine binding is
saturable with $K_D$ 1.1 nM while IC$_{50}$ of potassium-stimulated $^{45}$Ca uptake inhibition by nitrendipine is about 5.5 nM (210).

DA release from PC12 is dependent on external Ca$^{2+}$ and stimulated by activation of nicotinic ACh receptors with carbamylcholine or high concentrations of external KCl (15--56 mM). Therefore, DA release is probably due to both Ca$^{2+}$ influx through ACh channel and voltage dependent Ca$^{2+}$ channels (211). DA release in depolarized PC12 cells is inhibited by nitrendipine (212). While neurotransmitter release induced by monovalent cation transporting ionophore X537A and monensin is dependent on extracellular Ca$^{2+}$, neurotransmitter release induced by protein kinase C activators such as 12-O-tetradecanoylphorbol, 13-acetate and diacylglycerol is almost completely independent of extracellular [Ca$^{2+}$] and not accompanied by changes of intracellular [Ca$^{2+}$]. Therefore, neurotransmitter release from PC12 cells can be Ca$^{2+}$ dependent and independent (213).

Alpha-latrotoxin is a protein present in black widow spider venom which binds to presynaptic nerve terminals and stimulate release of neurotransmitters (214) in both Ca$^{2+}$-free and Ca$^{2+}$--containing media. According to Meldolesi et al. (215), α-latrotoxin releases neurotransmitters in PC12 in Ca$^{2+}$ free media and does not increase intracellular Ca$^{2+}$, while neurotransmitter release by membrane depolarization with high K$^+$ requires extracellular Ca$^{2+}$ and shows significant increase in intracellular Ca$^{2+}$. Thus the release evoked by α-latrotoxin in Ca$^{2+}$ -free medium is mediated by a factor(s) other than bulk redistribution of Ca$^{2+}$ from intracellular stores (215).
(R)-N6-Phenylisopropyladenosine (PIA) increases tyrosine hydroxylase (TH) activity and stimulate production of DOPA by 3- to 5-fold in PC12 cells, with a half-maximal effective concentration (EC$_{50}$) of 50 nM (216). Adenosine-dependent activation of TH is proposed due to increase of cAMP and phosphorylation of TH by cAMP-dependent protein kinase (216). High K$^+$ increase activation and phosphorylation of TH in PC12 and they are dependent on the presence of extracellular calcium (217). Nerve growth factor protein (NGF) was also found to promote the activation of tyrosine hydroxylase in PC12 cells. About 60% increased activity was observed in less than 1 h (218). Increasing Ca$^{2+}$ influx by high K$^+$, acetylcholine and ionophore A23187 was also shown to increase TH phosphorylation and activation (219).

2.1.9.3 MN9D Cells

MN9D is an immortalized, dopaminergic cell line that possess characteristics of mesencephalic (mid brain) neurons. This cell line is derived from the fusion of embryonic ventral mesencephalic cells (rostral mesencephalic tegmentum -RMT) and neuroblastoma cells (N18TG2) (220).

MN9D cells synthesize and store catecholamines and is used as a model to study neurodegenerative disorders. As shown in Table 2, MN9D contain high amounts of DA and DOPA (220). These cells express tyrosine hydroxylase, dopamine $\beta$ monooxygenase and aromatic amino acid decarboxylase (221). MN9D also express dopamine transporter (DAT), noephinephrine transporter (NET) and vesicular monoamine transporter-2 (VMAT-2) (221).
MN9D is used as a model of dopamine (DA) neurons. These cells are used to test mechanisms and potential therapeutics for Parkinson's disease. For example, it is being used to study the toxicity of neurotoxins MPP⁺ and 6-OHDA, which have been shown to reproduce several features of Parkinson's disease in animal and cell culture model toxicity (222-224).

**TABLE 2 - ENDOGENOUS AND CULTURE MEDIA CATECHOLAMINE LEVELS IN MN9D; CULTURE MEDIA CATECHOLAMINE LEVELS WERE MEASURED AFTER 24 H OF INCUBATION IN FRESH MEDIA BY HPLC-EC.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Endogenous catecholamine levels (ng/mg protein)</th>
<th>Culture media catecholamine levels (ng/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOPA</td>
<td>DA</td>
</tr>
<tr>
<td>MN9D</td>
<td>56.3 ± 3.2</td>
<td>104.7 ± 18.6</td>
</tr>
</tbody>
</table>

Choi et al. (225) have reported that voltage-activated Na⁺ channels are present in MN9D cells. However, recent studies by Rick et al. (226) suggest that Mn9D cells in undifferentiated form are electrophysiologically significantly different from midbrain DA neurons with respect to sodium, calcium and potassium currents. However, butyric acid differentiated cells partially resembles the electrophysiology of DA neurons.

**2.1.9.4 SH-SY5Y Neuroblastoma Cells**

SH-SY5Y is a thrice-cloned subline of human neuroblastoma cell line SK-N-SH. The parent cell line was 1ˢᵗ subcloned as SH-SY which subcloned again as SH-SY5. SH-SY5Y is a subclone of SH-SY5 (227). The original cell line was established in 1970 by tissue obtained bone marrow biopsy of a neuroblastoma patient (228). Biedler et al.
(227) have first reported the presence of DβM (3.47 nmol/h/mg protein) in these cells. These cells also contain TH which was 1st quantified by Ross et al. (229) as 6.8 ± 0.1 pmol/h/mg.

2.1.10 HepG2 Cells as a Model to Study Liver Metal Toxicity

HepG2 is a human liver carcinoma cell line derived from the liver tissue of a fifteen year old Caucasian American male with a well differentiated hepatocellular carcinoma. It is also called human hepatocytes. Since liver plays a major role of metal homeostasis and increase of liver metal concentration has been linked to number of diseases, metal uptake and toxicity to HepG2 cells have been studied extensively. Urani et al.(230) have observed a time-dependent accumulation of copper and zinc in HepG2 cells. They have also observed that co-incubation of these cells with copper and zinc resulted in a higher accumulation of zinc. StocKert et al.(231) have observed saturable and cellular energy-independent radio-Cu uptake in HepG2 cells ($V_{\text{max}}$, 7.1 ± 0.2 pmoles min$^{-1}$ mg protein$^{-1}$; Km, 3.3 μM). However, they have reported that tenfold molar excess of zinc did not affect the rate of copper uptake (231). These results indicate that both copper and zinc transport into HepG2 cells efficiently, but by two independent pathways where the zinc uptake is higher than copper uptake. Upregulation of metallothioneins (MT-I, MT-II) and ZnT-1 transporter were observed in Cd$^{2+}$ and Zn$^{2+}$ treated HepG2 cells (232).

Recent work have shown that copper increased lipid peroxidation, DNA fragmentation and decreased glutathione in HepG2 cells (233). It has also been shown that it was copper but not zinc that produced significant ROS in HepG2 cells (234).
Treatment of hepatocytes with zinc has been reported to decrease mitochondrial function by inhibiting mitochondrial dehydrogenases, and cytochrome c oxidase and deplete ATP (235). Effect of macrocyclic ligands on cytotoxic concentrations of Cu$^{2+}$ in HepG2 cells was tested using 1,4,7,10,13,16-hexathiacyclooctadecane (L3) and increased cellular toxicity and oxidative stress were observed in the presence of L3 compared to the metal control (236).

2.2 Part II

2.2.1 Biochemical Role of Cytochrome $b_{561}$

Cytochrome $b_{561}$ is found in a variety of neuronal and endocrine tissues (237), including hypothalamus, pituitary (238), erythrocytes (239) and retina (240). In addition to mammalian tissues, similar proteins have also been identified in plant tissues (241, 242) and in planarian tissues (243). It is a hemoprotein located in the membrane of secretory vesicles in adrenal medulla and CNS catecholaminergic neurons that are specialized in neurotransmitter storage and synthesis. Cyt $b_{561}$ supports a number of enzymatic activities by importing electrons through the membrane into vesicles from cytosolic Asc. In chromaffin granules Asc acts as the electron donor for DβM and SDA is being produced. SDA or Asc cannot penetrate through the granule membrane. Cyt $b_{561}$ imports electrons through the membrane into vesicles to reduce the SDA produced by the monooxygenases back to Asc. Therefore, cyt $b_{561}$ acts as a redox mediator between the extravesicullar and intravesicullar Asc pools (1-3 mM and 22 mM respectively) and its activity is crucial for the overall function of these vesicles. Asc also prevents the auto-oxidation of catecholamines present at high concentrations (500-600 mM) within cromaffin granules (244).
In addition to the electron translocation activity this protein may also be involved in iron metabolism. Cyt \( b_{561} \) expressed in yeast has showed Asc-dependent trans-membrane ferric-reductase activity \( (245, 246) \). This indicates that the cyt \( b_{561} \) isoform found in human erythrocyte membranes may function as a ferric –reductase; enzyme which catalyzes the conversion of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \). The observation that cyt \( b_{561} \) may be involved in iron absorption has opened new perspectives for its physiological function.

### 2.2.2 Adrenal Chromaffin Granule

Cyt \( b_{561} \) used in this project was isolated from bovine chromaffin granules. They have been extensively used as a model to study the molecular details of catecholamine metabolism \( (247) \). Chromaffin granules (CG) are spherical organelles with an average diameter of about 280 nm and each chromaffin cell contains 10,000-30,000 CG. A schematic diagram of CG is shown in Figure 6. Dopamine (DA) synthesized in the cytosol of chromaffin cells is transported into the granules by the vesicular monoamine transporter (VMAT). Inside the CG, DA is converted to norepinephrine (NE) by D\( \beta \)M using Asc as a reductant generating SDA and cyt \( b_{561} \) shuttles reducing equivalents into the granule from cytosolic Asc \( (248) \). Finally, semidehydroascorbate reductase in the outer mitochondrial membrane regenerates cytosolic Asc from SDA completing the redox cycle \( (249) \).

### 2.2.3 Dopamine-\( \beta \)-Monooxygenase (D\( \beta \)M)

D\( \beta \)M catalyzes the conversion of DA to NE in synaptic vesicles and adrenal medullary chromaffin vesicles. D\( \beta \)M is a copper containing glycoprotein \( (250) \) with an apparent molecular weight of 300 kDa. It is known to exist in soluble (sD\( \beta \)M) and
membrane bound (mDβM) forms and consists of four subunits which are arranged in pairs of dimers linked via disulfide bonds.

The catalytic mechanism of DβM is given in Scheme 4 which consumes one mole of oxygen, two moles of protons, and two moles of electrons. The electrons are produced by two moles of Asc (251, 252).

Figure 6 - Schematic diagram of catecholamine storage vesicle (247). VMAT- Vesicular monoamine transporter; m-DβM - Membrane bound DβM; s-DβM – Soluble DβM
Scheme 4- Reaction catalyzed by DβM

2.2.4 Structure of Cytochrome $b_{561}$

Cyt $b_{561}$ is named according to the wavelength maximum of its α band in the reduced form it has a high midpoint potential of +140 mV (253). Cyt $b_{561}$ is the only heme protein found in the chromaffin granule membrane (27) and it constitutes 15-20% of the total membrane proteins (254).

