An investigation into the possible neuroprotective role of melatonin in copper-loading

Thesis

Submitted in Fulfilment of
The Requirements for the Degree of

MASTERS OF SCIENCE (PHARMACY)

OF

RHODES UNIVERSITY

BY

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FEBRUARY 2001

DEDICATED IN THE MEMORY OF NIMAI

Many little deeds by many little people
In many little places
Can change the face of the world

Chinese proverb

ACKNOWLEDGEMENTS

I would like to thank the following people:

Prof. Santy Daya, my supervisor, for his guidance and support.

Shailendra “Shai” Anoopkumar-Dukie, for all his help, guidance and support. It was a pleasure to work with you.

Prof. Kanfer and the Pharmacy Department, for all their assistance within the Department.

Prof. Ben, for all his encouragement and “brainstorming”. It was an enlightening experience.

Dave and Sally Morley, and Andy Soper, for all their technical assistance.

Dr. Cheryl Sacht, Dr. Mike Datt, Dr. Kevin Wellington, Dr. Gary Watkins, Dr. Denzil Beukes, and Edith Antunes and Barbara, for all their expertise with regard to the chemistry. Thank you to Andre, for all the specialized glassware.

Dr. Janice Limson, for her expertise with the electrochemistry.

Prof. Pauline Hall and Joanne (UCT), for all their help and encouragement with regards to the Histology.

Purba Pal, Robin Cross, Shirley Pinchuck, and Marvin, for all their help.

Rob Van Der Hill, for the assistance with the atomic absorption spectrometer.

Prudence, Patrick and Atwell, for all their help regarding the animals and the Histology.

My fellow MSc collegues, the Colemere gang, and all my friends, for their constant encouragement and support.

Maisha, for all her encouragement and support. The ride was a rollercoaster, thank you for being there with me.

My parents and family, for all their encouragement and love, **THANK YOU!**

The MRC and Rhodes University for the financial assistance towards this study.

Finally, Shri Gauranga, without whom, nothing is possible.

ABBREVIATIONS

Aβ:	Amyloid-β
aHT:	N-acetylserotonin
aMT:	Melatonin
apoCp:	Apoceruloplasmin
ATP:	Adenosine triphosphate
BBB:	Blood brain barrier
BSA:	Bovine serum albumin
BHT:	Butylated hydroxytoluene
° C:	Degees Celsius
cAmp:	5’ cyclic adenosine monophosphate
CCO:	Cytochrome *c* oxidase
Ca^{2+}:	Calcium
Con:	Control
Cd:	Cadmium
cm:	Centimeter
Cp:	Ceruloplasmin
Co:	Cobalt
Cu:	Copper
Cu/mel:	Copper/melatonin
CV:	Cyclic voltammograms
CVS:	Cathodic adsorptive stripping voltammogram
d:	Day
DNA:	Deoxyribonucleic acid
DPI:	2,6-dichrorophenol-indophenol
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay

Fe^{2+}:	Iron
g:	Gram
G:	Glycogen
GCE:	Glassy carbon electrode
GSH:	Glutathione
µg:	Microgram
H:	Hydrogen
HIOMT:	Hydroxyindole-O-methyltransferase
HMDE:	Hanging mercury drop electrode
HoloCp:	Holoceruloplasmin
8-OhdG:	8-hydroxydeoxyguanosine
H_2O_2:	Hydrogen peroxide
HL:	5-hydroxytryptophol
HPLC:	High performance liquid chromatography
5-HT:	Serotonin
i.p.:	Intra peritoneal
K:	Potassium
KDa:	Kilodalton
Kg:	Kilogram
µl:	microlitre
LEC:	Long Evans Cinnamon Rats
M:	Molar
MA:	5-methoxyindole acetic acid
MAO:	Monoamine oxidase
MDA:	Malodiadehyde
Mel:	Melatonin
mg:	Milligram
ML:	5-methoytryptophol
ml:	Millilitre
µM:	Micromolar
mRNA:	Messenger ribonucleic acid

MT:	Metallothione
mol/L:	Moles per litre
n:	Sample size
N_2:	Nitrogen
NA:	Noradrenaline
Na:	Sodium
ng:	Nanogram
NADPH:	Nicotinamide adenine dinucleotide phosphate
NaCl:	Sodium chloride
NAT:	N-acetyltransferase
nm:	Nanometer
Ni:	Nickel
NOS:	Nitric oxide synthetase
O_2:	Oxygen
$O_2^{\cdot-}$:	Superoxide anion radical
p:	Probability
$^{\cdot}OH$:	Hydroxyl radical
PINA:	Pineal night-specific ATPase
pg:	Picogram
PBS:	Phosphate buffered saline
$ROO^{\cdot}$:	Peroxyl radical
ROS:	Reactive oxygen species
TBARS:	Thiobarbituric acid-reacting substance
Sec:	seconds
SEM:	Standard error of the mean
SCN:	Suprachaismatic nucleus
t:	Time
TBA:	Thiobarbituric acid
TCA:	Trichloroacetic acid
TLC:	Thin layer chromatography
USA:	United States of America

UV:	Ultraviolet
V:	Volts
w/v:	Weight per volume
Zn:	Zinc

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ABSTRACT

Copper is an extremely toxic metal in biological systems and thus, its availability to the system, must be effectively and efficiently controlled. Copper is vital for life, as it is essential for critical enzymes in biological systems. It is free copper in the biological systems that is toxic, as free copper induces free radical generation, which disrupts lipid membranes, interacts with DNA causing mutations, and eventually leads to cell death. Wilson's disease is a inherited copper disease, which results in hepatolenticular disease. Copper is unable to be excreted, and thus accumulates, eventually spilling over into the bloodstream from the liver, and "poisons" the patient. The Wilson's disease patient leads a short life, due to neurological and hepatological problems. There is no cure for Wilson's disease, only chelation therapy using potent chelators such as penicillamine and EDTA. Zinc, in high doses, can be used to compete with copper absorption. This has proved to be the only successful therapy at present. This study investigates the possible use of melatonin as a copper binder/chelator. Melatonin has been shown to interact with copper *in vitro*. By binding/chelating to copper, melatonin may inhibit copper-induced free radical generation, and thus prevent copper from interacting with DNA to cause mutations and act as a cytotoxin.

In vivo studies on copper (2mg/kg) administered for 2-weeks and 6-weeks were carried out on Wistar rats. The potential of melatonin (12mg/kg) to prevent copper-induced cellular damage was investigated. The results indicate that melatonin does not protect the lipid membranes from copper-induced lipid peroxidation. *In vitro* investigations using 1mM, 5mM and 10mM copper and 5mM melatonin, show that melatonin prevents copper-induced lipid peroxidation at a copper concentration of 1mM ($p<0.001$). The 5mM and 10mM copper induces less lipid peroxidation, compared to the 1mM copper. It has been reported that metal ions, antioxidants and chelating agents can influence peroxide decomposition during the assay. Melatonin (5mM) administration does not significantly prevent copper-induced lipid peroxidation at 5mM and 10mM copper. It is

possible that due to melatonin's relatively low concentration, it is unable to inhibit lipid peroxidation induced by the copper.

The chemical nature of the interaction between melatonin and copper was also investigated, using NMR, IR and electrochemistry techniques. The NMR and IR techniques show that melatonin coordinates with Cu^{2+} and not Cu^{1+}, at the carbonyl group of melatonin. The electrochemistry experiments using cyclic voltammetry and adsorptive stripping voltammetry, show that melatonin forms a strong bond with Cu^{1+}. Cu^{2+} prefers binding to oxygen, and that is clearly seen in the NMR and IR. Cu^{1+} prefers binding to nitrogen and then oxygen, and this is seen in the electrochemistry, as Cu^{1+} is forced to bind through one of the nitrogens on the melatonin. Previously, it has been shown that melatonin binds/chelates with Cu^{2+}.

Histochemical investigations show that copper administration for 2-weeks and 6-weeks, causes extensive mitochondrial damage in liver and kidney's proximal convoluted tubule epithelium cells. Melatonin (12mg/kg) co-administration with copper for 2-weeks and 6-weeks did not significantly protect the mitochondria from copper-induced damage. Copper-specific stains (rhodanine, silver sulphide and rubeanic acid) were used to stain liver, brain and kidney tissue samples. Rhodanine and silver sulphide were equally sensitive in staining copper in the 2-week samples, but not at all in the 6-week samples. This could not be explained. Rubeanic acid was ineffective in all samples tested. Thus, it appears that specific copper stains cannot be used in making a definitive diagnosis in cases of copper overload, and that specific copper stains do not always correlate with a high concentration of copper present in tissues.

Pineal organ culture was used to determine the effect of copper administration on pineal indole synthesis. Exogenous (^3H) tryptophan was administered to the pineal organ cultures, and the level of (^3H) pineal indoles synthesised, were measured. Pineals from 2-week and 6-week copper/melatonin treated animals exhibited paradoxical 5-methoxytryptophol (ML) levels, as compared to the 2-week and 6-week copper treated animals. The 2-week copper/melatonin administered animals, showed a decrease in the

ML level ($p<0.01$), and the copper/melatonin administered for 6-weeks, showed an increase in the ML levels ($p<0.01$). This indicates that melatonin interacts with the HIOMT enzyme. Pineals from 6-week copper/melatonin treated animals, as compared to the 6-week copper treated animals, showed an increase in N-acetylserotonin levels. This indicates that melatonin prevents the inhibition of the NAT enzyme.

The final experiment was to determine *in vitro*, the effect of Cu^{2+} and Cu^{1+} administration, on mitochondrial electron transport chain. Rat liver homogenate was incubated with and solutions of Cu^{2+} (10mM) and Cu^{1+} (10mM) and melatonin (10mM). Cu^{2+} administration caused an inhibition of the electron transport at t=0 and t=60, whereas Cu^{1+} administration at t=0 caused an inhibition of electron transport, but at t=60, Cu^{1+} administration stimulated electron transport. Melatonin administered with Cu^{2+}, resulted in an inhibition of the electron transport chain at t=0 and t=60.

The findings of this study indicate that melatonin might have a potentially beneficial effect in copper overloading, by binding/chelating copper.

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CHAPTER 1
LITERATURE REVIEW

1. INTRODUCTION

1.1. COPPER

1.1.1. History of Copper

The earliest recorded working with copper dates back to the 9^{th}-7^{th} millennia BC in Western Iran and Anatolia (Garper, 1987). By 3500 BC, smelting of copper, became common, with relatively pure copper extracted from oxidized copper ores. Around the time of Christ, Assyrian, Egyptian, and Hindu alchemists were preparing and prescribing copper compounds to treat a variety of human ailments. These included the use of copper sulfate as an antihelmintic, an astringent, and as an emetic. Today, copper toxicity arises from the use of "*spiritual green water*" in religious ceremonies. Copper is an essential trace element, and is needed in limited quantities, as it plays a vital role as a cofactor. Acute copper toxicity arises from food and beverage stored in copper vessels. This can result in an acute gastrointestinal illness. Copper has been commonly used as a suicidal agent in India, where the lethal dose is ≈ 1000 times normal dietary intakes. Its use as an emetic has been discontinued, due to its toxicity (Barceloux, 1999).

1.1.2. Physical and Chemical Properties of Copper

Copper lies in Group IB on the periodic table, between nickel and zinc. It is a noble metal, like gold and silver. It has industrial importance due to its useful properties of appearance, alloying, low corrosion, malleability, and high thermal and electrical conductivity. It does not dissolve in acidic environments, unless an oxidizing agent is present, and a green layer of copper carbonate forms over the metal, which protects it from further oxidation.

The chemical properties of copper allow it to exist in three stable oxidation states: Cu^{0}, Cu^{1+} and Cu^{2+}. The cuprous ion (Cu^{1+}) rapidly disassociates in aqueous solutions to cupric (Cu^{2+}) and metallic copper (Cu^{0}). Cupric compounds are blue or green in colour (Barceloux, 1999). Transition metal elements are characterized by having partially filled *d* orbitals in many of their compounds. Copper has the ability to mediate electron transfer by valence-shuttle mechanisms. The biological functions of copper are intimately related to its properties as a transition metal (Malmstr`m and Leckner, 1998).

1.1.3. Medicinal uses

Copper is used in dental crowns as an alloy with aluminium, cobalt, manganese, nickel and zinc. It is a constituent of intrauterine contraception devices, where the release of copper is necessary for the contraceptive effect. Copper bracelets are commonly worn for arthritis, but the efficacy of it is based on a folk remedy, with no scientific evidence to support it. *Spiritual water* or *green water*, is used as a cathartic in African rituals. The ingestion of this *green water* produces hemolysis, renal failure and death (Barceloux, 1999).

1.1.4. Copper homeostasis

Copper is an essential trace and transition metal. It is the third most abundant trace element found in the body, after iron and zinc. The cupric state (Cu^{2+}) is found most often in biological systems and copper plays a vital role as a cofactor and as an allosteric component of several cuproenzymes as shown in Table 1 (Uauy, 1998; Frieden, 1986).

1.1.4.1. Copper as an essential nutrient

Copper is essential for the survival of plants and animals, as copper is involved in the functioning of several enzymes.

In 1928, Hart *et al*., showed that copper was essential for erythropoiesis in rats fed a milk-based diet. The resulting anemia was corrected by adding ash from animal or vegetable sources to the diet. These authors showed consequently that, the hydrogen sulfide precipitated from the ash contained copper sulfide, and it was the copper sulfide that was responsible for the recovery of the animals. Similar findings established the basis of copper essentiality in humans (Hart *et al*., 1928; Mills, 1930).

Table 1. Mammalian copper enzymes (Frieden, 1986)

Protein	**Localization**	**Function**	**Expected results of deficiency**
Cytochrome *c* oxidase	Inner mitochondrial membrane	Electron transport	Deficient energy production and nerve conduction (seizures), and respiratory failure
Superoxide dismutase	Cytoplasm: brain, liver, heart, erythrocytes	Dismutation of superoxide radicals	Depigmentation
Tyrosinase	Melanocytes of eye, skin	Melanin production	CNS degeneration: convulsions
Dopamine-$-hydoxylase	Plasma, vesicle in adrenal medulla	Production of catecholamines	Imbalance in hypothalamic centers: hypothermia, anorexia, respiratory failure, somnolence, dehydration: ataxia
Amine oxidase	Plasma, brain, lung, kidney, intestine, placenta	Oxidation of mono- di- and polyamines	Increased histamine levels: urticaria
Lysyl oxidase	Extracellular, cartilage, bone, blood	Cross-linking of collagen and elastin	Connective tissue manifestations: joint hyperextensibility, vascular rupture, osseous abnormalities
Ceruloplasmin	Plasma	Cu transport, iron mobilization, antioxidant	Anemia, low copper enzymes

Cordano *et al*. in the 1960s demonstrated the essentiality of copper in malnourished children in Peru. These children had an anaemia refractory to iron therapy, neutropenia, and bone abnormalities that were responsive to copper supplementation. Copper is required for infant growth, host defense mechanisms, bone strength, red and white cell maturation, iron transport, cholesterol metabolism, myocardial contractility, glucose metabolism, and brain development (Danks, 1988).
Copper also functions in different redox enzymes and is thus essential for normal physiologic function such as cellular respiration, free radical defense, synthesis of melanin pigment, connective tissue biosynthesis and cellular iron metabolism (Uauy, 1998).

In vivo, any copper not bound to a specific copper-protein is toxic. Any acidic food or beverages in prolonged contact with copper vessels or tubing, can become contaminated with milligram quantities of Cu. In these cases, acute poisoning results, with nausea, vomiting and diarrhea and no treatment is necessary. If large quantities (gram quantities) of a Cu salt are ingested, then this could be fatal. The Cu-induced hemolytic anemia and anuria are generally fatal (Merck, 16^{ed}).

Deficiencies of copper can be of varied etiology (Table 2). The acquired deficiency is mainly a pathology of infants, however cases have been described in children and adults.
Clinical manifestations of acquired copper deficiencies are anemia, neutropenia and bone abnormalities. Other less frequent manifestations are abnormalities of cholesterol and glucose metabolism, and cardiovascular alterations, hypopigmentation of hair, hypotonia, impaired growth, increased incidence of infections and altered phagocytic capacity of neutrophils. All these are corrected on copper supplementation.

Copper overload can result in artherogenesis, due to the direct effect of copper on LDL-cholesterol oxidation. Copper is a prooxidant, and acts on cysteine residues of the LDL apolipoprotein B component, which results in the modification of the structure and binding properties of the LDL to the cell receptors, which affects the uptake of cholesterol by the cells (Olivares and Uauy, 1996). Also the excess copper results in

hepatolenticular degeneration, with a spill over effect into other tissues and organs, which results in tissue cirrhosis, and eventually organ failure and death (Linder, 1996).

Table 2 Etiology of acquired copper deficiency (Olivaries and Uauy 1996)

Decreased copper at birth
Low birth weight
Inadequate copper supply
Low dietary copper
Low bioavailability of dietary copper
Total parenteral nutrition with inadequate copper supplement
Inadequate copper absorption
Malabsorption syndrome
Increased requirements
High growth rate
Increased losses
Repeated or prolonged episodes of diarrhoea
Abnormal bile loss
Intestinal loss from small intestinal ostomies

1.1.4.2. Genetic copper deficiency/toxicity

Genetic copper deficiency is characterized by decreased levels of serum copper and ceruloplasmin (Cp), a degeneration of the vasculature showing the lack of elastin and collagen, depigmentation of the skin, kinky hair, brain damage, hypotonia and hyperthermia, hyperglycemia and hypercholesterolemia. It is an X-linked defect characterized by all the above overt symptoms, except with the absence of the anemia and the neutropenia. This X-linked genetic defect is called Menkes' Kinky hair disease (Frieden, 1986). The primary defect in Menkes syndrome is in the transfer of copper from intestinal mucosal cells into the blood, thus exhibiting low serum copper levels and an overall copper deficiency.

On the other extreme, Wilson's disease is also a X-linked defect, which is primarily due to the accumulation of copper in the liver, brain (causing hepatolenticular disease) and

other organs viz. kidneys and eyes (Evans, 1973; Frieden, 1986). In the brain copper toxicity causes movement disorders (which manifest as tremors, rigidity, dysarthria, dysphagia and abnormalities in gait) and psychiatric disturbances (inappropriate behaviour or psychoses) (Scheinberg and Sternlieb, 1984). The precise defect in Wilson's disease is caused by either a mutation or deletion of the gene for a P-type cation transporting ATPase of 1411 amino acids (Linder, 1996; Bull, 1993).

1.1.4.3. Copper Absorption

The adult human contains 60 ± 20 mg of copper, many times less than the biological levels of Fe and Zn (Frieden, 1986). The average adult human Western diet consumes between 0.6 to 1.6 mg Cu per day (Linder, 1991). Most of the copper consumed is from food rather than water. The copper content of different foods varies considerably. Shellfish and organ meats are the richest sources of copper, compared to muscle meat. Rich sources from plants are from seeds (which includes nuts and grains), whereas fruits and vegetables contain less copper (Linder, 1996). In the United States of America, 1.5-3.0 mg dietary copper has been designated as safe and adequate for adults (National Research Council, 1989).

In mammals, copper can be absorbed from the stomach to the distal small intestine. At least one-half of the amount of copper reaching the small intestine reappears in the bile as strongly bound compounds, and is lost in the stools. The distribution of copper throughout the body is mediated by Cp, albumin, transcuprein and other quantitatively less important copper binding proteins (Wapnir, 1998).

The acidic enviroment of the stomach, contributes to the freeing of copper bound to foodstuffs. The increase in luminal pH affects copper absorbability, once the gastric contents have been emptied into the duodenum. The increased pH, causes a decrease in free copper concentration and an increase in cupric hydroxide and basic copper salts with low dissociation constants (Wapnir, 1987).

Copper is absorbed across the brush border into cells of the intestinal mucosa, and is subsequently transferred across the basolateral membrane into interstitial fluid and blood (Figure 1).

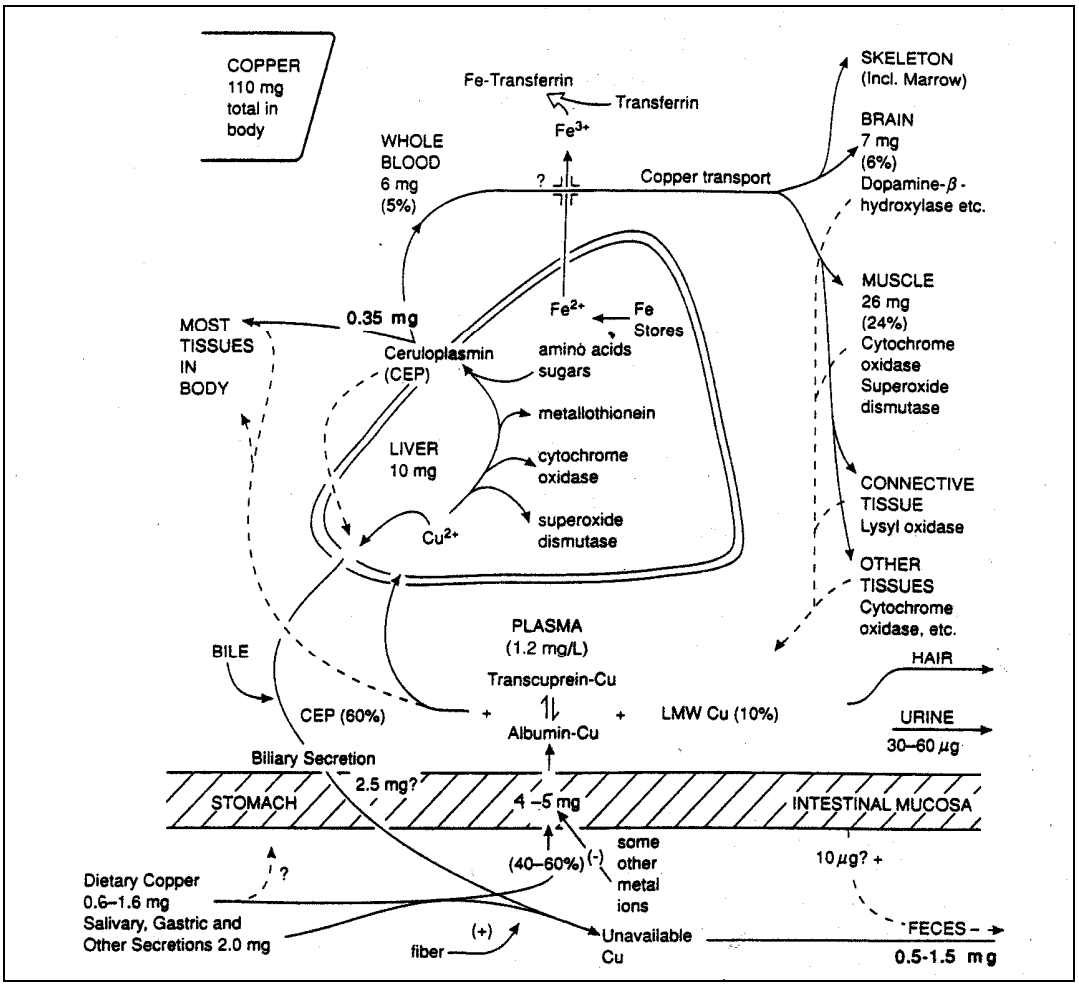

Figure 1. Summary of the nutritional biochemistry and metabolism of copper in adult humans (Modified from Linder, 1991b).

The transfer across the mucosal barrier occurs probably via non-energy-dependant diffusion. The transfer of copper across the basolateral membrane appears to be the rate-limiting step, and is mediated by a saturable, energy-dependent mechanism, for normally ingested amounts of copper. With higher copper intakes, additional diffusion or carrier-

mediated systems in the basolateral membrane come into play. It is at these sites, that competition for absorption between copper and other transition metals (zinc and iron) takes place (Linder, 1991a). Abnormally high concentrations of Zn^{2+} (30-100:1) and probably $Fe^{2+/3+}$ directly or indirectly inhibit the uptake or transfer of copper from the diet to the blood. A high Zn intake enhances the antagonism of copper absorption from the mucosa into the blood- thus playing a part in the therapeutic regime of Wilsons disease (Linder, 1991a; Wapnir, 1993; Yu, 1994).

Turnlund *et al*. (1985, 1989) convincingly demonstrated that the rate of copper absorption varied inversely with copper intake and can be as low as 12% with very high copper intake. A theoretical maximum absorptive capacity of 63-67% has been estimated (Figure 2). The typical diets from developing societies allow for an average true copper absorption in the 30-40% range. The key regulatory step in copper assimilation, is intestinal absorption.

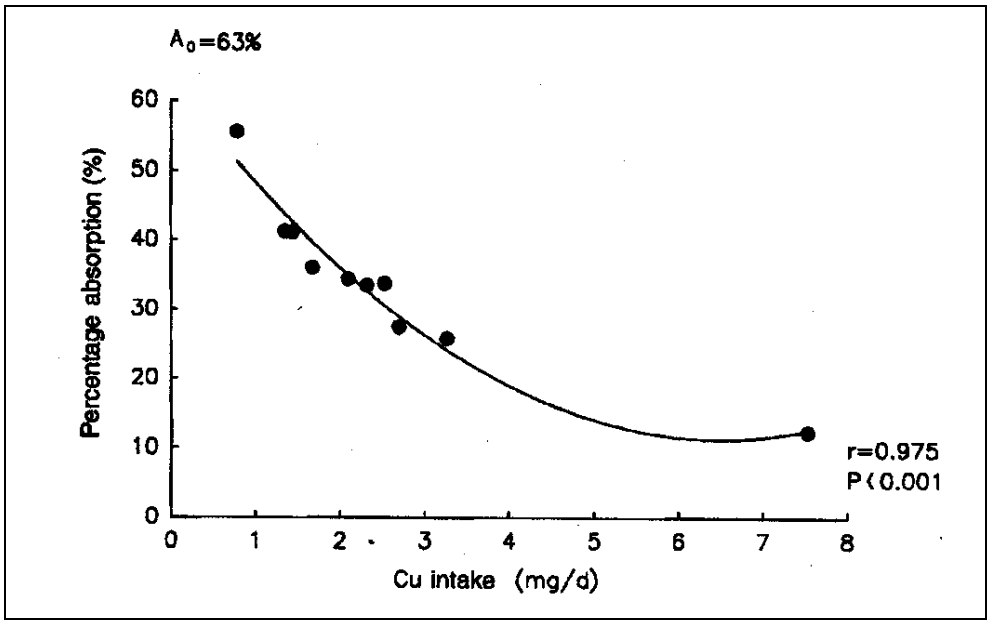

Figure 2. Aggregate results of human copper absorption studies at various copper daily intakes.
A_o = extrapolation of copper absorption rate at zero copper intake (Adapted from Turnlund, 1985; 1989)

The capacity for copper absorption is about equally distributed along the small intestine of rodents and, presumably, higher mammals. A small fraction of dietary copper however, is sufficiently solubilized in the stomach, thus its absorption at this site is considered to be nutritionally insignificant. In the small intestine of rodents, a rate-limiting, active-transport mechanism and a diffusion component have been reported (Crampton *et al.*, 1965). In humans, there are no comparable studies that provide evidence on the kinetics of the intestinal absorption of copper. A carrier-mediated transport process is attributed to the decrease in the proportion of copper absorbed at high dietary intakes. Dietary copper is incorporated into solid foods, and thus it appears less likely that the bulk flow across the intestinal mucosa is of quantitative significance (Wapnir, 1998).

Amino acids are suggested to be mandatory ligands for the uptake of copper by the brush border. They form complexes with the copper, and facilitates copper absorption (Kirchgessner, 1970). Cysteine is an effective chelating agent for copper, but it produces a reduction in bioavailabity, probably due to the reduction of copper from a divalent to a monovalent state (Baker, 1987).

Glutathione, an important biological peptide, plays an important role in postabsorptive copper transport. It has been postulated that glutathione forms an intermediary complex with copper in the erythrocyte, before copper is transferred to other proteins e.g. superoxide dismutase and metallothionein (Freedman, 1989; Harris, 1991).

Organic acids play a significant role in copper absorption. The most significant organic acid-copper interaction is between ascorbic acid and copper. Ascorbic acid reduces cupric (Cu^{2+}) to cuprous copper (Cu^{1+}), and thus reduces the bioavailability of copper (Van Campen, 1968). Harris and Percival (1991) showed that ascorbate in tissues reacts with ceruloplasmin, and thus facilitates the transfer of copper across certain membranes. It was proposed that monovalent copper, resulting from ascorbate electron transfer, is the form that is absorbed from ceruloplasmin by a select type of cells (DiSilvestro, 1981).

Suckling rats retain a greater proportion of copper in the small intestinal mucosa after copper perfusion than do weanling and adolescent rats. This is consistent with the notion that suckling rats have an immature distribution mechanism (Wapnir, 1987).
The absorption of copper in humans, at different ages, has only been indirectly assessed. Madaric *et al.* (1994), have noted that plasma concentrations of copper increases steadily from childhood to old age. The changes in circulating copper have been attributed to a decline of biliary secretion, as a component of the regulation of copper absorption, rather than to an increasingly efficient gastrointestinal absorption capacity throughout life.

Sex differences in plasma copper in humans for all ages is well documented. Females have higher plasma copper concentrations than males (Johnson *et al*., 1992).

1.1.4.4. Bioavailability

Mineral bioavailability has been defined as the efficiency with which a natural or manufactured source of an element delivers the element to storage or supplies it to metabolically active tissue or to a protein (Wapnir, 1998).

The ingestion of copper as a mineral salt is only relevant in humans when it is taken as a nutritional supplement. Thus, the potential of interactions with other minerals may have nutritional significance e.g. when copper is taken with zinc and/or iron, this affects the absorption of copper, and thus its bioavailability. Organomineral complexes of copper with amino acids or organic acids are used extensively in animal nutrition e.g. copper-lysine have been used as a supplement in chicks and in lambs. These complexes have been shown to be as effective as copper sulfate, but their bioavailability is only two-thirds of that of mineral salts, in lambs (Wapnir, 1998).

1.1.4.5. Transport

Copper homeostasis is achieved through metallochaperones, which guide and protect transition metal ions within the cell, and deliver them safely to the appropriate protein receptors (Pafahl *et al.*, 1997). Free Cu^{2+} is toxic, even in relatively low concentrations, thus transport within organisms occurs only in copper-complexes, usually in proteins. (Malmstr`m and Leckner, 1998).

The normal transport of copper is depicted in Figure 3 (Sarkar, 2000). The transport of copper across the intestinal lumen to the intestinal mucosa is a carrier-mediated process. The identity of the intestinal copper transporter is not known, but the Menkes disease copper transporter ATPase is a strong candidate, as it is a strongly expressed protein in the intestinal mucosa (Chelly, 1993; Mercer, 1993; Vulpe, 1993). On its entry into the blood, copper becomes bound to a variety of proteins and low molecular proteins. More than 90% of copper in blood plasma is present in the form of Cp, but it is not exchangeable in vivo. The remaining 5-10% of copper is exchangeable, and is bound to albumin and amino acids. Sarkar *et al*. (1966) showed that the main copper-amino acid complex in serum is a copper-histidine complex (1:2). This complex forms a ternary complex with albumin (Sarkar, 1968; Lau, 1971).