Cyt $b_{561}$ is a single polypeptide transmembrane protein with an apparent molecular weight of ~ 28 kDa. Bovine (255), mouse (256), and human chromaffin granule $b_{561}$ (257) have been cloned and sequenced. It is a highly hydrophobic protein with 273 amino acids and consists of six α helical membrane spans with both C and N terminals in the cytoplasmic side of the membrane as shown in Figure 7 (255). Srivastava et al. (258) have proposed a five-transmembrane segment model using human brain cyt $b_{561}$ cDNA and topological studies. However, considering experimental data such as heme content and topological accessibility, the six helix model is more plausible (31, 259).
Five mammalian cyt $b_{561}$ family members have been reported, and three are evolutionarily linked (260). They are chromaffin granule cyt $b_{561}$, duodenal brush border cell plasma membrane cyt $b_{561}$ and lysosomal membrane cyt $b_{561}$. Four isoforms of cyt $b_{561}$ were reported in plants (261). Low sequence similarities (30-45% identity) of this protein in the same species and phylogenetically distant species were reported (262). However their main structural features such as heme content, six transmembrane helices and Asc and SDA binding sites are well conserved.
2.2.4.1 Heme Prosthetic Group

Early studies suggested that purified cyt $b_{561}$ contains only one heme center (26, 27, 29, 255, 263, 264). However, low temperature EPR studies of Burbaev et al.(265) and Palmer and Kamensky (266) suggested the presence of upto three heme centers in cyt $b_{561}$. However, recent studies suggest that the protein contains two heme prosthetic groups with distinct reduction potentials (30, 259, 267-271). Based on circular dichroism (CD) studies of the granule membrane, a the first two heme model was proposed by Degli Eposti et al. (272). After analyzing EPR data of oxidized cyt $b_{561}$ and heme content, Tsubaki et al.(30) have also suggested the presence of two hemes in the protein located on either side of the membrane. Figure 8 illustrates the recently proposed two heme model for cyt $b_{561}$ by Nakanishi et al.(259).
His54 and His122 are the heme axial ligands on the intravesicular side, whereas His88 and His161 are the heme axial ligands on the cytosolic side. The heme b on the cytosolic side accepts electrons from Asc; the heme b on the intravesicular side may donate electrons equivalent to SDA radical to reproduce Asc.

2.2.5 Reduction of Cytochrome \( b_{561} \) with Ascorbate

Cyt \( b_{561} \) has long been known to serve as an electron conduit in maintaining the Asc concentrations in secretory vesicles. The reaction between Asc and oxidized cyt \( b_{561} \) is believed to proceed through a concerted of proton/electron transfer mechanism (273). Asc reduction kinetics of cyt \( b_{561} \) by Asc is much faster than that of cytochrome c and also it is mostly pH independent (at pH > 7) (274). Kelley and Njus (21) have
determined electron transfer rate between Asc trapped in vesicle ghosts and external ferricyanide through cyt \( b_{561} \). They were able to determine rate of SDA reduction by cyt \( b_{561} \) as \( 7 \times 10^{-4} \) M/s. The reduction of cyt \( b_{561} \) in chromaffin vesicle ghosts by external Asc was monitored by a stopped-flow method and the rate of reduction was reported as 450 (±190) M\(^{-1}\)s\(^{-1}\) at pH 7 (275). The rate of oxidation of cyt \( b_{561} \) by external SDA was 1.2 (±0.5) x 10\(^6\) M\(^{-1}\)s\(^{-1}\) at pH 7.0 and it decreased significantly with increasing pH (275).

2.2.5.1 Pre-Steady State Kinetics of Purified Cyt \( b_{561} \)

Takigami et al. (31) have conducted a stopped-flow study on the reaction of oxidized cyt \( b_{561} \) by Asc in the detergent-solubilized state. The time course has fitted to a linear combination of four exponential functions. The results indicated the presence of two heme \( b \) centers with different redox potentials. The fastest phase was assigned to the reduction of heme from the extravesicular side and it was analyzed further by a transient kinetic method based on a two-step bi-uni sequential ordered mechanism.

The \( K_a \) for Asc was calculated to be 2.2 mM at pH 6.0. The fastest phase of the reaction was completely lost by the treatment of diethylpyrocarbonate (DEPC), which specifically label the His residue near the extravesicular side heme center.

Stopped-flow analyses were also performed on purified CG, yeast and insect cells expressed cyt \( b_{561} \) by Liu et al. (32). The absorbance changes at 427 nm were measured with Asc at pH 7.0 for 15 s. Compared to four phase kinetics by Takigami et al. (31), in this study the absorbance changes were fitted to three exponential phases. The kinetic parameters obtained for three purified proteins in this study are given in Table 3.
TABLE 3 - KINETIC PARAMETERS FOR REACTION OF PURIFIED Cyt \( b_{561} \) WITH ASCORBATE (32)

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( A_1 ) (%)</th>
<th>( k_1 ) (s(^{-1}))</th>
<th>( A_2 ) (%)</th>
<th>( k_2 ) (s(^{-1}))</th>
<th>( A_3 ) (%)</th>
<th>( K_3 ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt ( b_{561} ) (CG)</td>
<td>42</td>
<td>11</td>
<td>37</td>
<td>2.1</td>
<td>22</td>
<td>0.25</td>
</tr>
<tr>
<td>His-cyt ( b_{561} ) (Sf9)</td>
<td>39</td>
<td>12</td>
<td>39</td>
<td>2.2</td>
<td>22</td>
<td>0.3</td>
</tr>
<tr>
<td>His-cyt ( b_{561} ) (Yeast)</td>
<td>45</td>
<td>34</td>
<td>28</td>
<td>4.9</td>
<td>27</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Notes: Reaction of purified protein (1 \( \mu \)M) with Asc (8 mM). \( A_1, A_2 \) and \( A_3 \) are % of overall absorbance change. \( k_1, k_2 \) and \( k_3 \) are apparent first order rate constants. His - tagged cyt \( b_{561} \) was purified from Sf9 and yeast cells.

2.2.6 Electron Transfer Process in Cyt \( b_{561} \)

Cyt \( b_{561} \) is rapidly oxidized and reduced by external electron acceptors and donors, respectively. The \( K_m \) of cyt \( b_{561} \) reduction by Asc is 340 \( \mu \)M (253), and under normal physiological conditions cyt \( b_{561} \) is mostly present in the reduced state (275).

Although the structure of cyt \( b_{561} \) is not resolved it believed that cyt \( b_{561} \) should have two specific sites, one located on the extravesicular side for Asc binding and the other on the intravesicular side for SDA binding (259). DEPC modification of the protein inhibited the reduction of heme by Asc (276) without affecting electron donating activity from reduced heme to SDA (277) indicating that an essential histidine residue in the Asc binding site. The major DEPC modification sites were identified as fully conserved.
His88, His161 and Lys85 located in the extravesicular side by MALDI-TOF analyses (277). The fact that Asc protects cyt $b_{561}$ from inactivation by DEPC further confirmed the presence of an Asc binding site of cyt $b_{561}$ located on the cytosolic side (278). Kobayashi et al. (279) have studied the electron transfer process of each individual heme center by pulse radiolysis and observed that only half of cyt $b_{561}$ is oxidized in the presence of excess SDA. After pretreating the cyt $b_{561}$ under mild alkaline conditions, the SDA radical could not oxidize the cyt $b_{561}$, but is reduced by Asc. This indicates that the two heme b centers have distinct roles in electron transfer and acceptance.

Nakanishi et al. (259) have recently proposed a novel mechanism for electron transfer from Asc to cyt $b_{561}$ (Figure 9). According to this mechanism, heme $b$ on the cytosolic side accepts an electron/proton from Asc and after releasing the product (SDA), the electron is transferred to the second heme on the intravesicular side and the proton on the axial imidazole group is transferred to the outer surface of cyt $b_{561}$ molecule via an unknown relay system.
Figure 9 - Proposed histidine cycle mechanism for the concerted proton/electron transfer reaction from Asc to heme b center of cyt b_{561}(259).
CHAPTER III

EXPERIMENTAL METHODS

3.1 Part I

3.1.1 Materials

Standard chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted. Protein assay reagents were obtained from Bio-Rad (Hercules, CA). Catecholamine standards were obtained from ESA (Chelmsford, MA). Fetal bovine serum (FBS) was purchased from Valley biomedical (Winchester, VA). F-12K medium and horse serum (HS) were purchased from American Type Culture Collection, ATCC (Manassas, VA). UltraPure 10X TBE buffer and SYBR Safe DNA gel stain in 0.5X TBE were purchased from Invitrogen (Eugene, OR). DNA marker was purchased from New England Biolabs Inc. (Ipswich, MA). Copper, volumetric standard from Aldrich and zinc reference solution from Fisher Scientific were used as the source of Cu (II) and Zn (II) in all experiments respectively. Stock solutions of bathocuproine (BCP), NCP, neocuproine (NCP), and 2’,7’-dichlorofluorescein diacetate (DCF-DA) were prepared in dimethyl sulfoxide (DMSO). All the solutions were prepared in Milli Q-deionized water (Millipore, Billerica, MA). KRB-HEPES contained 125 mM NaCl, 2 mM KCl, 1.4 mM MgSO\textsubscript{4}, 1.2 mM CaCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, 5 mM glucose, pH 7.4. KRB-bicarbonate contained 109.5 mM NaCl, 5.34 mM KCl, 0.81 mM MgSO\textsubscript{4}, 1.8 mM CaCl\textsubscript{2}, 0.77 mM NaH\textsubscript{2}PO\textsubscript{4}, 44 mM NaHCO\textsubscript{3}, 5.55 mM glucose.
3.1.2 Instrumentation

UV-visible spectra were recorded on a Cary Bio 300 UV-visible spectrophotometer (Varian, Inc). Fluorescence emission spectra were recorded on a Jobin Yvon-Spex Tau-3 spectrophotometer (ISA Instruments, Inc.). Dopamine levels were analyzed by reversed-phase HPLC with electrochemical detection (HPLC-EC) on a C$_{18}$ reversed phase column. HPLC-EC analyses were performed using ESA Model 582 solvent delivery module and Coulochem-II electrochemical detector with ESA 501 chromatographic software (ESA, Chelmsford, MA, USA).

3.1.3 Methods

3.1.3.1 Cell Culture

Neuroblastoma SH-SY5Y and rat pheochromocytoma (PC12) cell lines were obtained from ATCC. The rat hybridoma MN9D cell line was graciously provided by Drs. Alfred Heller and Lisa Won (University of Chicago). HepG2 cells were from Dr. Tom Wiese (Fort Hays University, Hays, KS). All cell lines except PC12 were grown in Dulbecco’s Modified Eagles Medium (DMEM) containing 10% FBS and maintained at 37 °C in an atmosphere of 5% CO$_2$ in humidified air. PC12 cells were grown in F-12K medium supplemented with 2.5% FBS and 15% horse serum at 37°C and 5% CO$_2$. Cells were fed three times weekly, passaged by incubation in Trypsin-Versene, and plated at the density of 2 × 10$^6$ cells per 10 cm tissue culture plate.