The mammalian cell utilizes at least two membrane transport pathways for copper uptake into the cell. These two pathways are mediated by energy-independent facilitated transport. The first pathway involves copper release and uptake from Cp. The released copper from Cp is reduced by a cell surface reductase and enters the cell through an energy-independent transport protein. Zhou and Gitschir (1997) have proposed that in humans the hCTR1 to be the high affinity copper uptake protein. Cp is the most abundant copper protein in plasma and contains 70-95% of plasma copper (Harris, 1993). Each Cp protein tightly binds 6 or 7 copper atoms to a variety of copper-binding sites, and thus makes copper available to cells and intracellular proteins (Dameron, 1987). Copper enters the cell, but the protein does not (Percival, 1989). A Cp receptor may facilitate copper uptake by the mammalian cell. It has been suggested that copper is taken up into the cell

as Cu^{1+}, rather than as Cu^{2+} (Harris, 1991). Stevens *et al.* (1984) indicated that specific Cp-binding sites on several cell types and tissues support the notion of a Cp receptor.
The second pathway involves the uptake of non-Cp-bound copper e.g. copper bound to albumin, histidine and other low molecular weight complexes (Harris, 1967). This pathway is referred to as " the free copper transport pathway" (Vulpe, 1995). This pathway is carrier-mediated, energy-independent, and saturable (Ettinger, 1986).

Copper transporters require plasma membrane reductases to reduce the Cu^{2+} to Cu^{1+}, with concomitant penetration of copper into the cell. Upon entering the cytoplasm, copper is complexed to a variety of ligands. The extracellular ligands do not enter the cell with copper (van den Berg, 1994). It has been suggested that the majority of cytoplasmic copper is complexed to Glutathione (GSH) as Cu^{1+} (Freedman, 1989). Copper can then be donated from the Cu^{1+}-GSH complex to various other intracellular proteins, like metallothionein (MT) (Ciriolo, 1990; Ferreira, 1993; Musci, 1996). Copper chaperone proteins (ATOX1, COX17, and Ccs1) have been suggested to deliver copper to copper-transporting ATPases (Klomp, 1997).

The Menkes ATPase is situated in the intestinal mucosal membrane, and thus in Menkes disease, the Menkes gene mutation results in the disruption in intestinal absorption of copper. The entrapment of copper within the intestinal cells leads to a copper deficiency in peripheral organs and tissues and a severe impairment of neurological and connective tissue function (Danks, 1988; Danks *et al.*, 1972).
The Wilson copper-ATPase (Figure 4) is positioned in the trans-golgi network, and is responsible in providing copper to proteins like Cp. This Wilsons copper-ATPase may also be involved in moving copper across the canalicular membrane and into the bile for excretion. A mutation in the Wilsons Copper-ATPase, causes a decrease in copper excretion out of the hepatocyte and into the bile. This results in copper toxicosis within the hepatocytes and in the brain (Sarkar, 2000).
Copper is distributed in all cellular organelles, e.g. the nucleus, mitochondria, lysosomes, endoplasmic reticulum and cytosol (Linder, 1991a). All cellular components contain copper proteins: superoxide dismutase is located in the cytosol, lysyl oxidase in the golgi

and secretory organelles, and MT in the cytosol, nucleus and lysosomes (Sarkar, 2000). Intracellualr copper trafficking, thus plays a vital role in the delivery of copper to copper-proteins.

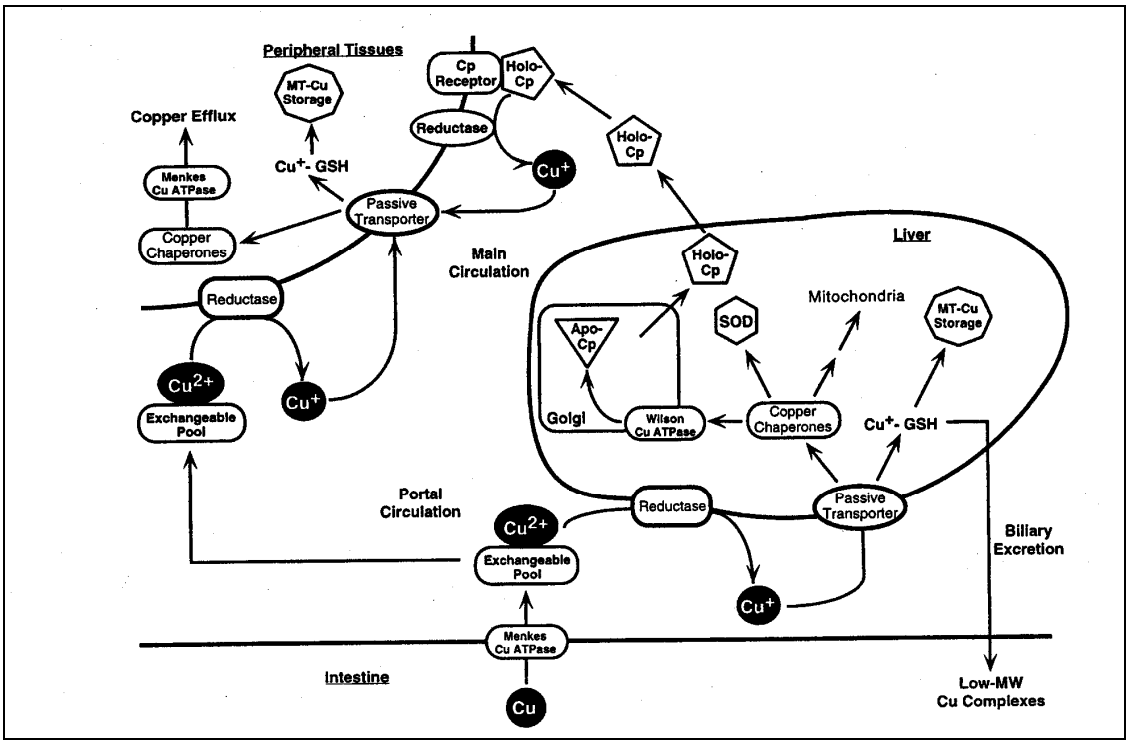

Figure 3. Normal copper transport (Sarkar, 2000)

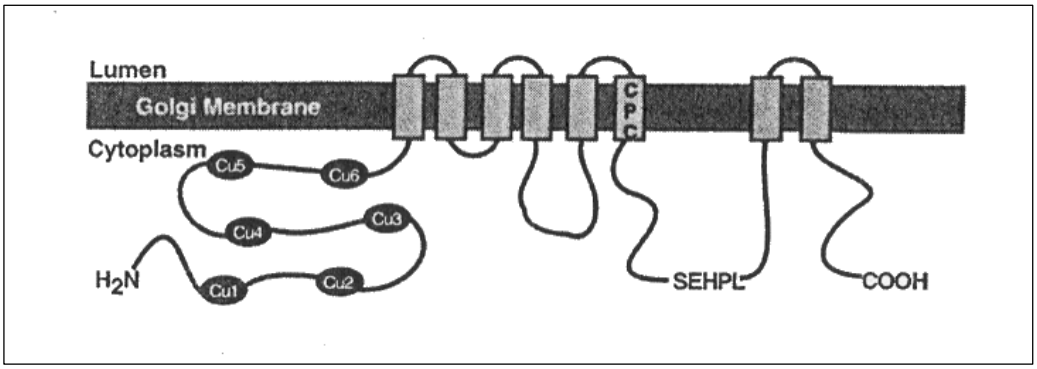

Figure 4. The proposed structure of the Wilsons disease copper ATPase. (Sarkar, 2000)

Using a minute tracer dose of radioactive copper (^{67}Cu) over time, various researchers have shown that two waves of copper distribution are seen after copper enters the blood (Linder, 1991b; Owen, 1965,1971). In the first wave, almost all of the copper rapidly leaves the blood and enters the liver and kidney. It then reemerges in the plasma. In other tissues and organs, it appears that the copper uptake takes place mainly in the second phase (Figure 5). The copper in the blood has been found to be bound to albumin and transcuprein, which carry the copper to the liver and kidney. The copper in the blood leaving the liver and kidney is associated with Cp.

These data obtained from rats are consistent with models of human copper distribution developed by Turnlund *et al.* (1989) and Scott & Turnlund (1994). It is suggested that under normal circumstances, copper entering the body from the diet, is carried by different transport proteins in two phases of distribution (Linder, 1998).

Darwish *et al.* (1986) state that histidine and other amino acids inhibit the uptake of copper by hepatocytes in vitro. Thus, in effect, ionic copper appears to be bound and firmly held by high-affinity protein carriers with high dissociation constants $\leq 10^{-11}$ mol/L (Lau, 1971; Masuoka, 1993). It appears more likely that specific protein receptors on the surfaces of recipient cells receive the copper from the protein carriers.

1.1.4.5.1. Albumin

Albumin carries incoming dietary copper in the circulation, and in most mammals, albumin has a high capacity for tight copper binding. About 40 :g of copper can bind to the high-affinity albumin sites in 1mL of human plasma, but only ≈ 180 ng of copper actually binds. Most albumins have a high-affinity site for Cu^{2+} at the amino terminus (Weiss, 1985). The rest of the ≈ 1000 :g of copper (per liter of plasma) is bound to Cp (≈ 65%), and trancuprein (≈ 12%) (Linder, 1991a).

In analbuminemia, where albumin is absent, there is no interference with copper distribution. Copper enters the liver and kidney (and later other tissues) just as rapidly as if albumin were present (Vargus, 1994). Albumin has been shown to inhibit the uptake of ionic copper by hepatocytes (Ettinger, 1986).

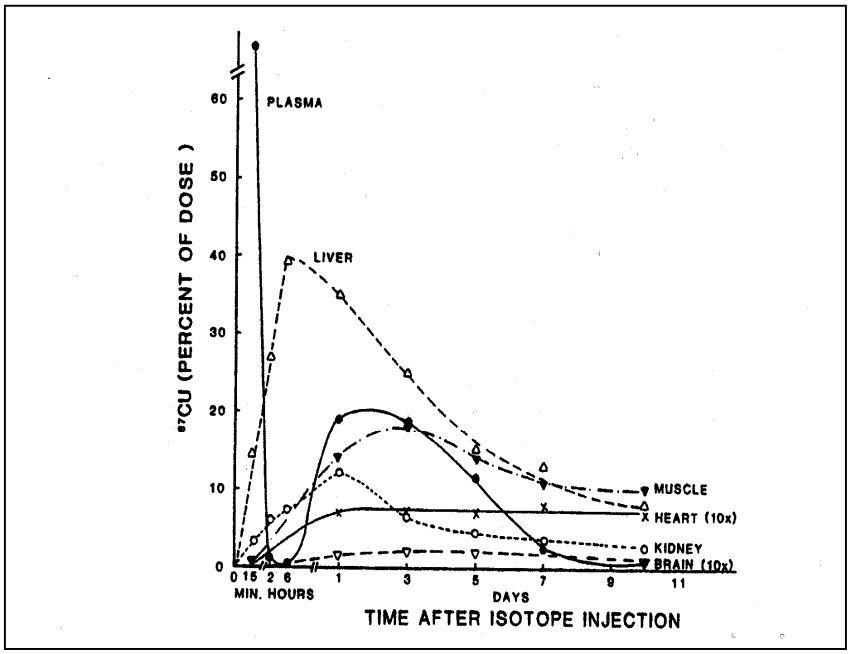

Figure 5. Distribution of tracer ^{67}Cu in plasma and tissues at various times after intraperitoneal Injection (Linder, 1998)

There are no hepatocyte cell membrane receptors for albumin that might facilitate the uptake of copper from albumin. As a result, it seems that albumin plays a passive role in

the transport of copper. It carries a large proportion of the exchangeable copper into circulation and releases it to other carriers for actual cell-specific uptake. Transcuprein-copper is rapidly exchangeable with copper from albumin, and transcuprein may be the specific carrier in question, at least in rodents (Weiss, 1985; Tsai, 1992a). However, there appears to be a redundancy in the system. In tissue culture, hepatocytes and other cell types, will take up copper from whatever source is offered (transcuprein, albumin, copper complexes, or Cp), although the rate may be slow at physiologically relevant concentrations (Linder, 1991a; Weiss, 1985; Ettinger, 1986; Percival, 1990; Harris, 1991).

There is a histidine at position 3 at the N-terminal site of albumin that binds Cu^{2+}. Histidine forms a ternary complex with albumin- Cu^{2+} (Sarkar, 1980), induces albumin to release copper as a histidine- Cu^{2+} complex. The histidine ligand itself never penetrates the cell, and thus histidine must present the copper to the cell surface (McArdle, 1990).

1.1.4.5.2. **Copper incorporation into the liver and kidney**

Once copper has bound to albumin and trancuprein, it is transported to the liver, via the portal blood, where it is deposited. Most of the incoming copper finds its way into the hepatocytes of the liver, with lesser amounts entering the kidney. On entry into the liver and kidney, newly absorbed copper is incorporated into several different compartments, which include endogenous copper enzymes, copper-requiring proteins that are secreted, and in the case of the liver, bile. A greater or lesser portion will go directly to the bile, depending on the dose of copper delivered i.e. more at higher doses and less at lower doses. This is crucial in the maintenance of copper homeostasis (Turnland *et al*., 1989). There appears to be two phases of distribution after copper enters the blood: entry into the liver (and kidney), and entry into other tissues. The first may be mediated by transcuprein, and the second by Cp (Figure 1) (Linder, 1991b).
Copper uptake is competitively inhibited by divalent metal ions of cadmium, manganese, zinc and cobalt (Linder, 1996).

A mathematical model proposed by Hazelrig *et al.* (1965), postulates at least three distinct hepatic processes: a) preparation of copper for excretion into the bile, b) temporary storage of copper, and c) incorporation of copper into Cp. The liver storage compartment may provide an emergency means for removing ionic copper from the blood, thus preventing its toxic buildup in tissues. The overriding concern of the liver is to remove "free" copper from the blood and prevent its diffusion into tissues. It is this "free" copper that is toxic and causes damage.

High concentrations of copper in the liver of rats occur in very young animals, iron-deficient animals, copper-treated adults, and hypophysectomised rats (Hermann and Kun, 1961). The mitochondria play a predominant role in the retention of copper. Gregoriadis and Soukes (1967) have shown that hepatic mitochondria, in copper-loaded rats, take up the largest amount of copper, followed by nuclei.

1.1.4.5.3. **Ceruloplasmin**

Cp is the most abundant copper protein in the plasma and contains >95 % of plasma copper (Harris, 1993; Ryden and Bjork, 1976). It is a 132-Kda multifunctional, monomeric, blue $\forall_2$-glycoprotein. It contains 1046 amino acids with ≈ 10% carbohydrates. It is a multi-copper oxidase that binds 6-7 copper atoms in at least three different ligand states and contains a trinuclear copper cluster responsible for its oxidase and free radical scavenging activity (Linder, 1991a).

Cp is present in normal human plasma at a concentration of approximately 300 :g/ ml (Holmberg and Laurell, 1948). Apoceruloplasmin (apoCp) [metal free form] is synthesized in the liver as a single polypeptide chain and secreted into the plasma as a holoceruloplasmin (holoCp), which is associated with 6-7 atoms of copper per molecule (Takahashi *et al.*, 1984). Copper-glutathione (Cu^{1+}-GSH) complex can mediate stable Cu^{1+} binding to apo-cuproproteins, e.g. apoCp (Smith *et al.*, 1988), apo-CuZnSOD (Ciriolo *et al.*, 1990), and to charge MT with Cu^{1+} (Ferreira *et al.*, 1993).

Each holoCp protein tightly binds 6 or 7 copper atoms to a variety of copper-binding sites (Musci *et al.*, 1993), and can donate copper to cells and to intracellular copper proteins (Dameron and Harris, 1987). Under normal dietary conditions, a considerable amount of the copper entering the liver and kidney from the diet re-emerges in the plasma on Cp. The liver and the kidney have the highest copper concentrations of mammalian organs [7 and 12 :g/g wet wt in liver and kidney, respectively]. This indicates that the liver and the kidney are the first tissues in which dietary copper is deposited, and that they both synthesize and secrete Cp (Linder, 1991a).