3.1.3.2 Cellular Viability

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay (280). Cells were seeded on Falcon 96-well plates and allowed to grow until 70-80% confluence. Cells were treated with the desired
concentration of Cu(II), Zn(II) or other reagents in KRB-HEPES or KRB-bicarbonate buffer and incubated for 6 h at 37 °C (see Figure Legends for further details). After the incubation, 10 μL of 5 mg/mL MTT solution was added to each well and was incubated for 2 h at 37 °C. The resulting formazan was solubilized by 210 μL of detergent containing 50% DMF and 20% SDS and incubated for 12 h at 37 °C. MTT reduction was quantified based on the difference in the absorbance at 570 nm and 650 nm.

Results are expressed as % viability of metal treated cells with respect to control cells which were treated under the same experimental conditions except in the absence of the metal. In the experiments where DMSO was used the final concentration was kept to a minimum usually <0.5% v/v.

3.1.3.3 Intracellular Copper Content

Cells grown to 70-80% confluence in 12-well plates and were washed with KRB-bicarbonate buffer and incubated with the desired concentrations of BCP and Cu(II) in the same buffer for 1 h at 37 °C. After the incubation, cells were washed twice with ice-cold KRB-HEPES, gently scraped and collected in 1.0 mL cold KRB-HEPES. Aliquots were removed for protein assay and the remainder was centrifuged for 3 min at 6000 rpm. The supernatant was discarded and the cell pellet was suspended in 45 μL of 6 M HNO₃. The coagulated proteins were removed by centrifugation and the supernatant was neutralized with 5 M KOH and the neutral solutions were buffered with 0.1 M KH₂PO₄, pH 7.4. Cu(II) in the extracts was reduced with sodium ascorbate (200 μM) and was quantified using Cu (I) specific chelator, bathocuproinedisulfonic acid (200 μM), by measuring the absorbance at 483 nm (281) using a calibration curve constructed
under similar conditions. All copper levels were normalized for the protein content of each sample.

### 3.1.3.4 Intracellular Zinc Content

Intracellular Zinc levels were also determined using a similar protocol, but using Zn(II) specific reagent 4-(2-pyridylazo)-resorcinol, (PAR) at pH 8.5 and quantifying the PAR-Zn (II) complex at 500 nm (282).

### 3.1.3.5 Intracellular Catecholamine Content

Cells were plated in 12-well plates and grown to 70-80% confluence. Cells were washed and incubated with KRB-HEPES buffer containing Cu(II) or Zn(II) in the presence or absence of BCP in KRB-HEPES for 60 min at 37°C. The incubation media was removed and cells were washed twice with ice-cold KRB-HEPES. Cells were pelleted by low-speed centrifugation and lysed with 75 µL of 0.1 M HClO₄. The catecholamines were separated and quantified by HPLC-EC as previously described (247) using ESA Model 582 solvent delivery module and Coulouchem-II electrochemical detector with ESA 501 chromatographic software (ESA, Chelmsford, MA). In brief, acidic cell extracts were separated on a C₁₈ reversed phase column (ESA, HR-80) using mobile phase composed of 75 mM NaH₂PO₄, 1.7 mM 1-octanesulfonic acid sodium salt, 25 µM Na₂EDTA, 100µL/L triethylamine, pH 3.0, with 10% CH₃CN at a flow rate of 0.8 ml/min. The corresponding control experiments were carried out using a similar protocol except that Zn(II), Cu(II) or BCP were excluded from the incubation media. All catecholamine concentrations were normalized for the protein content of each sample.
3.1.3.6 Measurement of Reactive Oxygen Species

Cells which were cultured in 12-well plates were rinsed with KRB-bicarbonate buffer and pre-incubated with 10 μM DCF-DA for 60 min. Then, DCF-DA loaded cells were washed with ice-cold buffer, and treated with Zn(II) and/or BCP for 60 min at 37°C. After the incubation cells were washed, harvested, solubilized with 0.1 mol/L Tris buffer (pH 7.5) containing 1% Triton X-100 and cell debris was removed by centrifugation. The 2’,7’-dichlorofluorescein (DCF) content in the supernatants was determined by fluorescence with excitation at 504 nm and emission at 526 nm using a Jobin Yvon-Spex Tau-3 spectrofluorimeter (ISA Instruments, Inc.). All fluorescence readings were normalized to protein content of individual samples.

3.1.3.7 Apoptotic DNA Fragmentation

Cells were grown in 12-well plates for 2 days and treated with 15 μM Cu (II)/Zn (II) and 2 μM BCP for 3 h. After the incubation time the media was carefully aspirated and 500μL of KRB was added to each well. Cells were collected into 1.5 mL eppendorf tubes (3 wells were combined) and centrifuged 2,000 rpm for 2 min. The supernatant was aspirated and cells were reconstituted in 200 μL of PBS. DNA was extracted using Roche Apoptotic DNA Ladder Kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. DNA samples were loaded onto a 1.2% agarose gel prepared in 0.5X TBE (Tris-borate-EDTA) containing SYBR Safe DNA gel stain. The gel was run at 70 V for 1-2 h and DNA bands were visualized under UV light and photographed using a Kodak Gel Logic 100 system (Eastman Kodak, Rochester, NY).
3.1.3.8 ATP Assay

Cells were cultured in 12-well plates for 2 days and treated with 4 μM BCP and 20 μM Cu(II)/Zn(II) for 1h in KRB-HEPES buffer. Following incubation period cells were collected to 1mL KRB buffer and 50 μL was taken out for the protein assay. The rest was centrifuged for 3 min at 6,000 rpm and supernatant was removed. ATP content was determined using BioVision ATP Colorimetric/Fluorometric Assay Kit. Total final volume was made to 1mL by cell lysis buffer (0.1 mol/L Tris, 1% Triton X-100, pH 7.5). ATP content was assayed by measuring fluorescence excitation at 535 nm and emission at 587 nm by Varian Cary Eclipse fluorescence spectrophotometer. The fluorescence reading was normalized to protein content of individual sample.

3.1.3.9 Protein Determination

Protein contents of various cell preparations were determined by the method of Bradford (283) using bovine serum albumin as the standard. Samples of cell suspensions (50 μL) in KRB-HEPES were incubated with 950 μL of Bradford protein reagent for 10 min and absorbance at 595 nm was measured.

3.1.3.10 Data Analysis

To control for minor variations in color development in the MTT assay, absorbance readings were converted to a percentage of parallel controls, which were treated identically except that external Cu(II), Zn(II) or other experimental reagents were excluded from the incubations. All quantitative uptake, catecholamine levels, and ROS data were normalized to protein content of individual incubations to correct the results for the variations of cell densities between individual experiments. All the experimental
were carried out in triplicates for 3-5 times and averages of all the data or a set of representative data are shown. The error bars represent the standard deviations of the data.

3.2 Part II

3.2.1 Materials

Asc, K$_3$Fe(CN)$_6$, and sodium dithionite were purchased from Aldrich. Tween-20 was purchased from Sigma. Dithiothreitol was obtained from Boehringer-Mannheim. SDS-PAGE reagents were from Bio-Rad, HEPES from Fisher and n-octyl β-D-glucopyranoside was from Calbiochem. Microcon YM-30 ultra-filtration devices were from Millipore Co, USA. Sucrose was purchased at the highest purity available from MP Biomedicals, LLC.

3.2.2 General Methods

All UV-visible spectra were recorded on Cary Bio 300 double beam UV-visible spectrophotometer (Varian, Inc.) equipped with a temperature-regulated multi-cell compartment at 25°C using 1.00 cm quartz cuvettes. Chromatographic protein separations were carried out on FPLC system (Pharmacia-LKB) equipped with a diode array detector at 4°C. The purities of the proteins were estimated by SDS-gel electrophoresis. All rapid kinetic experiments were carried out using OLIS RSM-1000 stopped-flow instrument at 25°C.

3.2.3 Isolation of Chromaffin Granules

Chromaffin granules were isolated from bovine adrenal glands collected at a local slaughterhouse. The glands were stored on ice during collection and transportation
to the laboratory and they were dissected the same day. Medullary tissue was collected from the glands and placed in ice cold 0.3 M sucrose, 10 mM HEPES buffer, pH 7.0. The tissues collected were diluted with the same buffer (1:5 w/v tissue: buffer ratio) and homogenized at low speed with a Biospec biohomogenizer [model M/33/1281-01]. The homogenate was placed in 250 mL centrifuge bottles and centrifuged at 3,600 rpm (1200 x g) for 10 min at 4°C in a pre-cooled JA-16.25 rotor (Beckman J2MC centrifuge). The supernatant was saved to be combined with the supernatant from the next centrifugation. The pellet was resuspended in 100 mL of buffer (0.3 M sucrose, 10 mM HEPES, pH 7.0) and homogenized with the biohomogenizer and centrifuged as before. Supernatants of first and second step were combined and centrifuged again as before. The resulting supernatant was placed in 50 mL centrifuge tubes and centrifuged at 17,000 rpm at 4°C for 30 min in a pre-cooled JA-17 rotor [Beckman J2MC centrifuge]. The supernatants were discarded and the fat (if any) sticking to the tube was removed with cotton swabs. The pellets were washed very carefully by swirling with ~1-2 mL of 0.3 M sucrose, 10 mM HEPES buffer, pH 7.0 with a gentle tap to remove the yellowish white fluffy upper layer. The washed pellets were resuspended (special caution was taken not to include any red blood cells appearing as a red dot at the bottom of the centrifuge tube) in 200-300 mL of the same buffer as before and homogenized using a Potter-Elvehjem homogenizer and the centrifugation step was repeated. The supernatant was discarded and the pellets were resuspended in a total volume of 85 mL of 0.3 M sucrose, 10 mM HEPES buffer, pH 7.0 and homogenized using the Potter-Elvehjem homogenizer. The homogenate (15 mL x 6) was layered on top of 1.6 M sucrose, 10 mM HEPES buffer, pH 7.0 (45 mL x 6) in ultracentrifuge tubes and
centrifuged at 27,500 rpm (58,700 x g) for 90 min at 4°C in a pre-cooled Sorvall A-641 rotor [Beckman Optima LE-80 Ultracentrifuge]. The supernatant and membranous material was removed under suction. The pink pellets obtained were washed with 3 x 1 mL of 1.6 M sucrose, 10 mM HEPES buffer, pH 7.0 to remove any remaining fat (usually appearing as a yellowish layer on top of the pellets). The pellets were then washed with 0.2 M Tris-phosphate, pH 7.0 to remove the sucrose and resuspended in 56 mL of 0.2 M Tris-phosphate buffer, pH 7.0 and homogenized using a Potter-Elvehjem homogenizer. Then the volume of the homogenate was measured and 1/7 volume of a solution of glycerol: 0.2 M Tris-phosphate buffer, pH 7.0 [3:7 (v/v)] was added to the homogenate and allowed to lyse for 20 min at room temperature. The chromaffin granules were transferred to a plastic bottle, frozen as a thin film using liquid nitrogen, and stored at -70°C.

3.2.4 Purification and Characterization of Cytochrome \( b_{561} \)

3.2.4.1 Purification of Cytochrome \( b_{561} \) from Chromaffin Granules

The lysed frozen granules were diluted in 50 mM KH\(_2\)PO\(_4\), pH 7.0 (1:1 (v/v)), and centrifuged at 17,000 rpm for 30 min at 4°C (Beckman J2MC centrifuge). The supernatant was discarded and the pellet was rinsed with KH\(_2\)PO\(_4\), pH 7.0 buffer. The pellet was re-suspended in 50 mL (for 50 g wet medulla tissue) of 50 mM potassium phosphate, pH 7.0 containing 2% (w/v) Tween-20 and 18% (w/v) Asc and stirred for 1 h at 4°C in the dark, under nitrogen. Then the suspension was centrifuged at 50,000 rpm for 1h in the Beckman Optima LE-80 ultracentrifuge using pre-cooled 70.1 Ti rotor at 4°C. The Tween-20 washed pellets were re-suspended in 50 mL (for 75 g of wet medulla tissue) of 25 mM diethanolamine.HCl, pH 9.3, containing 1% (w/v) n-octyl β-D-
glucopyranoside, and 0.5 mM dithiothreitol. The sample was stirred in the dark for 1 h at 4°C under nitrogen for complete solubilization and was centrifuged at 50,000 rpm for 1 h at 4°C. The supernatant was loaded onto a Mono-Q column (Pharmacia-LKB HR 10/10; pre-equilibrated with 25 mM diethanolamine-HCl, pH 9.3 containing 1% (w/v) n-octyl β-D-glucopyranoside, 0.5 mM dithiothreitol) controlled by a Pharmacia FPLC system equipped with a flow through cell assembly and a diode array UV-vis spectrophotometer (Hewlett Packard) as the detector, at 4°C, with a flow rate of 2 mL/min. The column was washed with the equilibration buffer at 2 mL/min until the A$_{280}$ returned to baseline. The Cyt b$_{561}$ was eluted at a flow rate of 2 mL/min using a step gradient of NaCl (0 - 62.5 mM for 5 min, 62.5 - 150 mM for 100 min, and 150 mM to 500 mM for 10 min) in 25 mM diethanolamine-HCl, pH 9.3, containing 1% n-octyl β-D-glucopyranoside, 0.5 mM dithiothreitol. In a typical experiment most of the cyt b$_{561}$ elutes at 85 -115 mM NaCl. Fractions with high 427 nm absorbance were combined, concentrated, and reconstituted into the storage buffer containing 20 mM potassium phosphate, pH 6.8, 20% (w/v) glycerol, 1% (w/v) n-octyl β-D-glucopyranoside, except when special storage conditions were necessary for subsequent experiments. The purified protein was transferred to eppendorf tubes, frozen using liquid nitrogen, and stored at -70°C.

3.2.4.2 Preparation of Oxidized Cytochrome b$_{561}$

Purified cyt b$_{561}$ was oxidized with a slight excess of K$_3$Fe(CN)$_6$ in 100 mM potassium phosphate buffer, pH 6.8, 20% (w/v) glycerol, 1% (w/v) n-octyl β-D-glucopyranoside. The excess K$_3$Fe(CN)$_6$ and K$_4$Fe(CN)$_6$ were removed by ultra-filtration in an Amicon Cell using a YM-30 membrane. The concentrated, oxidized Cyt b$_{561}$ was stored at -70°C in a storage buffer containing 20 mM potassium phosphate buffer, pH
6.8, 20% (w/v) glycerol, 1% (w/v) n-octyl β-D-glucopyranoside for the following experiments, unless otherwise stated.

3.2.4.3 UV-visible Spectral Characteristics of Oxidized and Reduced Cytochrome $b_{561}$

All spectra were recorded in 100 mM potassium phosphate buffer, pH 6.8, 20% (w/v) glycerol, 1% (w/v) n-octyl β-D-glucopyranoside. The spectra were recorded immediately after the reduction of the oxidized cyt $b_{561}$ with 2 mM Asc followed by 5 mM dithionite. The concentration of cyt $b_{561}$ was determined by using an extinction coefficient of 267.9 mM$^{-1}$ cm$^{-1}$ at 427 nm for the dithionite reduced protein (30).

3.2.4.4 Purity and Molecular Weight Determination of Cytochrome $b_{561}$

Purity of cyt $b_{561}$ was determined by SDS polyacrylamide gel electrophoresis. Gels were stained with silver stain which was purchased from Bio Rad. (general protocol is given in Appendix).

3.2.5 Pre-Steady State Kinetics

$K_3$Fe(CN)$_6$ oxidized cyt $b_{561}$ was used in the experiments. Excess oxidant was removed by ultrafiltration in an Amicon Cell using a YM-30 membrane. The protein was reconstituted in 20 mM potassium phosphate buffer with 1% (w/v) n-octyl β-D-glucopyranoside to a final concentration of 2 μM. Rapid kinetic experiments were carried out using OLIS RSM-1000 stopped-flow instrument at 25°C. The two sample syringes were filled with oxidized protein and an Asc solution prepared in the same buffer (2-20 mM). Equal amounts of the two solutions were mixed and absorbance spectra were recorded in 1 ms intervals or absorbance changes were measured as a function of time at fixed wave length of 427 nm.
3.2.5.1 Analysis of the Pre-Steady State Kinetic Data

Experimental data was fitted to a function of three exponential terms. The fastest phase of the reduction was considered as the first one-electron transfer reaction from Asc to heme $b$ center at the extravesicular side. The apparent rate of the fastest phase was further analyzed using transient kinetic method based on a two-step bi-uni sequential ordered mechanism [equation 5] (31). The apparent rate constants for the fastest phase ($k_{\text{app}1}$) versus Asc concentration showed saturation at higher Asc concentrations. Therefore, assuming that the second step is much slower than the first step, the equation 5 could be reduced to equation 6; where $K_s = k_1/k_2$, $[E]$ and $[S]$ are concentrations of E and S at equilibrium. As $[E]$ is much smaller than $[S]$, $[E]$ could be neglected. Also assuming $k_2$ is small, equation 6 could be reduced to a Michaelis-Menten-type equation and $K_s$ and $k_2$ were obtained by a double reciprocal plot of $k_{\text{app}1}$ and [Asc].

$$ E + S \xrightleftharpoons[k_1]{k_1} ES \xrightleftharpoons[k_2]{k_2} E \ast (+P) $$

(5)

$$ k_{\text{app}1} = \frac{k_2([E] + [S])}{K_s + ([E] + [S])} + k_2 $$

(6)
CHAPTER IV

RESULTS

4.1 Part I

4.1.1 Zn(II) and Cu(II) Uptake Experiments

Cells were incubated for short time periods with low concentration of Cu(II) and Zn(II) along with BCP and cellular copper and zinc levels were determined using UV-vis spectrophotometry. In copper uptake experiments, cell extracts were treated with excess sodium ascorbate to reduce Cu(II) to Cu(I) and bathocuproine disulphonic acid (BCD) was added. Cu(I) makes a red color complex with BCD which has an absorption maximum at 483 nm as shown in Figure 10A. A calibration curve was constructed using standard Cu(II) solution under similar conditions as the cell samples. Similarly, intracellular zinc contents were measured by 4-(2-pyridylazo)-resorcinol (PAR). Absorption spectrum of Zn(II)-PAR complex is shown in Figure 10B.

4.1.1.1 Zn(II) and Cu(II) Uptake into Cells in the Presence of BCP

BCP increases Cu(II) and Zn(II) uptake into all cells. The uptake of free Cu(II) and Zn(II) into both catecholaminergic and HepG2 cells is low under the experimental conditions. However, membrane permeable Cu(II) and Zn(II) chelator, BCP, increases both Zn(II) and Cu(II) uptake into catecholaminergic cells by about 5-10 fold (depending on the cell type) in the presence of 20 μM BCP with 30 μM Cu(II) or Zn(II) under similar conditions (Figures 11A & B). BCP mediated increase of metal uptake was most pronounced with PC12 cells and least pronounced with HepG2 cells. A similar and parallel behavior was observed with another membrane permeable Cu(II)/Zn(II)
chelator, NCP (Figure 11C), but uptake was not increased by the membrane non-permeable chelator, BCP disulfonate (data not shown).

Figure 10 – Electronic absorption spectra of Cu(I)-BCD and Zn(II)-PAR complexes. A, [Cu(I)(BCD)_2]^3⁻ 8 μM, pH 7.4; B, Zn(II)(PAR)_2 7.5 μM, pH 8.5.
Figure 11 – BCP & NCP-induced Cu(II) & Zn(II) uptake. Cells were grown to near confluence in 12-well plates and incubated with 20 µM BCP and 30 µM Zn(II) or Cu(II) in KRB-bicarbonate buffer for 1 h. Cells were washed, collected and intracellular copper and zinc levels were determined as detailed in Experimental Methods. A, Cu(II) uptake; B, Zn(II) uptake. Baseline copper and zinc levels of untreated cells were subtracted from all readings. D, Intracellular copper levels of MN9D cells treated with similar concentrations of Cu(II) and NCP as above. Data represent mean ± S.D. of triplicate samples.

4.1.1.2 Time Dependent and Concentration Dependent Metal Uptake

BCP mediated Cu(II) uptake is time and concentration dependent: As shown from the data in Figure 12A, uptake of Cu(II) into MN9D cells is increased by about 5
fold when extracellular free Cu(II) concentration is increased from 0 to 40 μM during 1 h incubation period. In the presence of constant 20 μM BCP, the uptake was increased by about 10-12 fold under the same conditions. The uptake of Cu(II) was apparently saturable in the absence of BCP, but linearly increased in the presence of BCP at this concentration range. Similarly, the uptake of Cu(II) (30 μM) in the presence of constant 20 μM BCP was increased as a function of time during the 1 h incubation period (Figure 12B) displaying apparent biphasic kinetics.

Figure 12 – Concentration- and time-dependence of Cu(II) uptake. A, Near confluent MN9D cells were incubated with 20 μM BCP and 0-40 μM Cu(II) for 60 min in KRB-bicarbonate buffer and intracellular copper levels were determined as detailed in Experimental Methods. B, MN9D cells were incubated with 20 μM BCP and 30 μM Cu(II) for the desired time period (0-60 min) and intracellular copper levels were determined as in A. Data represent mean ± S.D. of triplicate samples.
4.1.1.3 Effect of BCP Concentration on Copper and Zinc Uptake

Increase of Cu(II) and Zn(II) uptakes into MN9D and PC12 cells requires only substoichiometric (catalytic) amounts of BCP: At a fixed 30 μM extracellular concentration of Cu(II), the optimum BCP concentration required for maximum uptake into MN9D cells was about 2 μM (Figure 13A). A similar behavior was observed with BCP mediated increase of Zn(II) uptake into MN9D cells (Figure 13B) as well as both Cu(II) and Zn(II) uptake into SH-SY5Y and PC12 cells (data not shown). In contrast to the behavior of catecholaminergic cells, the effect of BCP on Cu(II) uptake into HepG2 cells was notably different. The uptake was biphasic and only slightly increased up to about 10 μM BCP and sharply increased above 10 μM at 20 μM fixed Cu(II) concentration (Figure 13C). However, Zn (II) uptake into HepG2 cells (Figure 13D) followed a trend similar to that was observed for MN9D cells with the exception that maximum uptake requires somewhat higher BCP (about 8 μM).
Figure 13 - Stoichiometry of BCP-induced Cu(II) & Zn(II) uptake. Cells were treated with KRB-bicarbonate buffer containing 0-30 µM BCP and 30 µM Cu(II) or Zn(II). Following 60 min incubation intracellular copper and zinc levels were determined as detailed in Experimental Methods. A, Cu(II) uptake of MN9D cells. B, Zn(II) uptake of Mn9D cells. C, Cu(II) uptake of HepG2 cells. D, Zn(II) uptake of HepG2 cells. Data represent mean ± S.D. of triplicate samples.
4.1.1.4  Involvement of Calcium Channels and Extracellular Calcium on the Cu(II) and Zn(II) Uptake

It has been previously suggested that Zn(II) can be transported through L-VGCCs (95). Therefore, we have examined the effect of extracellular Ca\(^{2+}\) and voltage-gated calcium channel inhibitors on Cu(II) and Zn(II) uptake.