Copper bound to the ceruloplasmin, is presented to the cell. This copper can then enter the cell, but the Cp can not (Percival and Harris, 1990). A Cp receptor may facilitate the uptake of copper within the mammalian cell, as this is supported by specific Cp-binding sites on various cell types and tissues (Stevens *et al.*, 1984). Maximum effective copper deliver at the membrane requires ascorbate (Percival, 1989).

In the liver, absorption of Cp molecule is part of a two-step delivery mechanism. Liver endothelium first removes the sialic acid residues from the Cp, which permits a second step in which the underlying hepatocytes absorb the modified Cp via the asialoglycoprotein receptor (Irie and Tavassoli, 1986). By absorbing and then destroying Cp, the liver maintains homeostatic control over copper levels in the blood. Cp fragments have been detected in the bile, supporting an internal proteolysis of the protein (Iyengar *et al.*, 1988).

Cp synthesis and secretion by the liver is regulated acutely by inflammatory hormones [IL-1, IL-6, and tissue necrosis factor] through an increase in transcription (Linder, 1991a), and in the long term by estrogen, probably through stabilization of mRNA (Middleton and Linder, 1993).

Wilsons disease is characterized by low Cp concentrations in the blood, and with corresponding high levels of copper in the liver and brain [hepatolenticular disease] (Brewer, 1992). In Wilsons disease patients, the process of incorporating copper into Cp in the liver is impaired, as is the excretion of copper into bile (due to a defect in the gene coding for a P-type ATPase). This leads to copper accumulation in the liver, which

results in copper overload and hepatic cirrhosis. Copper then spills over into the bloodstream, where it is then transported to other organs viz. the brain, eyes and kidneys (Linder, 1996).

1.1.4.5.4. Metallothionein

MTs are small polypeptides that bind metals. These have a molecular weight of ≈ 6500, and are present in most tissues and cell types (Kagi and Nordberg, 1979). MTs are expressed by cells in response to loading with divalent transition metal ions, especially Zn^{2+}, Cd^{2+}, and Cu^{2+} (but not Fe^{2+}). These contain about 30% cysteine residues, which are distributed in a fixed manner along the polypeptide chain (Hamer, 1986; Linder, 1996).

MT is a major copper-binding protein that is distributed between the soluble (cytosolic) and the insoluble (particulate) fractions of hepatocytes in copper-loaded animals. Wistar rats can recover from copper-induced liver and kidney damage, with ensuing hepatic unloading and possible redirection of the metal to the kidneys for excretion (Haywood *et al.*, 1985). This acquired tolerance may likely be associated with increased production of MT (Evering *et al.*, 1991).

In mammalian cells, binding of Cu^{1+} ions to MT mainly plays a role in copper sequestration in copper related diseases like Menkes and Wilsons disease. MTs are found in all vertebrates, and contain two or more distinct isoforms (MT-1 to MT-4). In mammals, MT-1 and MT-2 are found in all organs, whereas MT-3 is mainly expressed in the brain and MT-4 is most abundant in certain stratified tissues. MT-1 and MT-2 isoforms are inducible by a variety of stress conditions and compounds, which include glucocorticoids, cytokines, reactive oxygen species, and metal ions (MT-3 and MT-4 are relatively unresponsive to these inducers). The most potent inducers of MT-1/MT-2 biosynthesis are metal ions such as zinc, cadmium and copper (Va└<k and Hasler, 2000).

MT-1 and MT-2 are generally 61 amino acids long and contain 20 cysteines in the sequence Cys-Cys or Cys-X-Cys. The cysteines are the coordination sites for binding, and can bind 11 or 12 copper ions in the two domains (α and β domains)- utilizing 12 and 9 sulfhydryl groups, respectively, in an approximate tetrahedral geometry (Rosen *et al*., 1993). Nielson and Winge (1984) have shown that Cu^{1+} binds preferentially to the β domain of MT, with binding saturation near 6 copper ions, whereas cadmium binds preferentially to the α domain.

MT function as sites for metal ion storage and mechanisms for metal detoxification. Other functions include: donation of copper and zinc ions to the apo-forms of the enzymes that require these ions; and in hydroxyl and superoxide radical scavenging activity of copper-containing MTs (antioxidant defense). This latter function explains why MT expression is up-regulated in inflammation (Linder, 1996). Intestinal MT may serve as a control mechanism of copper absorption in the gut (Frieden, 1985).

MT has been shown to protect DNA against cleavage induced by Cu^{1+}-1,10-phenanthroline. 1,10-phenanthroline is a potential mutagen. In the presence of copper and a reducing agent, it is able to induce degradation of DNA via an oxidative mechanism. MT protects DNA by chelating copper, thus preventing the copper from partaking in the Fenton-like reaction, which produces hydroxyl radicals. MT also acts as a free radical scavenger, demonstrating an ability superior to other compounds e.g. GSH and cysteine, due to the free radicals binding at the cystenyl residues (Yang *et al, 2000*).

Binding of copper to MT is increased when zinc is administered either before or after copper loading in rats (Mehra and Bremner, 1983). In zinc-deficient animals of many species, copper-MT is absent from the livers (Bremner and Marshall, 1974; Bremner and Mehra, 1983).

MT works as an antioxidant, as long as zinc is present in Cu-containing MT, and as a prooxidant when zinc is not present. The hydroxyl radicals are produced in the presence of hydrogen peroxide. When MT is not bound to copper, it does not act as a prooxidant.

Excessive copper alters the role of normally functioning MT to a prooxidant, and it causes copper toxicity (Suzuki *et al,* 1996).

Mehra and Bremner (1984) showed that the route of administration of copper (Cu^{2+}) influenced the ability to induce the synthesis of MT. The induction of MT synthesis by Cu^{2+}, is dependent on a critical concentration of labile forms of the metal, and that it is readily obtained after Cu^{2+} injection. These findings also support the view that hepatotoxicity of copper is decreased when it is bound to MT in both the cytosol and particulate fractions of the liver.

1.1.4.6. P-type ATPases: Menkes and Wilsons P-type APTases

ATPase pumps are ubiquitous, being involved with the movement or translocation of ions such as H^+, Na^+, K^+, Ca^{2+}, and a variety of metal ions. Pumps that translocate metal ions are categorized as P-type ATPase (Tsai *et al*., 1992b), with the "P" originating from the covalent phosporylation of a conserved aspartic acid residue that is part of their reaction cycle. The P-type cation ATPases, which includes the copper ATPases, are highly conserved from bacteria to humans (Silver *et al*., 1993).

Menkes disease is caused by a mutation(s) in a gene that encodes the Menkes protein (MNK; ATP7A), that is a copper translocating P-type ATPase. It is found in most cell types, except in the liver (Paynter *et al*., 1994). A defect in this P-type ATPase leads to a fatal copper-deficiency in humans called Menkes disease. MKN is novel, as its activity appears to be regulated by the metal it exports, copper (Dameron, 1998). ATP7A is involved with transporting copper to intracellular compartments, and it is an ATP-dependent transfer. Strong expression of MNK mRNA is detected in muscles, kidney, lung, and brain; placenta and pancreas are weaker, and in the liver, only trace amounts are detected (Chelly *et al.*, 1993; Mercer *et al.*, 1993).
The Wilsons gene encodes the Wilson protein (WND; ATP7B), whose defects cause Wilsons disease, also encodes a copper translocating P-type ATPase which has a 57-65%

homology with MNK (Bull *et al*., 1993). WND is expressed predominantly in the liver and kidney (Bull *et al.*, 1993; Yamaguchi *et al.*, 1993), and is responsible for copper delivery to Cp and biliary efflux of copper (Terada *et al*., 1999).

MNK and WND are transmembrane proteins with 8 transmembrane domains, a long cytosolic N-terminal domain and two large cytosolic loops (Vulpe and Packman, 1995). MNK protein is regulated by a novel system of ligand-induced trafficking, so that, in the presence of raised (potentially toxic) copper levels, MNK rapidly relocalises from the trans-Golgi network, to the plasma membrane, where excess copper is effluxed (Camakaris *et al*., 1995). In polar cells, copper regulated trafficking of the MNK protein to the basolateral membrane of the gut epithelial cells may be responsible for copper absorption into the body. MNK at the trans-golgi network is likely to be the source of copper for copper-dependent enzymes in the secretory pathway. This is consistent with the findings in Menkes disease patients, where lysyl amine oxidase (an extracellular enzyme responsible for the cross-linking of collagen and elastin) is deficient, which results in severe arterial abnormalities (Danks, 1995). Therefore, MNK plays a vital role in copper homeostasis, as it performs the function of copper absorption, donation of copper to important copper-dependent enzymes, and copper detoxification.

The Wilsons protein (WND) is also localized in the trans-Golgi network and exhibits copper-regulated trafficking. It functions like MNK protein, but plays a special role in the liver. It results in the efflux of copper onto apoCp in either the endoplasmic reticulum or a Golgi compartment, or to force the release of copper via biliary excretion (Schaefer and Gitlin, 1999; Yang *et al.*, 1997). In the trans-golgi network, it delivers copper to apoCp, whilst elevated copper results in the exocytic movements of WND containing vesicles and the release of copper at the canalicular membrane. Biliary excretion of copper then ensues (Schaefer *et al.*, 1999).

In the brain, two splice variants of Wilsons disease gene codes for ATPase (PINA) that is found in the pineal gland and retina. The transcription start site of PINA resides between exon 8 and 9 and requires a *cis*-acting sequence that is common to two other pineal-

specific enzymes (Li *et al.*, 1998). The PINA variants are 100 times more common at night, and although lacking all six heavy-metal binding sites, the ATPase retains its ability to restore copper transport in a mutant that is *S. cerevisiae* deficient Ccc2p (Borjigin *et al.*, 1999).

ATP7A and ATP7B work in concert with a series of smaller peptides, the copper chaperones, that exchange copper at the ATPase sites or incorporate the copper directly into the structure of copper-dependent enzymes, like cytochrome *c* oxidase and Cu, Zn superoxide dismutase. These mechanisms come into operation in response to a high influx of copper or during the course of normal copper metabolism (Harris, 2000).

1.1.4.7. Copper chaperones

Recently, an element for the management of cellular copper has been identified viz. the 'copper chaperone' (Pufahl *et al.*, 1997). These ubiquitous proteins have an essential biological function: to transport copper in the cytoplasm to the site of utilization by copper-dependent proteins. Thus, copper chaperones prevent the inappropriate copper interactions with other cellular components.
Copper chaperones have been identified in species ranging from prokaryotes through to humans, and this suggests that copper chaperones are used throughout nature for intracellular copper routing and that they are highly conserved evolutionarily (Rosenzweig and O'Halloran, 2000). An invariant MXCXXC metal binding motif in the N-terminal region, is a structural feature in most copper chaperones (Valentine and Gralla, 1997).

The Gram-positive bacterium, *Enterococcus hirae* has a *cop* operon, which plays a commanding role in copper homeostasis. The *cop* operon consists of four genes, *cop*Y, *cop*Z, *cop*A and *cop*B. The latter two encode copper pumps that belong to the subgroup of the P-type ATPase family (Odermatt *et al*, 1993). They have been called CPx- or P_1-type ATPases (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996). CopA seems to be

responsible for Cu^{1+} uptake when copper is limiting, while CopB secretes Cu^{1+} when it is in excess (Solioz and Odermatt, 1996). The expression of the *cop* operon is regulated by the concerted action of the CopY repressor and CopZ, a copper-binding protein. When cytoplasmic copper concentrations increase, two Cu^{1+}CopZ molecules specifically transfer their copper to CopY to displace a structurally required Zn^{2+}, therefore releasing CopY from DNA and inducing the *cop* operon (Coline *et al.*, 1999). This mode of regulation involving a copper chaperone, to deliver the inducer to the repressor is new and unique.

In humans, HAH1 (also called ATOX1- is comparable to the bacterial CopZ and Atx1p in yeast) is a 68 amino acid protein. It is a member of a homologous family of metallochaperones, which binds and transports metals specifically (Klomp *et al*., 1997). It was found that the N-termini of cadmium, silver and copper pumping CPx-type ATPase contain domains that resemble Atx1p (in yeast) and CopZ (in bacteria). These domains occur in one or two copies in microbial enzymes and up to six copies in human Menkes and Wilson ATPases (Solioz and Vulpe, 1996).
HAH1 is synthesized as a single-chain protein, which is distributed throughout the cytoplasm and nuclei of cells. Hamza *et al*. (1999) proposed a model of the interaction of HAH1 with the ATPases in the trans-Golgi network. HAH1 binds cellular copper forming an HAH1-copper complex, which then trafficks the copper to transporting ATPases via direct protein interaction. Hamsa *et al.* (1999) have demonstrated a direct copper-dependent interaction between HAH1 and the WND and MNK proteins in vivo and in vitro. The transient nature of this interaction would permit diffusion-driven movement of cellular copper via rapid association and dissociation of HAH1 with the ATPases. This demonstrates the interaction of HAH1 with WND is essential for copper homeostasis.

Copper-dependent proteins in higher organisms can receive copper either during the maturation in the Golgi, as is the case of Cp (Sato and Gitlin, 1991), in the cytoplasm, as demonstrated for apo-cuperoxide dismutase (SOD1) (Culotta *et al.*, 1997) or in organellar locations (as in cytochrome *c* oxidase, CCO). The human APTases are distributed

between the *trans*-Golgi and plasma membrane. In the *trans*-Golgi, the metallochaperones deliver copper into this compartment for incorporation into cuproenzymes (Harrison *et al.*, 2000).

Rae *et al.* (1999) have shown that 'free' copper concentrations in the cell are less than one attomolar, which corresponds to less than one free copper ion per cell. Thus, it suggests a physiological requirement for metallochaperones: copper chaperones reserve copper from intracellular chelators so that it is available for incorporation into copper-containing enzymes. Also that metal-ion delivery must occur via a direct-transfer mechanism.

These copper chaperones transfer Cu^{1+}, rather than Cu^{2+}, in intracellular copper trafficking pathways. All the copper chaperones characterized to date preferentially stabilize and exchange Cu^{1+} (Rosenzweig and O'Halloran, 2000).

1.1.4.8. Manifestations of copper excess

The incidence of chronic copper toxicosis is low, despite the considerable variation in copper intakes. This reflects the efficiency of the homeostatic control mechanisms that operate to keep tissue copper levels within a narrow range. These mechanisms operate at both the intestinal absorption and biliary excretion levels. Yet, copper toxicity can develop under certain conditions, depending on factors such as species, genetics, age and diet (Bremner, 1979). For example, sheep are particularly susceptible to copper toxicity, as it appears that they can not increase their biliary copper excretion in response to increased copper intakes. Pigs, conversely, are very tolerant to copper, as they are accustomed, to be given diets containing 250 mg Cu/kg as growth stimulants.
Neonatal and milk-fed animals are more prone to copper poisoning, presumably because of their higher copper absorption efficiency and the immaturity of the biliary excretory mechanisms. This may partly explain why reports of copper-induced cirrhosis in humans, e.g. Indian childhood cirrhosis, are restricted mainly to young children (Tanner, 1997).

The genetic component to copper toxicosis is illustrated by the occurrence of Wilsons disease in humans and the increased sensitivity of Long Evans Cinnamon (LEC) rats to copper poisoning. [The LEC rat is a model for Wilsons disease.]

The clinical manifestations of copper toxicosis are not constant and vary between species. Pigs who are relatively tolerant to copper, exhibit weakness, dullness, respiratory distress, pulmonary edema, anemia, jaundice and ulceration in the esophageal region of the stomach, with excessive copper intakes (Allen, 1962). Rats are also relatively tolerant of copper. High dietary intakes of copper (> 500 mg/kg), results in growth impairment, extensive necrosis of hepatocytes, and widespread necrosis of the proximal convoluted tubule epithelial cells of the kidney. Nonetheless, rats can adapt to prolonged exposure to copper, thus developing tolerance, as the liver copper concentrations decrease in association with liver regeneration. The extrusion of apoptotic bodies and Mallory-like structures also accompany these changes (Fuentealba *et al*., 1989, 1993).
Under normal circumstances, much of the copper in the liver appears in the cytosol, but as copper accumulates, an increasing proportion occurs in the particulate fraction- the nucleus and lysosomes. The lysosomal accumulation is most likely linked to copper-induced autophagy. This has generally been assumed to be part of a detoxification process and a prelude to biliary excretion of copper (Bremner, 1998). Lysosomal copper accumulation has been reported in neonates (Goldfischer and Bernstein, 1969), Bedlington terriers (Johnson *et al*., 1981), Wilsons disease patients (Goldfischer, 1967) and copper-loaded rats (Haywood et al, 1985).