4.1.1.4.1  Effect of L-VGCC Inhibitors

*Calcium channel blockers partially inhibit copper(II) and zinc(II) uptake:* L-VGCC blocker, nitrendipine, partially inhibits the uptake of both Zn(II) and Cu(II) into PC12 (Figures 14A & B) and MN9D (Figures 14C & D) cells in the presence of BCP. The effect of nitrendipine in the absence of BCP could not be accurately determined since the uptake levels are too small. However, nitrendipine had no significant effect on the Cu(II) uptake into HepG2 cells (Figure 15A), while Zn(II) uptake was partially inhibited similar to that was observed with PC12 cells (Figure 15B).
Figure 14 - Inhibitory effect of nitrendipine on Cu(II) & Zn(II) uptake in PC12 and MN9D cells. Cells were treated with 50 µM nitrendipine for 10 min prior to the incubation with 2 µM BCP and 20 µM Zn(II) or Cu(II) 1 h. A & B, PC12 copper uptake and zinc uptake in KRB-HEPES buffer. C & D MN9D copper and zinc uptake in KRB-bicarbonate buffer. Data represent mean ± S.D. of triplicate samples.
Figure 15 - Effect of nitrendipine on BCP-induced Cu(II) & Zn(II) uptakes in HepG2 cells. Cells in near confluence were pre-treated with 50 µM nitrendipine for 10 min and another 1 h with 2 µM BCP and 20 µM Zn(II) or Cu(II) for 1 h in KRB-HEPES buffer. Zinc and copper uptakes were measured spectrophotometrically as described in Experimental Methods. A, Cu uptake; B, Zn uptake.
Another commonly used L-VGCC blocker, nifedipine, also inhibited Zn(II) uptake in the presence of BCP similar to nitrendipine. However, nifedipine increased Cu(II) uptake and the uptake values were similar in the presence and absence of 2 µM BCP as shown in Figure 16.

![Figure 16 - Effect of nifedipine on copper uptake. MN9D cells in near confluence were pre-treated with 50 µM nifedipine for 10 min and another 1 h with 20 µM BCP or BCP and 30 µM Cu(II) for 1 h in KRB-HEPES buffer. Copper uptake was measured spectrophotometrically as described in Experimental Methods.](image)

4.1.1.4.2 Effect of Extracellular Calcium

The exclusion of extracellular calcium in the incubation medium increased both Cu(II) and Zn(II) uptake into PC12 cells significantly (Figures 17A & B). The experiments were carried out in the presence or absence of 1.8 mM Ca²⁺ in the KRB-bicarbonate buffer. Cell culture media was carefully aspirated and cells were washed 2 or 3 times with Ca²⁺ free buffer and incubated with 2 µM BCP and Cu(II)/Zn(II). Ca²⁺ chelators could not be used in the buffer since they can chelate Cu(II) and Zn(II) also.
Figure 17 - Effect of extracellular Ca$^{2+}$ on BCP-induced Cu(II) and Zn(II) uptake. Cells in near confluence were treated with 2 µM BCP and Zn(II) or Cu(II) in regular or Ca$^{2+}$-free KRB-bicarbonate buffer for 1 h. Zn(II) and Cu(II) uptakes were measured as described in *Experimental Methods*. Data represent mean ± S.D. of triplicate samples.

4.1.2 Characterization of Cellular Toxicity of Copper and Zinc

It was previously reported that higher concentrations of copper and zinc were toxic to various cell lines (13, 19, 93). We tested copper and zinc toxicity to three catecholaminergic cell lines PC12, MN9D, and SH-SY5Y and compared with hepatic...
HepG2 cells. The cells were treated with 10-60 µM of Cu(II) or Zn(II) for 6 h in KRB-HEPES buffer. The cell viability was tested spectrophotometrically by the MTT assay.

Figure 18 - Effect of free Cu(II) and Zn(II) on cell viability. Cells were grown in 96-well plates and were incubated with 0-60 µM Cu(II) or Zn(II) in KRB-HEPES buffer for 6 h. Cell viabilities were determined by MTT assay and were expressed as a percentage of control where cells were not exposed to Cu(II) or Zn(II) as detailed in Experimental Methods. A, Cu(II) toxicity; B, Zn(II) toxicity. Data represent mean ± S.D. of five samples.
Micromolar concentrations of Cu(II) and Zn(II) are toxic to catecholaminergic cells: The data presented in Figures 18A & B show that viability of all catecholaminergic cells decreased with increasing concentration of the metal ion in the concentration range of 0-60 µM during 6 h incubations in KRB. The data also show that Cu(II) is significantly more toxic to the catecholaminergic cells in comparison to Zn(II). Furthermore, MN9D cells appear to be more sensitive to both Cu(II) and Zn(II) especially with concentrations above 20 µM, and HepG2 cells were more resistant to both metals under similar experimental conditions. For example, while 50 µM Cu(II) reduced MN9D cell viability by about 80-90% HepG2 viability was reduced only by about 25-30% after 6 h incubation.

4.1.2.1 Effect of BCP on Cu(II) and Zn(II) Toxicity

Effect of BCP on copper and zinc induced cell toxicity was tested. Cells were pre-incubated with 20 µM BCP for 1 h and treated with 0-25 µM Cu(II) and Zn(II) for 6 h.

Membrane permeable chelators increase Cu(II) and Zn(II) toxicities: BCP markedly increases the toxicities of both Zn(II) and Cu(II) to all three catecholaminergic cells (Figures 19A-C). However the effect of BCP on HepG2 cell toxicity was much less pronounced under similar experimental conditions (Figure 19D). Interestingly, in the presence of BCP, Zn(II) was more toxic to PC12 and HepG2 cells in comparison to Cu(II) (Figures 19C & D).
Figure 19 – Effect of BCP on Cu(II) and Zn(II) toxicities. Cells were incubated with 20 µM BCP in KRB-bicarbonate buffer for 60 min. Then the media was replaced with Cu(II) or Zn(II) in and incubated for 6 h. Cell viabilities were then assessed using the MTT assay and were expressed as a percentage of control, where cells were not exposed to Zn(II) or Cu(II) as detailed in Experimental Methods. A, SH-SY5Y cells; B, MN9D cells; C, PC12 cells; D, HepG2 cells. Data represent mean ± S.D. of five samples.
Similar to BCP, NCP also increased Cu(II) and Zn(II) toxicity. Figure 20 shows increase in Cu(II) toxicity when MN9D cells were treated with NCP and Cu(II) for 6 h, under similar experimental conditions as in Figure 19.

![Graph showing effect of NCP on Cu(II) toxicity](image)

Figure 20 – Effect of NCP on Cu(II) toxicity. MN9D cells were grown to near confluence in 96-well plates. Growth media was replaced with KRB-bicarbonate buffer containing 20 µmol/L NCP. After 60 min, media was aspirated and incubated with Cu(II) for 6 h. Cell viabilities were then assessed using the MTT assay and were expressed as a percentage of control, where cells were not exposed to NCP or copper. Data represent mean ± S.D. of five samples.

While membrane permeable chelator, BCP increased the Cu(II) and Zn(II)-induced cell death, the membrane non-permeable metal chelator, bathocuproine disulfonate (BCD) protected cells from metal toxicity (Figure 21).
Figure 21 – Effect of BCD on Cu(II) toxicity. MN9D cells grown in 96-well plates were incubated with 20 µM BCD, 20 µM Cu(II) or Cu+BCD in KRB-HEPES buffer for 5 h. Cell viabilities were then assessed using the MTT assay and were expressed as a percentage of control, where cells were not exposed to BCD or Cu(II). Data represent mean ± S.D. of five samples.

4.1.2.2 Effect of BCP Concentration on Copper and Zinc Induced Cell Death

Sub-stoichiometric (catalytic) amounts of BCP cause maximum Cu(II) and zinc(II) toxicity to catecholaminergic cells. Parallel to the uptake characteristics, only catalytic amounts of BCP are required for the maximum toxicity of Cu(II) or Zn(II) in catecholaminergic cells. Experiments with constant 20 µM Cu(II) with increasing concentrations of BCP showed that only about 2 µM BCP is required to exert the maximum toxicity in MN9D cells and a further increase of BCP had no significant effect on the toxicity (Figure 18A). A similar behavior was also observed with Zn(II) and BCP (Figure 22A), although the overall toxicity of Zn(II) was somewhat less pronounced than Cu (II). However, while HepG2 cells were resistant to 20 µM Cu(II) at all BCP concentrations up to 30 µM (Figure 22B), Zn(II) toxicity appears to slowly increase with increasing BCP concentration at the same Zn(II) concentration (Figure 22B).
Figure 22 – Effect of BCP concentration on Cu(II) and Zn(II) toxicity. Cells were treated with 0-50 µM BCP and 20 µM Cu(II) or Zn(II) in KRB-bicarbonate buffer for 6 h. Cell viabilities were then assessed using the MTT assay and were expressed as a percentage of control, where cells were not exposed to Cu(II) or Zn(II). A, MN9D cells; B, HepG2 cells. Data represent mean ± S.D. of five samples.
Figure 23A & B shows NCP dependent Zn(II) toxicity to SH-SY5Y cells and NCP dependent Cu(II) toxicity to MN9D cells. NCP itself is not toxic to cells (Figure 23B). Similar to BCP, substoichiometric amount of NCP required for maximum toxicity.

Figure 23 – Effect of NCP concentration on Cu(II) & Zn(II) toxicity. Cells were grown to near confluence in 96-well plates and washed with bicarbonate buffer, and replaced with same buffer containing 0-60 µM NCP, incubated 30 min and added 20 µM Cu(II)/Zn(II) and incubated for 6 h. Cell viabilities were then assessed using the MTT assay and were expressed as a percentage of control. A, SH-SY5Y cells treated with Zn(II); B, MN9D cells treated with Cu(II).
4.1.2.3 Effect of Extracellular Ca\(^{2+}\) and L-VGCCs on Toxicity

*Nitrendipine and extracellular calcium partially protect Zn(II) and Cu(II) toxicities to catecholaminergic cells:* Toxicities of both Cu(II) and Zn(II) were significantly decreased by the presence of extracellular Ca\(^{2+}\) (1.8 mM) as shown Figures 24 A & B, parallel to the effects on metal uptakes. Similar to uptake extracellular Ca\(^{2+}\) has more effect on Zn(II) toxicity (Figure 24B).

![Figure 24](image)

Figure 24 – Effect Ca\(^{2+}\)-free media on Zn(II) and Cu(II) -induced toxicities. PC12 cells in near confluence were treated with various concentrations of Zn(II) or Cu(II) in regular or Ca\(^{2+}\)-free KRB-bicarbonate buffer for 6 h. Cell viabilities were determined by MTT assay and were expressed as a percentage of control, where cells were not exposed to Cu(II) or Zn(II). A, Cu(II) toxicity. B, Zn(II) toxicity. Data represent mean ± S.D. of five samples.

Parallel to the effects on uptake, nitrendipine partially protects the PC12 cells from Zn(II) toxicity in the absence and presence of BCP (Figure 25). However, nitrendipine does not significantly protect PC12 or MN9D cells from Cu(II) toxicity (data not shown) although Cu(II) uptake was also inhibited by nitrendipine similar to that of Zn(II).
Figure 25 – Inhibitory effect of Nitrendipine on Zn(II)-induced toxicity. A, PC12 cells in near confluence were incubated with 10 µM nitrendipine for 10 min followed by 40-60 µM Zn(II) in KRB-HEPES buffer for 6 h. Cell viability was assessed by MTT assay. B & C, Cells in near confluence were treated with 0, 10, 25 & 50 µM nitrendipine for 10 min followed by 2 µM BCP and 15 µM Zn(II) for 6 h in KRB-HEPES buffer. Cell viabilities were determined by MTT assay and were expressed as a percentage of control, where cells were not exposed to nitrendipine or Zn(II). B, PC12 cells; C, MN9D cells. Data represent mean ± S.D. of five samples.

4.1.3 Catecholamine Perturbation

Perturbation of chatecholamines is closely related with neurodegenerative disorders such as Parkinson’s disease which is characterized by degradation of
dopaminergic neurons. To test the catecholamine perturbation we measured dopamine depletion in MN9D and PC12 cells by Cu(II) and Zn(II) by HPLC-EC. SH-SY5Y cells contain relatively lesser amount of DA compared to other two cell lines. After 1 h incubation with different concentrations of by Cu(II) and Zn(II) in KRB-HEPES buffer, intracellular DA levels were determined and compared with DA levels of cells incubated in KRB-HEPES buffer without adding copper or zinc.

Incubation of PC12 cells with varying concentrations of Cu(II) or Zn(II) alone for 1 h caused a significant, concentration-dependent depletion of intracellular DA levels (note that PC12 cells contain largely DA, but only small amounts of NE and E and their changes were also parallel to that of dopamine) (Figures 22A & B). Parallel to the toxicities, DA depletion was more pronounced with Cu(II) compared to Zn(II) in both cell lines. A similar behavior was also observed with MN9D cells for both metals (Figures 23A & B).

Intracellular DA levels of PC12 cells were drastically reduced by the inclusion of 2 μM BCP with Zn(II) in the incubation medium parallel to the observed effects on the trends of uptake and toxicities (Figure 26A). A smaller but reproducible DA depletion was also observed in MN9D cells in the presence of 2 μM BCP (Figure 27A). However, while BCP had no significant effect on Cu(II) mediated depletion of DA in MN9D cells (Figure 27B); the effect on PC12 cells was somewhat unexpected. At low Cu(II) concentrations DA levels were significantly increased in the presence of 2 μM BCP and increasing concentrations of Cu(II) gradually decreased the DA levels. These results
were fully reproducible and only observed with Cu(II)/ BCP and PC12 cells (Figure 26B).

Figure 26 - Dopamine depletion by Cu(II) & Zn(II) in PC12 cells. Cells in near confluence were treated with different concentrations of Zn(II)/Cu(II) with or without 2 µM BCP for 60 min in KRB buffer. Following the 60 min incubation, cells were collected and protein and the DA contents were determined by HPLC-EC as detailed in Experimental Methods. Panel A, DA depletion by Zn or Zn+BCP; Panel B, DA depletion by Cu or Cu+BCP Values are expressed as the mean ± S. D. for three trials.
Figure 27 - Dopamine depletion by Cu(II) & Zn(II) in MN9D cells. Cells in near confluence were treated with different concentrations of zinc/copper with or without 2 µmol/L BCP for 60 min in KRB buffer. Following the 60 min incubation, cells were collected and protein and the DA contents (HPLC-EC) were determined as detailed in Experimental Methods. Panel A, DA depletion by Zn or Zn+BCP; Panel B, DA depletion by Cu or Cu+BCP. Values are expressed as the mean ± S. D. for three trials.
4.1.3.1 Pathway of DA Depletion

Neurotransmitters stored in secretory vesicles can deplete by either exocytosis or reversed action of monoamine transporters. In exocytosis, membrane bound vesicles move to the cell surface and then fuse with the plasma membrane and secrete molecules stored in the vesicles to extracellular fluid. Kiss-and-run is another process of exocytosis that takes place in some cells such as synapses. As indicated by the name, in this process the vesicles briefly contact the cell membrane and release neurotransmitters to the outside and retreat back into the cytosol.

The purpose of dopamine transporter (DAT) and norepinephrine transporter (NET) is to transport DA & NE secreted by synapses back to the neurons. These transporters can also act as a reverse transporter to efflux their substrates from the cytosol to the extracellular space (284, 285). Therefore, the possibility that DA depletion is due to the back transport of cytosolic DA through DAT and/or NET was tested by commonly used DAT & NET inhibitors (GRB 12909 and desipramine, respectively). DA depletion by Cu(II) in MN9D cells did not inhibit by pre-incubation of 10 μM DAT or NET inhibitor (Figure 28A). Similarly, as shown from the data in Figure 28B, pre-incubation of PC12 cells with 1 μM DAT & NET inhibitor for 10 min prior to the incubation with Cu(II) did not affect the DA depletion.
Figure 28 - Effect of DAT and NET inhibitors on DA depletion. A, MN9D cells in near confluence were treated with 10 µM GBR12909 or 10 µM desipramine hydrochloride for 10 min followed by 30 µM copper in KRB-HEPES buffer for 60 min. Following the 60 min incubation, cells were collected and the dopamine contents were determined as detailed in Experimental Methods. B. Similarly in A, PC12 cells were treated with 1 µM GBR12909 and 1µM desipramine hydrochloride for 10 min followed by 20 µM copper in KRB-HEPES buffer for 60 min. Values are expressed as the mean ± S. D. of three samples.
While the exclusion of extracellular Ca\(^{2+}\) completely inhibited the DA depletion by Cu(II), nitrendipine inhibited it only partially (Figure 29). DA depletion by Zn(II) also follows a similar trend with respect to extracellular calcium and nitrendipine, but was less pronounced (data not shown).

![Bar chart](image)

**Figure 29 – Effect of extracellular Ca\(^{2+}\) on Cu(II)-induced DA depletion.** Cells in near confluence were treated with 20 µM copper in KRB-HEPES buffer with or without 1.2 mM Ca\(^{2+}\) for 60 min. To determine the effect of voltage-gated Ca\(^{2+}\) channels cells were treated with 10 µM nitrendipine for 10 min followed by 20 µM Cu(II) for 60 min. Values are expressed as the mean ± S. D. of three samples.

### 4.1.4 BCP Increase Zinc-Induced ROS Production

**Zn(II) Increases the ROS Levels in Catecholaminergic Cells:** To determine whether the toxicity of Cu(II) and Zn(II) and the enhancement of toxicity by BCP were associated with the increase of ROS production, a series of experiments were carried out with the ROS sensitive reagent DFC-DA. Preliminary experiments revealed that the ROS levels could not be determined by DFC-DA in Cu(II) -BCP incubates. In the presence of BCP, Cu(II) efficiently oxidizes DFC-DA, producing large quantities of DFC,
independent of the presence or absence of ROS [Although many authors have reported the measurement of ROS using DFC-DA in the presence of Cu (II) and chelators our observations are consistent with the detailed study of Laggner et al. (286)]. Therefore, the ROS measurements were restricted to Zn(II). The preliminary experiments have shown that increasing concentrations of Zn(II) increase the intracellular ROS during 1 h incubation period in all three catecholaminergic cells. In addition, the inclusion of 2 μM BCP sharply increased the ROS levels in comparison to the levels in Zn(II) alone conditions in catecholaminergic cells parallel to the increase of uptake and toxicity (Figure 30).
Figure 30 – Effect of Zn(II)-BCP on intracellular ROS production. Cells in near confluence were treated with KRB-bicarbonate buffer containing 10 µM DFC-DA for 60 min. After the incubation the solution was aspirated, the cells were rinsed with cold buffer and incubated with 2 µM BCP and 0-20 µM Zn(II) for 60 min. Following the incubation cells were collected and DFC fluorescence was measured as detailed in Experimental Methods. A, SH-SY5Y cells; B, MN9D cells; C, PC12 cells treated with 20 µM BCP and 30 µM Zn(II). Values are expressed as the mean ± S. D. of three samples.

4.1.5 ATP Depletion

Effects of Copper (II) and Zinc (II) on the Intracellular ATP levels: The effect of copper (II) and zinc (II) on the intracellular ATP levels of PC12 cells were determined using BioVision ATP Colorimetric/Fluorometric Assay Kit. While both 20 µM Cu(II) and Zn(II) reduced the ATP levels by about 30% inclusion of 4 µM BCP further reduced the
ATP levels by about 80% for Zn(II) and 40% for Cu(II) (Figure 27A). The drastic reduction of ATP parallels the high toxicity of zinc towards PC12 cells in the presence of BCP. However ATP depletion by Zn(II) in the presence of BCP in HepG2 cells was also high similar to PC12 cells (Figure 31A). Even though the results were unexpected, it follows the trend that HepG2 cells also showed higher sensitivity to Zn(II) the presence of BCP compared to Cu(II).

![Graph A](image)

**Figure 31 – Effect of Zn(II) & Cu(II) on intracellular ATP levels.** Cells in near confluence were treated with KRB buffer containing 4 μM BCP and 20 μM Cu\(^{2+}\) or Zn\(^{2+}\) for 60 min. Following the incubation period cells were collected and ATP content was measured as detailed in *Experimental Methods*. A, PC12 cells; B, HepG2 cells.

### 4.1.6 Apoptotic DNA Ladder

Both Cu(II) and Zn (II) induce apoptotic DNA fragmentation in PC12 cells: As shown in Fig 32, 15 μM Cu(II) or Zn(II) and 2 μM BCP treatment of PC12 cells for 3 h produced a pattern of DNA fragmentation consistent with an apoptotic cell death with respect to a control in the absence of the metal.
Figure 32 - Cu-BCP and Zn-BCP induce nucleosomal DNA fragmentation. PC12 cells in near confluence were treated with KRB-HEPES buffer containing 2 μM BCP and 15 μM Cu²⁺ or Zn²⁺ for 3 h. Following the incubation period cells were collected, samples were prepared, electrophoresed on a 1.2% agarose gel and visualized as detailed in Experimental Methods. Lane A, positive control from the kit; Lane B, Cu+BCP; Lane C, Zn+BCP; Lane D, control; Lane E, DNA marker.

4.2 Part II

4.2.1 Purification of Cytochrome b₅₆₁ from Bovine Adrenal Glands

Chromaffin granules were isolated from bovine adrenal glands. The sucrose gradient step was used to remove fat and other contaminants and obtain a clean pink granule pellet. The granules were washed with 2% Tween-20 to remove loosely bound proteins and lipids in the membrane. They were further solubilized with n-octyl β-D-glucopyranoside resulting in complete solubilization of membrane proteins. Purification of cyt b₅₆₁ from the protein mixture was accomplished using an ion exchange (Mono Q, Pharmacia LKB) column according to a procedure developed in our laboratory (287).
In most preparations, cyt \( b_{561} \) eluted in two pools and the two fractions eluted very closely at a salt concentration between 82.5 to 115 mM. These two fractions were spectroscopically identical and were usually combined, and the concentrated proteins were stored in the storage buffer (20 mM potassium phosphate, pH 6.8, 20% (w/v) glycerol, 1% (w/v) n-octyl \( \beta \)-D-glucopyranoside), at \(-70^\circ\)C.