1.1.4.9. Mechanisms for protection against copper toxicity

Cellular mechanisms utilized by cells to detoxify metals are shown in Figure 6. The detoxifying mechanisms can be subdivided into, reduction of metal uptake, enhanced metal exportation, and metal sequestration.
Limiting toxicity through the reduction of metal importation is achieved by inhibiting the import machinery or by making the extracellular metal unavailable for absorption.

Exportation of excess metal ions to limit the intracellular toxicity is an important process. The cation translocating P-type ATPases are used to export the metal ions out of the cells or into organelles. These ATPases are frequently modified to increase the specificity and efficiency for a given metal. Often the metal has to be reduced to a lesser charged species, so that it can be transported more easily.

The intracellular chelation or sequestration of metals into relatively innocuous complexes or organelles to limit their toxicity, is a commonly utilized mechanism. The chelating agents are peptides or proteins that form stable complexes that reduce and limit the element's reactivity and aids in its excretion out of the cell. Mammalian pathways for detoxifying copper include MTs and ATP translocases (Figure 7). MT synthesis is induced by copper ions. The primary route for excretion is the bile and may involve MT (Hamer, 1986).

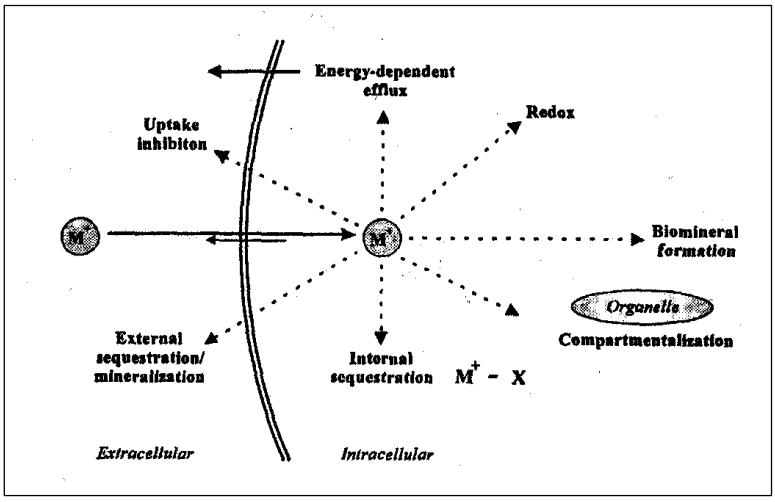

Figure 6. General mechanisms for metal ion detoxification. Three categories of mechanisms are used to limit intracellular toxicity of metal ions: 1) reduced import through uptake inhibition or external chelation; 2) sequestration into peptide or protein complexes, organelles, or both, or biomineral formation; 3) increased exportation out of cells by pumps or into organelles that subsequently extrude (Dameron and Harrison, 1998).

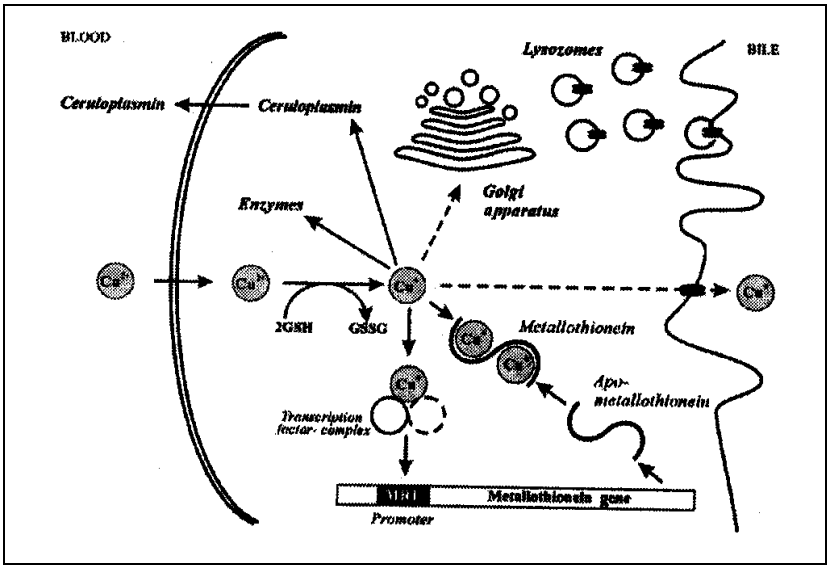

Figure 7. Metal detoxification in mammalian cells. Copper is sequestered by MT, whose synthesis is regulated by copper. Copper is also exported by copper-regulated translocating ATPases. (From Dameron and Harrison, 1998)

1.1.4.10. Mechanisms of copper conservation in organs

During periods of copper restriction, copper is conserved. Organ-specific mechanisms of copper metabolism results in organ-specific turnover curves (Owen, 1971). The liver and possibly the kidney require a significant decrease in copper concentration before conservation mechanisms are induced. But, once these mechanisms are induced, these are almost complete (Levenson and Janghorbani, 1994).

There are numerous conservation mechanisms, and these include: alterations in cuproprotein synthesis and degradation rates, changes in the uptake and export of copper from specific cell types, and changes in the utilization of copper storage forms. Turnland *et al.*, (1989) have shown that copper absorption is dose dependent, which indicates that during periods of dietary restriction, where copper availability is low, the efficiency of copper absorption is increased.

Several genes that are involved in the metabolism of copper have been shown not to be regulated by copper. An example is Cp, where hepatic Cp mRNA amounts are not altered by changes in copper status (Gitlin *et al.*, 1992).

Whole-body copper turnover has two kinetic compartments with half-lives of 2.8 and 9.2 days (Linder and Roboz, 1986).

Dunn *et al*. (1991) developed a 16-compartment model based on 3-d ^{64}Cu turnover data, that included organ copper, plasma, bile, feces and hepatic subcellular fractions. To date, this model is the most comprehensive kinetic model of copper metabolism.

1.1.5. Copper as a prooxidant

Copper overload results in many pathologic conditions that are consistent with oxidative damage to membranes or molecules. Copper ions are active in oxidation-reduction reactions. Copper ions are able to catalyze the formation of hydroxyl radicals via the Haber-Weiss reaction (in vitro):

$$O_2^{\cdot-} + Cu^{2+} \rightarrow O_2 + Cu^{1+} \quad \text{… Equation 1.1}$$

$$Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^{\cdot} \quad \text{… Equation 1.2}$$

Cu^{2+} can also react with hydrogen peroxide to yield Cu^{1+} and superoxide radical. The Cu^{1+} can then partake in the reaction in equation 1.2.

A common consequence of copper-induced production of reactive oxygen species (ROS) is increased lipid peroxidation. Dietary copper overload in rats, result in in vivo peroxidation of mitochondrial membrane lipids, as shown by increased concentration of conjugated dienes and thiobarbituric acid-reacting substances (TBARS). This results in dilated, crystic cristae of inner mitochondrial membranes in the liver and other biochemical and histological signs of hepatocyte injury. The mitochondria may be the specific target of copper-induced liver damage, as no comparable effects are seen in microsomes (Sokol *et al*., 1990).

Mitochondrial function is also impaired in copper-loaded rat livers, with decreases in state 3 respiration and the respiratory control ratios in the mitochondria when several electron donors were used (Sokol *et al*., 1993). Mitochondrial electron transport proteins

are also affected, as analysis of the oxidoreductase activities, indicate a reduction in complex IV (CCO) activity. Such changes in mitochondrial function contributes to hepatocellular dysfunction by reducing cellular energy charge, increasing mitochondrial leakage of calcium into the cytosol, or exposing the cell to increasing amounts of superoxide generated by the disruption of normal electron flow (Bremner, 1998). These changes in mitochondrial function result, due to oxidative stress.

Oxidative stress is defined as the imbalance between biochemical processes leading to the production of reactive oxygen species and the cellular antioxidant cascade, causing molecular damage that can lead to a critical failure of biological functions and ultimately cell death (Sayre *et al.*, 1999).

The result of impaired mitochondrial function is the increased generation of free radicals e.g. superoxide and hydroxyl radicals. These radicals are normally produced as by-products of oxidative metabolism. Mitochondria are known to be the most important physiological source of superoxide radicals in animal cells. Impaired mitochondrial function also impairs intracellular Ca^{2+} buffering. An increase in intracellular Ca^{2+} leads to increased free radical generation, and to the activation of nitric oxide synthase (NOS) (Beal, 1996).

Hepatic lysosomes in copper-loaded rats are affected due to increased lipid peroxidation as a direct effect of copper-catalyzed reactions. The concentrations of TBARS in isolated lysosomal membranes of these rats were doubled, with an increase in the membrane fragility and a decrease in their fluidity. With an increase in lysosomal pH, these membrane alterations may affect the function of the proton ATPase pumps (Myers *et al.*, 1993).

The presence of apoptotic bodies in the livers of copper-loaded animals is indicative of copper-induced damage to DNA (King and Bremner, 1979). The hydroxyl radical indiscriminantly reacts at diffusion-controlled rates with many biomolecules and are probably scavenged by intracellular antioxidants, before it reaches the DNA. However, copper binds readily to DNA to form adducts, and is involved in chromatin condensation (Sangripathi *et al.*, 1991).

Glutathione (GSH), a reducing agent, reduces Cu^{2+} to Cu^{1+}, which can then result in copper binding to DNA. It is found in high concentrations within the nuclei, and was shown to actually inhibit the free radical formation caused by Cu^{1+}. GSH stabilizes the copper in the Cu^{1+} state, and prevents it from participating in free radical generation. Thus, GSH may protect against copper-induced DNA damage (Milne *et al.*, 1993). GSH is required for biliary excretion of copper in adult rats (Houwens *et al.*, 1990). However, the GSH system may be saturated in copper-loading, thus resulting in free Cu^{1+} interacting with intracellular organelles, resulting in peroxidation and DNA damage.

Transition metals usually promote free radical reactions. For example, several transition metal salts reduce H_2O_2 in a one-electron reaction to generate $OH^{\cdot}$ via the Fenton reaction (Equation 1.3):

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\cdot} + OH^{-} \quad \text{… Equation 1.3}$$

Where M^{n+} can be Cu^{1+}, Ti^{3+}, Fe^{2+}, Co^{2+} and Ni^{2+}.

Cu^{2+} ions can react with $O_2^{\cdot -}$ to yield Cu^{1+} (Equation 1.1), which in turn reacts with hydrogen peroxide. Cu^{1+}, via the Fenton reaction, reacts with hydrogen peroxide to yield the reactive secondary hydroxyl radicals. The overproduction of free radicals due Cu^{2+} and Fe^{3+} have been implicated as a causal factor in the death of nigral cells associated to Parkinson's disease (Bush, 2000).

Samuni *et al.* (1981) proposed the binding of copper (Cu^{2+}) to a target protein, which then forms a protein-copper complex. This complex then reacts with superoxide radicals, which results in the Cu^{2+} being reduced to Cu^{1+}-protein complex. Cu^{1+}-protein complex appears to be a relatively stable, long life complex (Equation 1.4). This complex then reacts with hydrogen peroxide to yield hydroxyl radicals (Equation 1.5). These secondary hydroxyl radicals formed from copper ions bound near vital targets would induce local damage, and impair the biological function of the molecule.

$$\text{protein-}Cu^{2+} + O_2^{\cdot -} \rightarrow \text{protein-}Cu^{1+} + O_2 \quad \text{… Equation 1.4}$$

$$\text{protein-}Cu^{1+} + H_2O_2 \rightarrow \quad \text{protein-}Cu^{2+} + OH^{\cdot} + OH^{-} \quad \ldots \text{ Equation 1.5}$$

Thus, complexed copper in the presence of hydrogen peroxide can bring about site-specific damage. Hydroxyl radicals are the most reactive amongst oxygen species. It cause oxidative damage to cell membranes, proteins and DNA- thus the radicals are cytotoxic (Gutteridge and Wilkens, 1983). Toyokuni and Sagripanti (1994) demonstrated that rats continuously exposed to copper, had elevated levels of 8-hydroxydeoxyguanosine (8-OHdG) in DNA from liver, kidney and tissues surrounding the site of delivery. Production of 8-OHdG has been associated with mutagenesis and carcinogenesis. This effect, if extrapolated to humans, indicates that humans require a continuous daily delivery of approximately 280mg of copper.

1.1.5.1. Oxidative stress and Beta-receptors

Beta-adrenoreceptor ($-receptor) function is impaired by oxidative stress, in various tissues. The pineal gland in the brain, which secretes melatonin, is innervated with beta-receptors. Beta-receptors are stimulated by noradrenaline, which then results in the rapid synthesis of N-acetyltransferase (NAT). NAT is the rate-limiting step in the production of melatonin (Feuer, 1990). In experiments with the administration of hydrogen peroxide, beta-adrenergic responsiveness is reduced. Peroxidation of lipid membranes, lead to altered membrane viscosity, which affects the coupling of the receptor to the effector system. Oxidative stress, acting on membranes, increases the accessibility of beta-receptors to hydrophobic, but not hydrophilic ligands. However, more extensive oxidative stress decreases maximal binding to beta-receptors for both hydrophobic and hydrophilic ligands (Kaneko *et al.*, 1991). Thus it can be postulated that copper toxicity, could lead to a decrease in NAT activity, which would result in a decrease in melatonin production.

1.1.6. Copper induced Ca^{2+}-dependent neurotransmitter release

Wang (1999) demonstrated that Cu^{2+} could induce a massive Ca^{2+}-dependent neurotransmitter release from brain catecholaminergic nerve terminals. The effect of Cu^{2+} on catecholamine release is ultimately dependent on an opening of the voltage-gated Ca^{2+} channels, the influx of Ca^{2+}, and the activation of the Ca^{2+}-dependent synaptic vesicular secretory machinery. A possible mechanism by which Cu^{2+} can induce neurotransmitter release could be through the modulation of the nerve terminal membrane potential. The primary effect of Cu^{2+} on the catecholaminergic terminals may be membrane depolarization, which furthermore to causing transmitter release, can also secondarily inhibit transmitter reuptake. The increase in the levels of Cu^{2+} in vivo, is likely to cause a net increase in transmitter release from catecholaminergic terminals. Dopamine is toxic at higher concentrations to striatal neurons. This is also potentiated by compromised mitochondria (McLaughlin *et al.,* 1998). It is likely that, chronic Cu^{2+} toxicity in Wilsons disease can impair catecholaminergic transmission due to sustained catecholamine release uncoupled from synaptic activity. Over time, a sustained increase in striatal dopamine levels may lead to neuronal damage and death, and this could contribute to the neuropathological processes associated with Cu^{2+} toxicity in Wilsons disease.

1.1.7. The role of copper in Neurodegenerative disease

Oxidative stress has been frequently implicated in neurodegeneration, and it reflects the selective vulnerability of the central nervous system to the increased utilization of dioxygen. Hydrogen peroxide, produced by oxidases can result in greater oxidative stress susceptibility in tissues enriched in these enzymes. The principal ROS culprit of oxidative stress is the hydroxyl radical.

Copper has been found to be involved in the neuropathology of neurodegenerative disorders e.g. Wilsons disease, Menkes disease, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Prion disease (Sayre *et al.,* 1999; Waggoner *et al.*, 1999).

1.1.7.1. Role of copper in Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative disorder. It is characterized pathologically by the presence of neurofibrillary tangles (NFT), senile plaques, neurophil threads, amyloid-$ (A$) deposition, and a selective loss of neurons. A$ is proteolytically derived from the transmembrane amyloid precursor protein and the mutations in this protein result in familial Alzheimer's disease, with a marked increase in A$ accumulation in the brain (Price and Sisodia, 1998). The accumulation of A$ appears to trigger events leading to free radical mediated neurotoxicity and oxidative damage (Wang, 1998).

A$ is self-aggregating and can disrupt cellular ion homeostatis, induce cytotoxic cellular oxidant stress, and promote local microglial and astrocytic activation (Selkoe, 1998). Atwood *et al.* (1998) suggested that copper may play a role in the promotion of A$ aggregation, under mildly acidic conditions. The amyloid precursor protein has been found to bind and reduce copper (Hesse *et al.*, 1994; Multhaup *et al.*, 1996). The reduced copper is bound to the amyloid protein as it is delivered from the cell body to the axonal cell surface and the dendritic plasma membrane (Selkoe, 1998). It is thus possible that amyloid is involved in the transcellular transport of copper. This bound copper is rapidly reoxidized in the presence of hydrogen peroxide and this is accompanied by fragmentation of the amyloid precursor protein into distinct peptides, which includes the amino terminus of A$. This suggests an additional mechanism whereby copper may mediate A$ aggregation, and thus potentiate oxygen radical injury in Alzheimer's disease (Multhaup *et al.*, 1996). Atwood *at al.* (2000) demonstrated that multiple affinity cooperative binding sites of Cu^{2+} exist on A$, which promotes precipitation. The results indicate that small changes in the free or exchangeable Cu^{2+} concentration may have an impact on the solubility of A$, in vivo.

1.1.7.2. Prion disease

Human prion disease is characterized by a rapidly progressive dementia and cerebellar ataxia resulting from neuronal spongiform degeneration and astrocytic gliosis. Prion diseases are caused by the accumulation of a posttranslationally modified form (PrP^{sc}) of the normal cellular prion protein (PrP^{c}) (Prusiner, 1997). Miura *et al.* (1996) demonstrated that an octapeptide in the amino terminus of the PrP^{c} binds copper and that this binding appears to confer a more defined structure on this region. It promotes a conformational shift from an " helical to a $-sheet structure (Waggoner, 1999)

1.1.8. Role of copper-drug complexes in disease

Copper complexes of nonsteroidal antiinflammatory drugs (NSAIDS) have been synthesized (Sorenson, 1976) and have demonstrated antiulcer, anticonvulsant, anticancer, and antidiabetic properties (Sorenson, 1989). These complexes, have been found to be more effective than the parent drugs, in man and in animal models of inflammation (Sorenson, 1989; Roch-Arveiler *et al.*, 1990). Bertrand *et al.* (1999) demonstrated that the copper-complexes exerted a protective effect probably due to a free radical scavenging effect.

1.2. The Pineal Gland

1.2.1. History of the Pineal Gland

The famous anatomist, Herophilos (325-280 B.C.), at the University of Alexander in Egypt, first discovered the pineal organ in man. He was the also the first in localizing the soul in the brain ventricular system, and as a tap for regulating the stream of the "pneuma" (the human spirit) from the third to the fourth ventricles of the brain (AriNns Kappers, 1979).

Galenos of Pergamon (∀130-300 A.D.) agreed with the notion of the "pneuma", but not with the tap function of the pineal, as he observed that this was situated outside the brain ventricular system. Galenos stated that the pineal is just a "gland", like any other gland filling the gap between branching vessels. He coined the term "soma konoeides" or "konareion" for the human pineal, because it resembled the cone of a pine tree (AriNns Kappers, 1979).

RenJ Descartes (1596-1650) described the pineal as the "seat of the soul". This was particularly important during that period, as both scientists and philosophers where questioning the importance of the soul. He based this on the fact that the pineal was the only unpaired part of the brain, and thus the soul could exercise its function more particularly from the pineal. Niels Steensen (1638-1686) refuted the ideas of Descartes in regard to the functions of the pineal (AriNns Kappers, 1979).

In the late 19th century and the beginning of the 20th century, there was an explosion in pineal organ research. Bernard (1813-1878) and Brown-Sequard (1817-1894) discovered the endocrine glands and hormone producing organs, and this led to greater interest of physiologists in the pineal gland. At the end of the 19th century, the mammalian pineal gland was seriously considered as a candidate for hormonal production.

In 1898, Heuber was the first to diagnose and describe a patient (a boy) suffering from a pinealoma, and showing signs of precocious puberty.
Bargmann (1943) produced a survey that dealt with the microscopic anatomy and histology of the pineal gland in all invertebrates. He stated that unknown external factors could induce changes in the mammalian pineal, and underlined the necessity for research on the influence of light on the pineal (AriNns Kappers, 1979).

In the last 50 years, scientists have confirmed that the pineal gland is an endocrine organ, and determined the neuronal connections between the pineal gland and the hypothalamus. The discovery of melatonin, the chief hormone produced by the pineal, has stimulated worldwide research in this field.

1.2.2. Pineal location and anatomy

The pineal gland is located within the brain. It develops from an evagination of the neural tube, which becomes the diencephalon (AriNns Kappers, 1979). It varies in size, shape and location in different species. In humans, the gland is located on the dorsal surface of the hypothalamus, occupying a central position between the two cerebral hemispheres (Yu and Reiter, 1993).
It is a highly vascularized tissue and consists of two types of cells: pinealocytes and neuroglia. In humans, pinealocytes predominate and produce both melatonin and peptides e.g. arginine-vasotocin (Brzezinski, 1997).
In the rat, the pineal is located in the superior part of the *sulcus transversus cerebri* at the surface of the brain. It lies between the cerebral hemispheres, anteriorly, and the cerebellum posteriorly. The pineal gland is connected to the commissural region by a thin filament-like stalk. It is formed by the fusing and growing out of the anterior and posterior epiphyseal peduncles originating from the most proximal parts of the rostro-dorsal and caudo-ventral walls of the original epiphyseal evaginations (Ariens KappNrs, 1960-*o*) (Figure 8). The pineal gland is attached to the brain by the pineal stalk, and it consists of pinealocytes, pinealoblasts and fibrocytes.

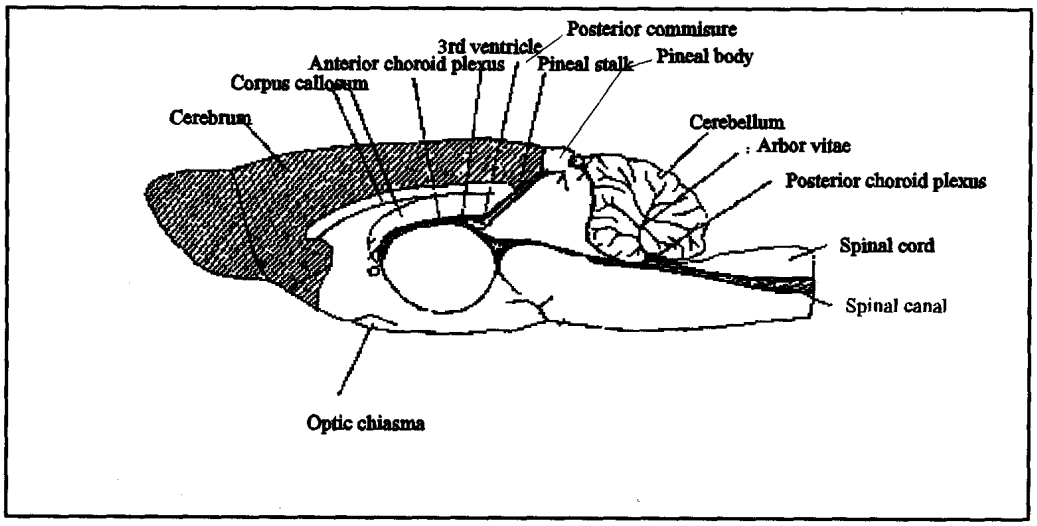

Figure 8. Traverse section of the rat brain showing the relative position of the pineal body and pineal stalk (Rowett, 1962).

1.2.3. Pineal innervation

The pineal gland in mammals is heavily innervated by nor-adrenaline containing sympathetic fibres (Pfister *et al.*, 1978). Wolfe *et al.* (1962) demonstrated that the secretory function of the mammalian pineal gland was linked to its sympathetic innervation. The pineal gland is also referred to as the "neuro-endocrine transducer organ", due to its ability to synthesize and release melatonin in response to a neuronal stimulus. An external light stimulus is converted to photic signals in the retina. These signals pass via the suprachiasmatic nucleus (SCN), to the tubural hypothalamus, over the medial forebrain bundle, reticular formation and upper thoracic intermediolateral cell column, to the superior cervical ganglion. The superior cervical ganglion conveys the signal via the postganglionic sympathetic fibres, to the conarian nerve. The conarian nerve passes the signal to the pineal gland, which then releases noradrenaline (NA). NA

then binds to $-adrenergic receptors on the pineal cell surface. This results in a cascade of events that lead to the production of melatonin (Klein, 1979; Ebadi *et al.* 1986).

1.2.4. Pineal indole metabolism

The pineal gland secretes an indolamine, melatonin (so termed, due to its blanching effect on melanophores) (Lerner *et al.*, 1959). Tryptophan is taken up from the blood stream into the pinealocytes, where it is utilized in the synthesis of pineal proteins. Most of the tryptophan is converted to 5-hydroxytryptophan via tryptophan hydroxylase in the mitochondria (Hori *et al.*, 1976). In the synthesis of seratonin from tryptophan, this appears to be the rate-limiting step. Trytophan hydroxylase requires the presence of oxygen, ferrous iron, and a reduced pteridine co-factor to function. A high concentration of the pteridine co-factor is found in the pineal gland (Lovenberg *et al.*, 1967; Levine *et al.*, 1979).

5-hydroxytryptophan is converted via L-amino acid decarboxylase to serotonin (5HT), also called 5-hydroxytryptamine. This enzyme is located in the cytosol and requires a pyridoxal phosphate to function (Snyder *et al.*, 1965).

Serotonin can undergo three different metabolic pathways (Figure 9):

(1) It can be acetylated to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (NAT), with acetyl coenzyme A, a cofactor, being the acetyl donor (Weissbach *et al.*, 1960). N-acetylserotonin is the precursor of melatonin (Klein *et al.*, 1971). N-acetylserotonin is then converted to melatonin by O-methylation in the 5-position by the enzyme hydroxyindole-O-methyltransferase (HIOMT).

(2) It may undergo deamination and oxidation reactions, where serotonin is oxidized by the enzyme monoamine oxidase to 5-hydroxyindole acetaldehyde. 5-hydroxyindole acetaldehyde is an unstable intermediate and undergoes further metabolism (Axelrod *et al.*, 1969). The acetaldehyde is converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968). A proportion of the 5-hydroxyindole

acetaldehyde is converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is then methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman and Axelrod, 1967).

(3) It may be methoxylated by HIOMT to form 5-methoxytryptamine. S-adenosylmethionine donates the methyl to the serotonin.

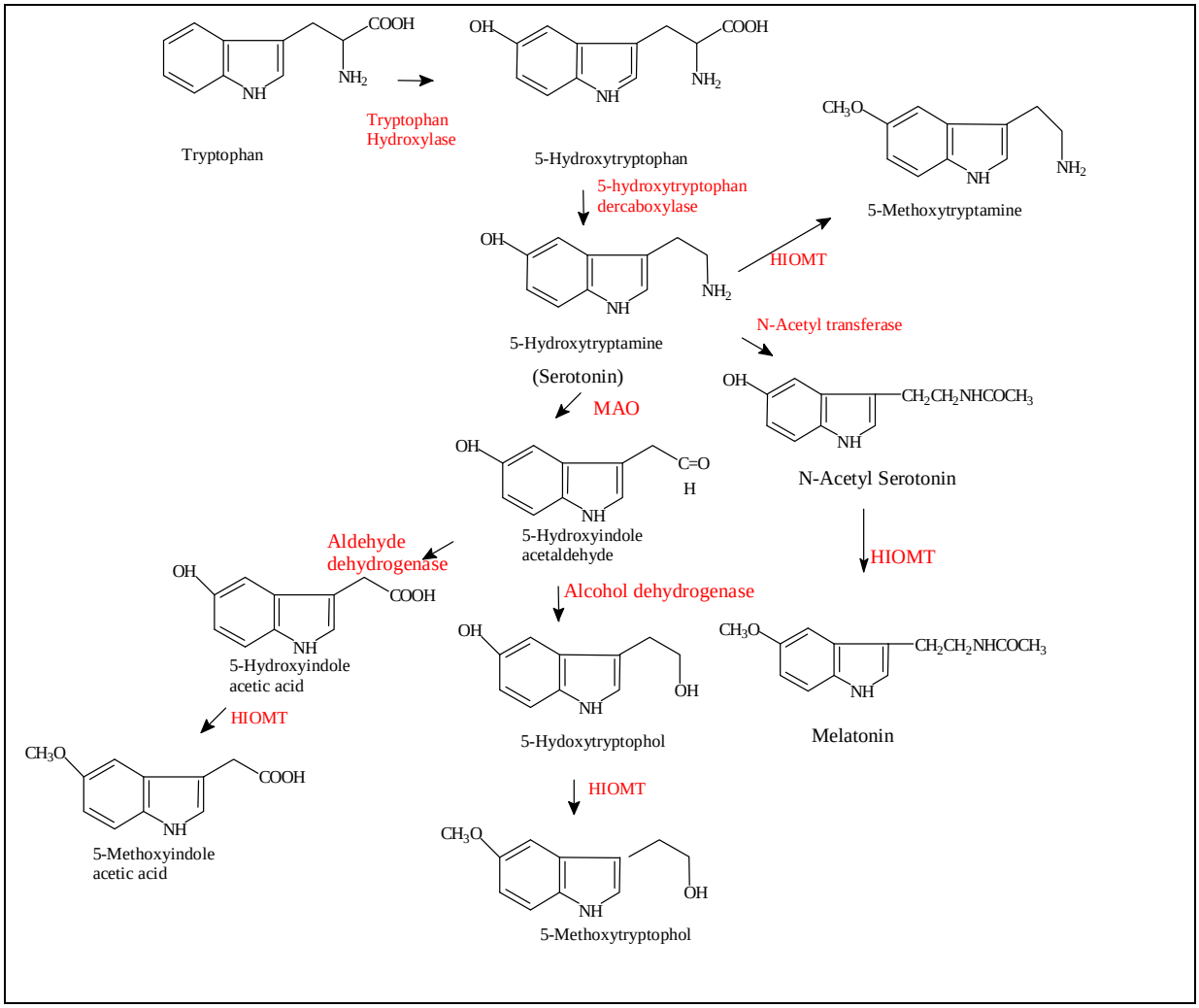

Figure 9. Schematic representation of pineal indole metabolism (modified from Young and Silman, 1982)

1.2.5. Melatonin

1.2.5.1. History of melatonin

It was known for a long time that a substance from the mammalian pineal gland caused the blanching of melanophores in amphibian skin. In 1917, McCord and Allen reported that amphibians fed with bovine pineal extracts exhibited skin lightening. Lerner and co-workers (1958) isolated the compound responsible for this action from bovine pineals, and it was identified to be N-acetyl-5-methoxytryptamine. Due to its blanching effect on melanophores, it was called melatonin. The chemical structure of melatonin is shown in Figure 10.