4.2.2 Spectroscopic Characteristics of Purified Cytochrome \( b_{561} \)

The purification process yielded cyt \( b_{561} \) in high purity. The absorption spectrum of purified protein is shown in Figure 33. The fractions collected by the purification process were combined depending on \( A_{427}/A_{280} \) ratio, where higher the ratio, more purity of the sample.

![UV-vis spectra of fractions collected from FPLC](image)

Figure 33 - UV-vis spectra of fractions collected from FPLC. Fractions with high 427 nm absorbance were combined.
The oxidized cyt $b_{561}$ has maximum absorption at 415 nm and the reduced form displayed the characteristic UV-vis spectra with a Soret band at 427 nm and $\alpha$- and $\beta$-bands at 561 and 530 nm, respectively. The $K_3Fe(CN)_6$ oxidized cyt $b_{561}$ can be reduced by Asc (2 mM) and the remaining portion can only be reduced by dithionite (5 mM) at pH 6.8 as indicated by relative intensities of the 427 nm absorption bands (Figure 34). The concentration of cyt $b_{561}$ was determined by using $A_{427}$ of dithionite reduced protein (molar absorptivity of 27,700 $M^{-1}cm^{-1}$).

![Figure 34 - UV-vis absorption spectra of the purified cyt $b_{561}$ in various redox states. $K_3Fe(CN)_6$ oxidized (---); 2 mM Asc reduced (---); 5 mM dithionite reduced (---). Spectra were recorded in 20 mM potassium phosphate buffer (pH 6.8) containing 20% (w/v) glycerol, 1% (w/v) n-octyl $\beta$-D-glucopyranoside at 25°C.](image-url)
4.2.3 SDS-PAGE of purified Cytochrome b$_{561}$

Purity of the isolated protein was tested by SDS-PAGE and a typical gel pattern of a purified protein samples shown in the Figure 35. Protein samples taken from different steps of the purification process were also included in the same gel to show the efficiency of purification. The cyt b$_{561}$ fractions collected after passing through Mono-Q column (E) was a single band with an apparent molecular weight of ~28 KDa.

![SDS-PAGE analysis of purified cyt b$_{561}$. Gel composition: 15% acrylamide. Gel was stained with the silver stain. Samples taken from different stages of the purification process were included in the gel. (A) Solubilized granule; (B) Tween washed; (C) Detergent solubilized loading sample; (D) Molecular weight marker; (E) purified b$_{561}$.](image)

Figure 35 - SDS-PAGE analysis of purified cyt b$_{561}$. Gel composition: 15% acrylamide. Gel was stained with the silver stain. Samples taken from different stages of the purification process were included in the gel. (A) Solubilized granule; (B) Tween washed; (C) Detergent solubilized loading sample; (D) Molecular weight marker; (E) purified b$_{561}$. 
4.2.4 Pre-Steady State Kinetic Analysis of Purified Cytochrome $b_{561}$ with Ascorbate

The rate of reaction of purified CG $b_{561}$ with the physiological reductant, Asc was followed using stopped-flow apparatus. Rapid mixing of oxidized cyt $b_{561}$ with Asc produced a fast change of the electronic spectrum. Figure 36 clearly shows the time dependent shift of the Soret band at 415 nm (characteristic for native cyt $b_{561,ox}$) to 427 nm and the increase of $\alpha$- and $\beta$-bands at 561 and 530 nm respectively.
Figure 36 - Spectral changes for reaction of oxidized cyt $b_{561}$ with Asc. Reduction of oxidized cyt $b_{561}$ which was observed by Olis stopped-flow spectrophotometer in millisecond time scale. A, 2-D view, B, 3-D view.

4.2.4.1 Single-Wavelength Studies

When oxidized cyt $b_{561}$ and sodium Asc were mixed in the stopped-flow apparatus, it was possible to follow the reduction of the protein conveniently at 427 nm (Figure 37).
Figure 37 - Reduction kinetics of cyt b$_{561}$ (8 $\mu$M) with Asc (5mM). All solutions were prepared in 20 mM potassium phosphate buffer, pH 6.8, containing 1% (w/v) n-octyl $\beta$-D-glucopyranoside. Equal amounts of b$_{561}$ and Asc were rapidly mixed and changes of absorption at 427 nm were observed at 20°C for a period of 20 sec.

The time course of cyt b$_{561}$ reduction could not be fitted to a single exponential function but would be best fitted to a function with three exponential terms. The data was collected for different Asc concentrations and the kinetic parameters obtained by fitting the data to equation 2 were summarized in Table 4.
TABLE 4 - APPARENT RATE CONSTANTS FOR THE REACTION OF Cyt b\textsubscript{561} WITH VARIOUS CONCENTRATIONS OF ASCORBATE AT pH 7.0

<table>
<thead>
<tr>
<th>Asc (mM)</th>
<th>k\textsubscript{app1}</th>
<th>k\textsubscript{app2}</th>
<th>k\textsubscript{app3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>8.1</td>
<td>0.49</td>
<td>0.078</td>
</tr>
<tr>
<td>1.25</td>
<td>10.9</td>
<td>0.58</td>
<td>0.087</td>
</tr>
<tr>
<td>2.58</td>
<td>15.0</td>
<td>0.88</td>
<td>0.051</td>
</tr>
<tr>
<td>5.00</td>
<td>17.3</td>
<td>0.68</td>
<td>0.054</td>
</tr>
</tbody>
</table>

The apparent rate constants of the fastest phase k\textsubscript{app1} and the Asc concentrations were fitted to a double reciprocal plot (Figure 38) as described in Materials and Methods.

Figure 38 - Plot of the app. k vs Asc, pH 7.0 at 20\textdegree C. The K\textsubscript{s} and k\textsubscript{2} values were obtained as 880 \textmu M and 19 s\textsuperscript{-1}. 
CHAPTER V

DISCUSSION

5.1 Part I

Cu(II) and Zn(II) uptake into catecholaminergic cells is increased by about 5-10 fold (Fig. 7) by membrane permeable chelators, BCP and NCP. These are strong Cu(II) and Zn(II) chelators with optimum stoichiometry of metal : ligand of 1:2. Therefore the maximum metal uptake is expected to be at or above 1:2 stoichiometry with respect to the metal and BCP, if increased uptake was due to the non-specific transport of the pre-formed metal-chelator complex through the plasma membrane. However, the requirement of sub-stoichiometric amounts of the chelators for the optimum uptake shows that chelator assisted metal uptake does not fit into this simple model, and suggests a catalytic role of the chelators (Fig. 13). The requirement of sub-stoichiometric levels of chelators for maximum copper uptake has also been previously shown with rat cortical astrocytes (288) and APP-CHO cells (289).

A rough estimate shows that a 200-300 fold concentration gradient is generated across the plasma membrane of catecholaminergic cells at 2 μM BCP in the presence of 20-30 μM extracellular metal (assuming a 3 μL/mg protein) suggesting that these chelators mediate an active uptake of the metal. While simple ionophoric effects of chelators are insufficient to explain this observation, an attractive proposal is that the metal chelator complex enters the cell through diffusion and then the metal is exchanged with intracellular high affinity metal binding proteins such as metallothioneins.
resulting in active accumulation. The requirement of sub-stoichiometric chelator for the optimal uptake could be a consequence of the slow and rate limiting exchange of the metal between the chelator and metal binding proteins under cellular conditions. A relatively high, yet sub-stoichiometric BCP requirement for optimum metal uptake into HepG2 cells [especially Cu(II); Fig. 13C] could be the consequence of intracellular metal ion content and the binding characteristics and expression levels of specific metal binding proteins in these cells in comparison to catecholaminergic cells.

Free Cu(II) enters the neuronal cells through copper transporter 1 (290) and divalent metal transporter 1 (291), and zinc is believed to enter through the ZIP family of proteins (94). However, the partial inhibition of Cu(II) and Zn(II) uptakes into PC12 cells in the presence of sub-stoichiometric amounts of BCP by L-type VGCC inhibitor, nitrendipine (Fig. 14), and by extracellular calcium (Fig. 17) suggest that VGCC are at least partially involved in the transport of these metals into catecholaminergic cells. Although the effect of nitrendipine could not be accurately determined in the absence of BCP due to the low uptake and limitations of the detectability under our experimental conditions, previous studies have shown that free Zn(II) could enter into neuronal cells through VGCC (95), transient receptor potential melastatin 7 (TRPM7) (97), NMDA receptor-gated, and Ca\(^{2+}\)-permeable AMPA/kinate (98) channels. The Cu(II) uptake through VGCC has also been documented (292). Therefore, we propose that both free Zn(II) and Cu(II) could enter catecholaminergic cells through VGCC and other channels under the experimental conditions (see below). Although the ion passage through VGCC are not allowed under the resting conditions, the accumulation of positively charged free metal or metal-chelator complex that will exceed the metal buffering
capacity of intracellular environment may cause the partial membrane depolarization and opening of VGCC and other voltage sensitive cation channels, allowing further accumulation of the free metal. Nitrendipine insensitivity of Cu(II) uptake into HepG2 cells is consistent with this proposal, since VGCC channels are likely to be absent in HepG2 cells (Fig. 15A). However, the noticeable inhibition of Zn(II) uptake into HepG2 cells by nitrendipine is somewhat unexpected and we believe that some other non-specific nitrendipine sensitive channel(s) may be responsible (Fig. 15B).

Nifedipine is a voltage gated calcium channel (VGCC) blocker, which has similar structure to nifedipine commonly used as an antianginal and antihypertensive therapeutic agent. My preliminary studies show that the incubation of cells with Cu (II) and nifedipine increase intracellular Cu (II) level drastically (Fig. 16). Also nifedipine does not increase zinc uptake. These and some preliminary evidence suggest that redox activity of Cu (II) may play a role in this behavior and it will be interesting to investigating this further.

Sub-stoichiometric levels of BCP and NCP increase the toxicities of both metals, parallel to their increasing uptakes (Fig.18 A). Exclusion of extracellular calcium significantly increases the toxicity of Zn(II) and moderately increases the Cu(II) toxicity, again parallel to the effects on corresponding uptakes (Fig. 20). Both in the presence and absence of the metal chelators, nitrendipine partially protects Zn(II) toxicity to catecholaminergic cells but shows only a minor effect on the Cu(II) toxicity (Fig. 21). These results suggest that with minor variations catecholaminergic toxicity profiles of Zn(II) and Cu(II) are parallel to the uptake levels rather than to the chemical characteristics of the two metals in the presence of metal chelators. In contrast to
catecholaminergic cells, BCP had no significant effect on the HepG2 Cu(II) toxicity; but Zn(II) toxicity appears to slightly increase with increasing concentrations of BCP, indicating a noticeable difference between the toxicities of two metals to HepG2 in comparison to catecholaminergic cells.

Both free Cu(II) and Zn(II) deplete intracellular catecholamine levels in a concentration dependent manner and Cu(II) is more effective in comparison to Zn(II) under similar conditions. In the presence of BCP, Zn(II) depletes the catecholamines of PC12 cells dramatically (Fig. 26A). However, in the presence of BCP Cu(II) had an unexpected effect on the catechoamine levels of PC12 cells i.e. at low Cu(II), catecholamine levels were increased apparently due to the activation of tyrosine hydroxylase activity (which we have not exploited further) and then decreased with increasing concentration of copper, as expected (Fig.26B). Apart from this difference two metals show a similar effect on intracellular catecholamine levels in the presence and absence of BCP, again parallel to their respective uptakes. Free Cu(II) mediated catecholamine depletion was not affected by DAT or NET inhibitors (Fig. 28), but completely abolished by the exclusion of extracellular Ca^{2+} (Fig. 29). Nitrendipine partially inhibits catecholamine depletion by Cu(II) (Fig. 29). These findings are consistent with a previous report that Cu(II) induces a calcium-dependent neurotransmitter release from brain catecholaminergic nerve terminals (293). The effects of calcium or DAT (or NET) inhibitors on catecholamine depletion by free Zn(II) could not be accurately determined because the catecholamine depletion by Zn(II) is relatively small. However, previous studies have shown that Zn(II) treatment of PC12 cells induces rapid calcium influx (93) implying that the catecholamine depletion by
Zn(II) is also dependent on extracellular calcium. Therefore, catecholamine depletion by both Cu(II) and Zn(II) is most likely to be mediated by a process in which both extracellular calcium and VGCC are involved (i.e. calcium mediated exocytosis).

5.2 Part II

Cyt $b_{561}$ in the present study was isolated from bovine adrenal glands and purified by single column isolation method developed in our lab (287) and satisfactory yields in high purity were obtained. The Uv-vis spectral analysis of purified protein gave an expected A427/A280 ratio of about 3.7 and SDS-PAGE showed a clean single band around 28 kDa indicating high purity of the protein.

In the present study, stopped-flow analyses were conducted on the reaction of cyt $b_{561}$ with Asc. Multiple-wavelength stopped-flow analysis of cyt $b_{561}$ reduction by Asc shows gradual change in electronic spectra with multiple isobestic points indicating the formation of several species in the solution. The rate of reduction was very complex (Figure 9) and could not be fitted to a single exponential equation. The data were fitted to a linear combination of three exponential functions. The fastest phase was assigned to the one electron reduction of heme $b$ center at extravascular side. This phase showed Michaelis-Menten-type kinetics and $K_s$ value calculated for Asc was 0.88 mM at pH 7.0. This was lower than the value (4.5 mM) reported by Takigami et al.(31) [they have fitted the reduction kinetics of cyt $b_{561}$ to four exponential functions instead of three] but comparable with the $K_m$ of 0.34 mM obtained by Flatmark and Terland (253). The $k_2$ value obtained in the present study was 19 s$^{-1}$, however the value obtained by Takigami et al. (31) was ~90 s$^{-1}$. On the other hand Njus et al. (274) have reported that
Asc reduces cyt $b_{561}$ with a $K_m$ of $1.0 \pm 0.2$ mM and $V_{max}$ of $4.0 \pm 0.8$ s$^{-1}$ at pH 7 in chromaffin ghosts.

Fitting of Asc reduction kinetics of cyt $b_{561}$ to three phases indicates the reduction occurs in multiple steps and that showed cyt $b_{561}$ contain more than one heme $b$ center. Based on the two heme model, the three phases can be assigned as follows. The fastest phase may be the reduction of heme $b$ in extravesicular side and the second fast phase may be the electron transfer from extravesicular heme to intravesicular heme $b$. Takigami et al. (31) have observed a loss of the fastest phase in DEPC modification due to $N$-carbethoxylation of the heme-coordinating histidyl residues on the extravesicular side. Thus the lowest phase may be the slow reduction of intravesicular heme $b$ by Asc. Tsubaki et al. (277) have also reported a reduction of DEPC modified cyt $b_{561}$ by Asc at a very slow rate and the slow reduction was assigned as electron backflow from Asc to the oxidized heme at intravesiculal side which is supposed to donate electrons to SDA.
CHAPTER VI

CONCLUSIONS

6.1 Part I

The involvement of copper (II) and zinc (II) in the pathophysiology of neurodegeneration has been well documented, but the molecular mechanism of the toxicity is not fully understood. In the present study Cu (II) and Zn (II) toxicity to catecholaminergic PC12, MN9D and SH-SY5Y cells and hepatic HepG2 cells have been examined.

Previous studies have proposed that the high catecholaminergic toxicity of copper is primarily due to its redox activity and oxidative interaction with catecholamines leading to the increased production of ROS. However, present parallel studies with non-redox active Zn(II) indicate that, with minor differences, the toxicity profiles of two metals correlate well with the relative uptakes rather than their redox or other chemical properties. While free copper is somewhat more toxic to catecholaminergic cells than zinc, in the presence of membrane permeable metal chelators, both metals are comparably and highly toxic to catecholaminergic cells. More strikingly, in the presence of BCP, Zn(II) is even more toxic to PC12 in comparison to Cu(II). Parallel to toxicity, ATP depletion by Zn(II) in PC12 cells in the presence of BCP also higher compared to that of Cu(II). In addition, the characteristics of catecholamine depletion by both metals are parallel and similar suggesting that the redox interaction between the cytosolic catecholamines and Cu(II) could not be the cause of DA depletion toxicity as previously reported (294). This evidence and the lack of protection from toxicity with high
concentrations of redox active Cu(I) stabilizer BCP support the notion that the redox properties of Cu(II) may not be the primary cause of its catecholaminergic toxicity.

We propose that the active accumulation of the metal in the presence of membrane permeable metal chelators is initially driven by the inside negative membrane potential and the facile exchange of the metal with the high affinity intracellular metal binding proteins. The accumulation of the metal (or metal complex) above the metal buffering capacity of the cell causes the partial membrane depolarization and opening of VGCC and other voltage sensitive cation channels. Under these conditions, the free metals could pass through these open channels, and further increase the intracellular concentrations. The sharp increase of the ROS and DNA fragmentation patterns by both metals in the presence of BCP suggest an apoptotic cell death due to the increased intracellular ROS levels. Thus, the interference with the functions of VGCC and other ion channels leading to the perturbation of intracellular calcium levels and the ionic gradients results in the excessive ROS production which appears to be the major cause of the high toxicity of these metals to catecholaminergic cells (295). The minor differences in toxicities of two metals could be due to the differences in their interactions with cellular components or the minor contributions to the toxicity from the redox properties of Cu(II). The low and moderate toxicity of these metals to HepG2 cells must be due to the absence of VGCC and other voltage sensitive channels in these cells. The relatively high sensitivity of these cells to Zn(II) in comparison to Cu(II) could be due to the more efficient interaction of Zn(II) with vital intracellular calcium binding proteins in these cells.
Finally, we note that the use of membrane permeable metal chelators for the treatment of neurodegenerative diseases has been widely discussed in recent years (177, 178). However, in light of the above findings, the efficacy of this approach should be carefully and critically evaluated. Similarly, membrane permeable metal chelators in the environment and consumable products could contribute to the etiology of neurodegenerative diseases. Therefore further studies are certainly necessary to fully determine the CNS effects of these agents.

6.2 Part II

In conclusion, pre-steady state kinetic analysis of the reduction of cyt $b_{561}$ with Asc was conducted and spectral changes were observed in millisecond time scale, for the first time. The reduction kinetics of cyt $b_{561}$ was complex and has been found to proceed in multiple kinetic phases. Single-wavelength absorbance changes at 427 nm were monitored and the data could be fitted to three distinct exponential phases. The results support the two heme model. The fastest phase was reasonably assigned to electron transfer from Asc to extravesicular heme $b$ and $K_s$ was obtained as $880 \mu$M (pH 7.0). The slowest phase is assignable to the slow reduction of heme $b$ on the intravesicular side.
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REFERENCES


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Oxidatively generated DNA damage after Cu(II) catalysis of dopamine and
related catecholamine neurotransmitters and neurotoxins: Role of reactive

APPENDIX

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

SDS-PAGE was carried out using the Biorad Mini-Protein II Electrophoresis Cell.

Manufacturer’s protocol based on Laemmli\(^1\) buffer system was used to separate the proteins without any modifications.

Following stock solutions were made and stored at 4\(^\circ\)C unless otherwise stated.

1.5M Tris HCl pH 8.8

18.15 g Tris base was dissolved in 100 mL d.d. H\(_2\)O

pH was adjusted to 8.8 with 1N HCl.

0.5 M Tris HCl pH 6.8

6 g Tris base was dissolved 100 mL d.d. H\(_2\)O

pH was adjusted to 6.8 with 1 N HCl.

10\% SDS

1.01 g SDS in 10 mL d.d. H\(_2\)O

Store @ room temperature

5X Running buffer pH 8.3

9 g Tris base

43.2 g Glycine

3.0 g SDS in 600 mL d.d. H\(_2\)O

APPENDIX (continued)

**SDS Reducing sample loading buffer**

4.0 mL distilled H₂O  
1.0 mL 0.5 M Tris-HCl, pH 6.8  
0.8 mL Glycerol  
1.6 mL 10% SDS  
400 mg Dithiothreitol (Cleland’s Reagent)  
4 mg Bromophenol blue  

Store @ room temperature

Acrylamide/bis (37.5:1 mixture) stock solutions were purchased from Biorad.

<table>
<thead>
<tr>
<th></th>
<th>15% resolving</th>
<th>4% stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 40% Acrylamide /Bis</td>
<td>3.75 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>2. 0.5M Tris-HCl, pH 6.8</td>
<td>-</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>3. 1.5M Tris-HCl, pH 8.8</td>
<td>2.5 mL</td>
<td>-</td>
</tr>
<tr>
<td>4. 10% SDS</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>5. dd H₂O</td>
<td>3.6 mL</td>
<td>6.4 mL</td>
</tr>
<tr>
<td>6. TEMED</td>
<td>10 μL</td>
<td>10 μL</td>
</tr>
<tr>
<td>7. 10% Ammonium persulfate</td>
<td>50 μL</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

*Total volume of resolving gel is ~10 ml*
Resolving gels were made according to the following recipe.

Appropriate amounts of 1, 2 & 3 were added into a round bottomed flask and degassed for 10 min. Then 4 was added and degassed for another 5 min. Then 5 & 6 were added and mixed well. The gel was poured as described in the manufacturer's manual and kept for 1 h for polymerization.

Stacking gel was made according to the following recipe.

1, 2 & 3 were added into a round bottomed flask and degassed for 10 min. Then 4 was added and degassed for 5 min. Finally, 6 & 7 were added and mixed well. The gel was poured and kept for 45 min for polymerization.

Sample preparation

All samples were made in the loading buffer and then placed in a water bath for 5 min at 95°C. Then 5 μL of each sample was applied on to the gel noting the respective lanes. A molecular weight marker in the low range was used to determine the molecular weight of the unknown protein. Gels were run for 45 min at a constant voltage of 200 V. Depending on the acrylamide percentage the time usually varied. Finally the gels were developed with Biorad silver stain according to the manufacturer's protocol.