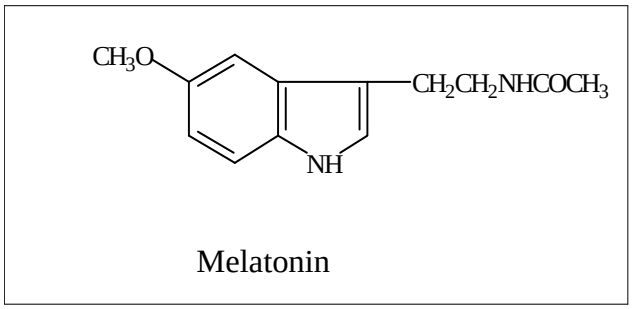

Figure 10. The chemical structure of the pineal indole, melatonin (Feuer, 1990)

1.2.5.2. Synthesis of melatonin

Melatonin is synthesized primarily in the pineal gland from serotonin via acetylation reactions catalysed by NAT and HIOMT. NAT is regulated by specific cAMP-dependent transcription factors, which ultimately regulate the oscillatory synthesis of melatonin (Foulkes *et al.*, 1997).

The melatonin biosynthetic pathway is regulated by light, such that when light falls apon the retina, neuronal impulses originating from the retina are transmitted to the SCN of the hypothalamus and other hypothalamic structures. From the SCN a projection descends to the intermediolateral cell column in the upper thoracic region of the spinal cord, where fibers project to the superior (third) cervical ganglion. From here postganglionic sympathetic fibers terminate at the pineal gland (Haines, 1997). With the onset of darkness, these sympathetic fibers release noradrenaline, which bind predominately to postsynaptic $1-adrenergic receptors (and to a lesser extent to "1-adrenergic receptors). $1-adrenergic receptors are coupled to adenylate cyclase. $-adrenergic stimulation of the pinealocytes activate the adenylate cyclase via G_s (a stimulatory guanine nucleotide-binding regulatory protein), which catalyses the conversion of ATP to cAMP (Strada *et al.*, 1972).

The increase intracellular cAMP results in elevated NAT activity. It increases the transcription of mRNA and increases NAT (Reiter, 1991; Brezezinski, 1997). Serotonin is then converted to N-acetylserotonin via NAT. N-acetylserotonin is then converted to melatonin via HIOMT. NAT activity is lower during the day (Reiter, 1997), with a resultant decrease in melatonin production during the day. At night, blood melatonin levels reach values of 150 pg/ml.

This cascade of neuronal and intrapineal signals facilitate melatonin anabolism and is responsible for the circadian synthesis and release of melatonin. Melatonin concentrations in the pineal gland increase with the onset of darkness. It passively diffuses into the cerebrospinal fluid and into circulation. In humans, melatonin secretion peaks at night between 2:00 am and 4:00 am, and gradually decreases thereafter throughout the proceeding 24-hour period (Waldhauser *et al.*, 1984).

Melatonin synthesis is not confined only to the pineal gland. It is also synthesized in the retina, the Harderian gland, the intra-orbital lacrimal glands, hypothalamus, the gut, the inner ears, peripheral mononuclear cells, and platelets (Menendez-Pelaez and Reiter, 1993). These extrapineal synthesizing sites are proposed to synthesize melatonin for use within their respective sites. Pineal synthesized melatonin is predominately responsible for the circulating melatonin in the blood, and hence it is regarded as an endocrine organ. Reiter (1989) has proposed that extrapineal synthesized melatonin sites compensate for melatonin production in pinealectomized animals.

1.2.5.3. Physicochemical properties of melatonin

Melatonin is a small, non-charged molecule that is both highly lipophilic, as well as, relatively water soluble (Shida *et al.*, 1994). It can easily traverse the blood-brain barrier and penetrate any biological membrane, due to its lipophilicity, and appears in every tissue and body fluid (Menendez-Palaez and Reiter, 1993).

The 5-methoxy group offers melatonin a considerable increase in radical-trapping capacity at physiological pH as compared to 5-hydroxylated and nonsubstituted indoles. It shields the molecule from dimerization and prevents it from forming quinone imine structures. The N-acetyl group prevents melatonin from binding to serotonin receptors. It protects melatonin from degradation by monoamine oxidase (MAO). In comparison to 5-methoxytryptamine, the acetyl group contributes to melatonin's radical scavenging capacity. The pyrrole ring is one of the most important reactive parts of the molecule. It

can be opened by peroxidative cleavage, either through enzymatic, or nonenzymatic mechanisms involving free radicals. Opening of the pyrrole ring results in the formation of a class of biogenic amines called kynuramines (Hardeland *et al.*, 1993).

1.2.5.4. Functions of melatonin

Melatonin has many and varied functions in mammalian systems. These are:

i. Free radical scavenger: melatonin is the most potent hydroxyl radical scavenger (Reiter *et al.*, 1994). It has also been shown that melatonin scavenges the peroxyl radical (ROO^{q}) (Melchiorri *et al.*, 1995). This is discussed in section 2.5.7.

ii. Reproduction: melatonin causes a marked decrease in sperm motility and at high concentrations (and still within physiological values), completely abolishes its movement (Oosthuizen *et al.*, 1986).

iii. Immunocompetence: it has been shown that melatonin administration enhances the immune response (Pierpaoli and Maestroni, 1987). Melatonin administration restores the immune dysfunction from soft-tissue trauma and hemorrhagic shock (Wichman *et al.*, 1996).

iv. Jet-lag: Turek (1987) demonstrated that melatonin supplementation appeared to shorten the re-entrainment of the circadian rhythm.

v. Aging: relative melatonin deficiencies with persistent serotonin have been reported to promote aging (Rozencwaig *et al.*, 1987)

vi. Metal binding: Limson *et al.* (1998) reported that melatonin binds/complexes with metals. This has important consequences in the possible role of melatonin in metal toxicity e.g. in Alzheimer's disease, where an accumulation of aluminium is suggested to be a contributing factor in the eitiology of the disease; Wilsons disease, where a accumulation of copper results in hepatolenticular degeneration; and in iron toxicity.

1.2.5.5. Melatonin secretion and distribution

Melatonin is secreted from the pineal gland (pinealocytes) directly into the bloodstream via the pineal capillaries, which drain into the surrounding venous sinuses. Plasma melatonin levels are higher than the melatonin levels in the cerebrospinal fluid. This confirms that the primary secretory route of melatonin is the bloodstream (Feuer, 1990). Approximately 60-70% of the circulating melatonin is plasma bound, mainly to albumin. In the cerebrospinal fluid, melatonin is found in its free state (Cardinali *et al.*, 1972). There is no equilibrium in the melatonin level between the plasma and the cerebrospinal fluid. About 90% of secreted melatonin is taken up by tissues, during a single passage through the body (Lewis *et al.*, 1990). Melatonin being both highly lipophilic and relatively water-soluble, is able, to be readily taken up by cells. It can easily traverse the blood-brain barrier as well as other morphophysiological barriers, and is believed to have ready access to every cell in the organism (Menendez-Pelaez and Reiter, 1993)

1.2.5.6. Melatonin metabolism

Melatonin turnover rate is short, as in rats, the half-life is about 20 minutes (Gibbs and Vriend, 1981). In young animals the half-life of melatonin is longer, due to low metabolizing enzyme activity in the liver (Weinberg *et al.*, 1981). The majority of melatonin metabolism takes place in the liver. Melatonin metabolism follows a bi-phasic course. The initial half-life is short, lasting a few minutes, followed by a second longer phase (Gibbs and Vriend, 1981). Approximately 75% of the melatonin taken up by the liver is converted by oxidation to 6-hydroxymelatonin by cytochrome P-450-dependent microsomal mixed-function oxidase enzymes. Approximately 70% of the 6-hydroxymelatonin is then further conjugated to sulphate, and to a lesser extent to glucuronide. These conjugates are excreted in the urine and feces (Kopin *et al.*, 1961; Lerner and Nordlund, 1978).

In the brain, indoleamine-2,3-dioxygenase cleaves the pyrrole ring to form N-acetyl-N-formyl-5-methoxykynurenamine. This is then converted to N-acetyl-5-methoxykynurenamine (Feuer, 1990).

Exogenously administered melatonin has a serum half-life of 0.5-5.6 minutes (Brezezinski, 1997).

1.2.5.7. Melatonin receptors

Two pharmacologically distinct families of melatonin receptors have been identified, ML1 and ML2. These receptors are membrane-bound. ML1 receptors are believed to be involved in renal function, sleep induction, circadian rhythms, reproduction, and the contractility of cerebral arteries (Feuer, 1990). ML2 receptor functions are unknown at present.

Recent reports indicate that melatonin may readily diffuse into cells to activate intracellular sites through binding to cytosolic calmodulin (Brezezinski, 1997). Once melatonin has bound to calmodulin, it can influence calcium signaling through interactions with downstream effector enzymes e.g. adenylyl cyclase and phosphodiesterases. Melatonin may also interact with a nuclear family of orphan receptors called retinoid-Z receptors. This implies that once melatonin is in the nucleus, it may regulate gene expression and also function as a free radical scavenger (Brezezinski, 1997).

1.2.5.8. Melatonin and free radicals

1.2.5.8.1. Free radical production

Oxygen comprises 21% of the earth's atmosphere, and is essential for the existence of aerobic organisms. A major paradox is that oxygen is lethal in certain states to organisms

that depend on it for life. Oxygen is utilized by the aerobic organisms to generate energy in the form of ATP (Reiter, 1995). However, about 5% of the inspired oxygen is converted to free radicals, many which are very toxic (Reiter, 1996).

Oxidative stress is the phrase given to describe damage done by free radicals. The degree of oxidative stress a cell endures dictates whether the cell remains healthy or becomes diseased. A variety of conditions increase oxidative stress, including ingestion of toxins, excessive exercise, ionizing radiation, and infection. Under conditions of severe oxidative damage, many cells undergo necrosis or apoptosis (Reiter *et al.*, 1995).

Radicals are formed from molecules that posses an unpaired electron in their orbitals. Oxygen qualifies as a radical as it possesses two unpaired electrons in different orbitals, maintaining a parallel spin. Oxygen is able to accept electrons, due to its unpaired electrons and unfilled orbitals. It thus makes oxygen a powerful oxidizing agent. Oxygen accepts electrons and maintains them in antiparallel spin, in reference to its two unpaired electrons in the given orbitals. Molecular oxygen (O_2) accepts electrons and maintains them in a spin conversion. A result of this is that O_2 is monovalently reduced to $O_2^{\cdot -}$. In the presence of SOD, $O_2^{\cdot -}$ can be converted to the reactive intermediate H_2O_2. H_2O_2 is detoxified by catalase (CAT) and GSH peroxidase. In the presence of transition metals e.g. copper or iron, H_2O_2 may undergo a “Fenton” reaction to yield the extremely toxic hydroxyl radical ($OH^{\cdot}$) (Coyle and Puttfarcken, 1993; Suzuki *et al.*, 1997). This is illustrated in Figure 11.

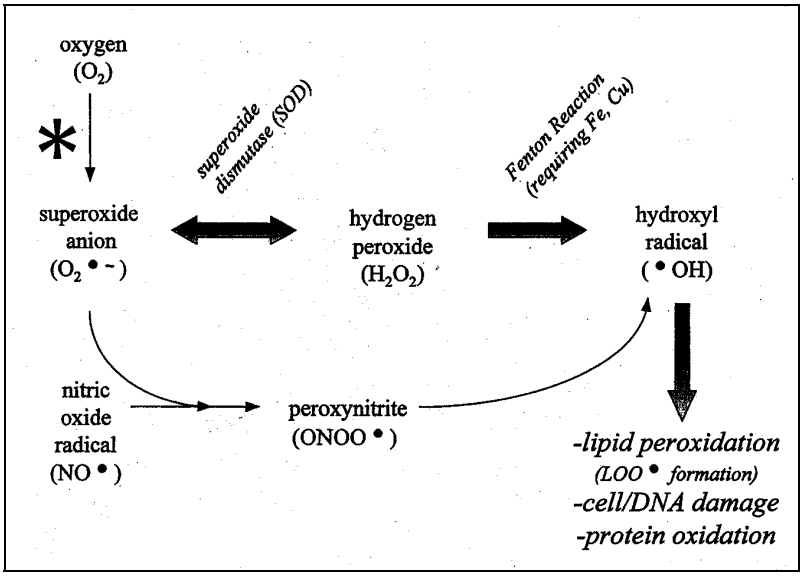

Figure 11. Generation of oxygen free radicals (Beyer, 1998)

H_2O_2 is a relatively stable oxidant. H_2O_2 can cross membranes readily, but it is toxic at relatively high concentrations (probably unphysiological). $OH^{\cdot}$ is an extremely powerful oxidant that reacts at near diffusion-limited rates with most organic metabolites. The $OH^{\cdot}$ radical formation in vivo is "site specific", depending on the location of transition metals (especially iron and copper). $OH^{\cdot}$ radical reacts at great speed with almost every molecule found in living cells, e.g. it causes strand breakage and chemical alterations of deoxyribose and of purine and pyrimidine bases of DNA, membrane lipids, and carbohydrates (Halliwell and Gutteridge, 1986,1992; Halliwell, 1992; Halliwell *et al.*, 1992).

The nature of the damage done to the cells by the formation of H_2O_2 and $O_2^{\cdot -}$ will be affected by the location of metal ion catalysts within the cells. Thus, if no catalytic metal ions are available, the H_2O_2 and $O_2^{\cdot -}$ will have limited, if any damaging effects (Halliwell, 1992).

When H_2O_2 in the presence of copper or iron react with lipid membranes (equation 1.6), the process of lipid peroxidation takes place, as a hydrogen atom is abstracted from a

polyunsaturated fatty acid chain in the lipid membrane. This results in the formation of a carbon-centered radical in the membrane (equation 1.7).

$$H_2O_2 + Cu^{1+} \rightarrow Cu^{2+} + OH^{\cdot} + OH^{-} \quad \ldots \text{ Equation 1.6}$$

$$\text{Lipid-H} + \text{radical} \rightarrow \text{lipid*} + \text{radical-H} \quad \ldots \text{ Equation 1.7}$$

$$\text{Lipid*} + O_2 \rightarrow \text{lipid-}O_2\text{*} \quad \ldots \text{ Equation 1.8}$$

$$\text{lipid-}O_2\text{*} + \text{lipid-H} \rightarrow \text{lipid-}O_2\text{H} + \text{lipid*} \quad \ldots \text{ Equation 1.9}$$

Carbon-centered radicals in vivo can react with O_2 to form peroxyl radicals (equation 1.8). Peroxyl radicals can attack membrane proteins, resulting in damaged receptors and enzymes. The abstraction of a single hydrogen (H) can set off a cascade of free radical chain reactions. This leads to the conversion of many membrane lipids into lipid hydroperoxides (lipid-O_2H) (equation 1.9). Lipid peroxides within membranes severely disrupts its functioning, as membrane fluidity is altered and this allows Ca^{2+} to leak across the membrane. Copper and iron can contribute to lipid peroxidation in two ways: Firstly, they catalyse the formation of initiating H-abstracting species. Secondly, they stimulate peroxidation by reacting with lipid hydroperoxides and decomposing them to peroxyl radicals and alkoxyl radicals (lipid-O_2*), which can then abstract H and lead to further peroxidation (Halliwell and Gutteridge, 1989, 1990).

Ascorbic acid in the presence of copper or iron accelerates the oxidative damage towards DNA, proteins and lipids. Ascorbic acid demonstrates prooxidant properties, as mixtures with copper and iron salts, are well known to stimulate lipid peroxidation and the formation of $OH^{\cdot}$ from H_2O_2 (Walling, 1982). All the reported prooxidant properties of ascorbate probably involve its interaction with transition metal ions (Halliwell, 1990). Sequestration of transition metal ions is an effective antioxidant defense mechanism and thus, ascorbate's antioxidant properties in the human body will normally predominate. In disease states, metal ions become more "available", and in these cases ascorbate's antioxidant properties may not predominate (Bendich *et al.*, 1986).

The availability, in vivo, of free "catalytic" copper may be more restricted than iron, as copper is a more powerful promoter of free radical damage to lipids (Evans *et al.*, 1989).

1.2.5.9. Melatonin as a free radical scavenger

1.2.5.9.1. In vitro evidence

Ianas *et al.* (1991) was the first to report melatonin's antioxidant actions. However, these authors also reported that melatonin had prooxidant actions. Melatonin exhibited antioxidant actions when its concentration exceeded 0.25 mM, and at concentrations below that, melatonin was reported to be prooxidative.

Tan *et al.* (1993) reported the free radical scavenging actions of melatonin using a different system to Ianas. Hydroxyl radicals ($OH^{\cdot}$) were generated, by exposing H_2O_2 to ultraviolet light (UV). Due to $OH^{\cdot}$ very short half-life (1×10^{-9} sec at 37 °C), a spin trapping agent, 5,5-dimethylpyrroline N-oxide (DMPO), was utilized. The resultant $OH^{\cdot}$-DMPO adducts, which have a longer half-life and can be quantified as an index of $OH^{\cdot}$ generation, were identified by HPLC with electrochemical detection and electron spin resonance spectroscopy. Mannitol and GSH (both known scavengers) were compared against melatonin. The results indicated that melatonin was a more effective free radical scavenger, and no prooxidant actions of melatonin were uncovered.

Tan *et al.* further showed that melatonin was a more effective $OH^{\cdot}$ scavenger, when compared to serotonin, N-acetylserotonin or 5-methoxytryptamine (all chemically related to melatonin). These structure-activity studies showed that the 5-methoxy group on melatonin's indole nucleus and the N-acetyl group on the side chain, are both necessary for the efficient scavenging activity of melatonin.

Poeggeler *et al.* (1994) showed that in a system with melatonin, $FeSO_4$ and H_2O_2, melatonin was quickly oxidized in the presence of $OH^{\cdot}$ (generated by $FeSO_4$ and H_2O_2). This was based on the fact that melatonin rapidly lost its fluorescence.

Melatonin's free radical scavenging actions were attributed to donation of an electron to the free radical, and in the process becoming a free radical. Melatonin donates an electron and becomes oxidized to produce an indolyl cation radical. Melatonin's free radical scavenging action occurs in a two step process (Figure 12): In the first step, the melatonin donates an electron to the $OH^{\cdot}$ radical, to from the indolyl cation radical. In the second step, the indolyl cation radical is quickly oxidized by the $O_2^{\cdot -}$ to form 5-methoxy-N-acetyl-N-formyl-kynuramine. Thus melatonin is irreversibly oxidized and cannot be regenerated. Melatonin acts definitively to terminate free radical reaction chains and does not participate in redox cycling (Hardeland *et al.*, 1993).

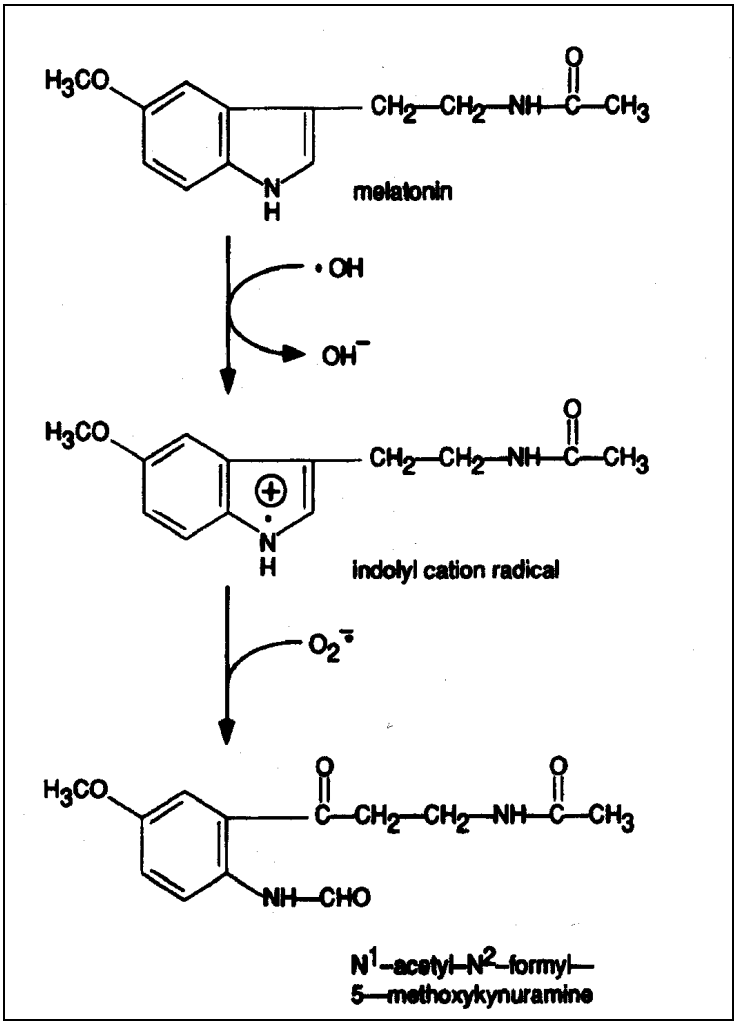

Figure 12: The presumed mechanism by which melatonin acts as a free radical scavenger (Hardeland *et al.*, 1993)

1.2.5.9.2. In vivo evidence

Reiter *et al.* (1997) following the reports of Tan *et al.* examined the antioxidant properties of melatonin in vivo. Rats were treated with the carcinogen, Safrole. Safrole in vivo results in DNA damage, as a consequence of free radical generation. The Safrole treated rats, exhibited extensive hepatic DNA adduct production 24 hours later, after Safrole administration. When melatonin was co-administered, it reduced the DNA adduct formation. This result confirmed melatonin's antioxidant actions.

Tan *et al*. (1994) undertook experiments to determine whether melatonin's circadian rhythm afforded protection against Safrole. Rats were administered Safrole during the day (when melatonin levels were low) and at night (when melatonin levels were high). The results showed that when Safrole was administered at night, there was less DNA damage than when rats were administered Safrole during the day. This was also confirmed by administering Safrole to pinealectomized rats. Pinealectomized rats have a greatly reduced melatonin output. When melatonin was supplemented to pinealectomized rats treated with Safrole, the DNA adduct formation was greatly reduced. Tan *et al.* concluded that melatonin, even at amounts normally produced in the organism, was sufficient to provide significant antioxidative protection.

1.2.5.9.3. Additional effects of melatonin on the antioxidant defense system

Melatonin stimulates the activity in the brain of glutathione peroxidase (GSHPx). GSHPx metabolizes H_2O_2 to H_2O, thereby reducing the generation of $OH^{\cdot}$. Melatonin thus promotes the metabolism of H_2O_2 and hydroperoxidases, thereby reducing the generation of toxic-free radicals (Reiter, 1997).

Pierrefiche and Laborit (1995) reported that melatonin also stimulates hepatic and cerebral glucose-6-phosphate dehydrogenase activity in mice, and thus NADPH levels increase. This promotes the enzymatic production of GSH via glutathione reductase. Glutathione is a necessary co-factor for GSHPx. Thus melatonin also functions indirectly as an antioxidant.

1.2.6. Melatonin and Alzheimer's disease

As discussed in section 1.4.7.1., copper may promote the precipitation of A$ protein in Alzheimer's disease, resulting in senile plaques. High concentrations of $ amyloid are toxic and damage biological membranes. Daniels *et al.* (1998) reported that $ amyloid and aluminium, both caused lipid peroxidation. These authors showed that prior administration with melatonin, decreased lipid peroxidation induced by $ amyloid and aluminium, in a dose-dependent manner. These authors suggested that melatonin supplementation to Alzheimer's patients may be beneficial in reducing lipid peroxidation induced by $ amyloid and aluminium.

Limson *et al.* (1998) reported that melatonin binds/complexes with heavy metals: copper, aluminium, zinc, cadmium, iron and lead. These experiments were performed in vitro utilizing adsorptive cathodic stripping voltammetry. The results indicate that melatonin shows binding affinity for copper and aluminium. This may indicate that melatonin, besides acting as an antioxidant, may bind these metals and prevent them from partaking in free radical production. It is also important to note that any free metal ions in the brain are toxic, and may participate in free radical production, especially copper and iron. Thus, melatonin may function as a metal ion binder, and thus remove "free" metal ions from participating in the generation of free radicals.

1.2.7. A novel pineal night-specific ATPase

Borjigin *et al.* (1999) identified a novel pineal night-specific ATPase (PINA) encoded by the Wilsons disease gene. The pineal gland is a functional component of the circadian timing system that measures and translates the duration of environmental light into rhythmic neuronal signals and in some species it has been designated as a "third eye". The SCN is a central component of this system that is entrained by light input from the retina and by melatonin secreted from the pineal gland. The diurnal rhythm of melatonin formation is driven by a complexed network of neurons, controlled by the SCN that is

modulated by light impacting on the retina. The rate-limiting enzyme in melatonin synthesis is NAT. The investigators have reported a novel PINA, that share identical temporal expression patterns and tissue distribution with NAT. PINA is generated from alternative splicing of the Wilson disease gene, and can function as a copper transporter (ATP7B). In LEC rats where the PINA is deleted, melatonin synthesis was investigated. LEC pineals display a defect in NAT protein and activity. It was demonstrated that the PINA mutation was independent to the NAT defect. The NAT mutation was caused by a germ-line mutation in the NAT gene. As yet, it is not known if PINA affects the regulation of melatonin synthesis, and is currently being investigated.

1.3. Research objectives

The first objective of this study was to investigate melatonin's ability to protect cells against copper-induced toxicity. Melatonin is a known neuroprotectant, and this part of the study is based on the assumption that melatonin might be able to protect against copper-induced cellular damage. In Wilsons disease, copper causes cellular damage in the brain and liver. Wilsons disease is characterised by chronic copper toxicity that results in hepatolenticular disease. In this study, copper toxicity was induced in an animal model. A determination of lipid peroxidation of the liver and brain is a reliable indicator of free radical-induced damage. Melatonin could be an ideal candidate to prevent copper-induced lipid peroxidation, as melatonin is a potent free radical scavenger. The mechanism by which melatonin would prevent free radical damage would be investigated.

The second objective was to determine the chemical interaction between melatonin and copper. Melatonin has been shown to bind/chelate to Cu^{2+} *in vitro* by electrochemistry. Thus, the interaction between melatonin and Cu^{1+} needs to be investigated using electrochemistry, nuclear magnetic resonance and infrared techniques.

1.3.1. Proposed experiments

1.3.1.1. Evaluation of copper loading on cellular processes

Copper's ability to induce cellular damage and melatonin's ability to prevent cellular damage, would be established by:

- Lipid peroxidation assay (*in vivo* and *in vitro)*
- To determine which oxidation state of copper generates free radicals (*in vitro*)
- To determine through electron microscopy and light microscopy techniques, cellular damage caused by copper (*in vivo*)
- Electron transport chain (*in vitro*)
- Pineal organ culture to determine the effect of copper on pineal indolamine synthesis (*in vivo*)

1.3.1.2. *In vitro* evaluation of the chemical interactions between melatonin and copper

- To determine which oxidative state of copper interacts with melatonin through nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and electrochemistry techniques

CHAPTER 2

Lipid Peroxidation
An *in vitro* and *in vivo* examination of copper-induced lipid peroxidation in rat organs

2.1. Introduction

Biological membranes are vital for the existence of cellular integrity. These serve as barriers, protecting the cells from harmful compounds in the surrounding environment, and function as compartmentalising structures, which are essential for cellular function.

Singer and Nicholson (1972) described biological membranes with the 'Fluid Mosaic Model'. These membranes are dynamic, irregular lipid mixtures of phospholipids and cholesterol, with globular proteins embedded within it (Voet and Voet, 1990). The globular proteins embedded in the membranes are receptors, channels, and pores. These form part of the cellular machinery that are necessary for the recognition of molecules and to transport these across the membrane and into the cell. Lipid peroxidation lowers the fluidity of the mitochondrial membrane with a consequent alteration of the ion flux around it. It was hypothesized that lipid peroxidation may bring about the formation of peroxide clusters that may act as channels across the membrane making it permeable to ions (Bindoli, 1988).

Membrane function can be affected by physical or chemical means, which alter the membrane structure, causing changes in membrane fluidity. This results in increased permeability of Ca^{2+} ions into the cell, which leads to cell destruction and ultimately cell death. Membrane lipids are very rich in polyunsaturated fatty acids (PUFA) side chains, which are especially sensitive to free radical attack. Free radicals are able to cause alterations in membrane integrity, resulting in lipid peroxidation. Lipid peroxidation is an irreversible reaction, and may be considered a result of some pathological process.

Products of peroxidation are also toxic and could cause further pathological changes (Barber and Bernheim, 1967).

Peroxidation is initiated by the attack of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain (Figure 13). The hydrogen atom is a free radical and its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbonyl centred radical can react with O_2 to form a peroxyl radical. Peroxyl radicals can combine with each other or they can attack membrane proteins.

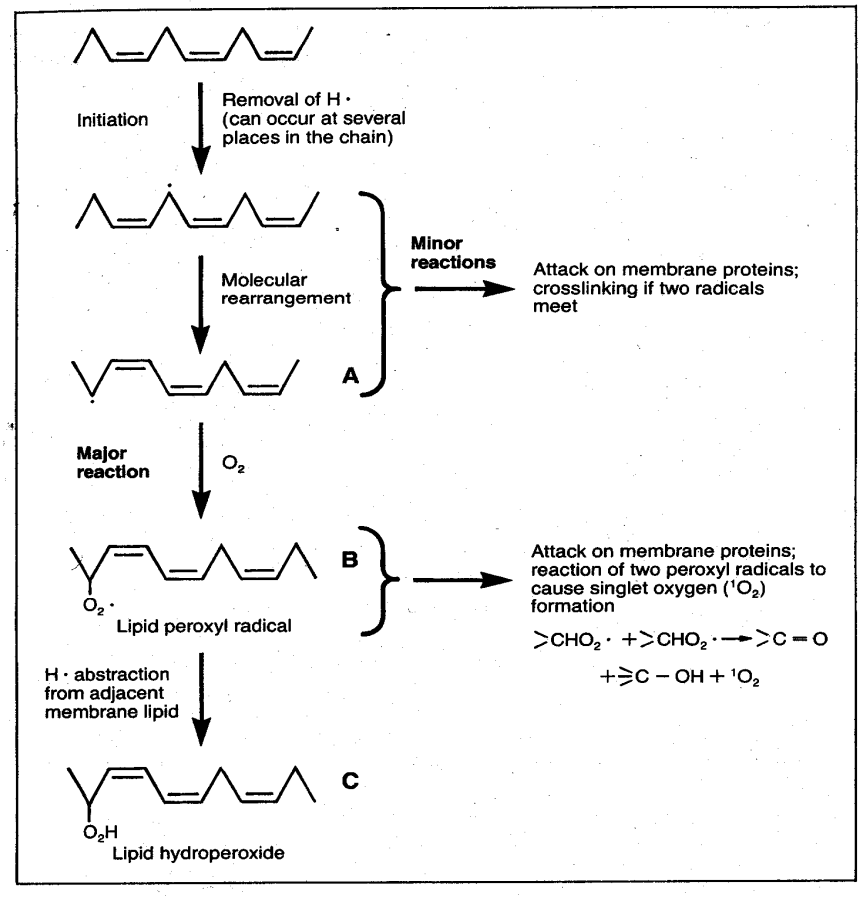

Figure 13. Outline of lipid peroxidation mechanism. A hydrogen atom is abstracted from a fatty acid with three double bonds. A, B and C are conjugated diene double bond structures (Gutteridge and Halliwell, 1990).

It is able to abstract hydrogen from adjacent fatty acid side chains in the membrane, and thus the chain reaction of lipid peroxidation is propagated. Thus, a single initiating event can result in the conversion of fatty acid side chains into lipid hydroperoxides (Gutteridge and Halliwell, 1990).

Lipid peroxidation in biological membranes cause impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and an increased non-specific permeability to ions like Ca^{2+}. Free, ligand-bound and vesicular copper is present throughout the brain. Copper is a rapidly acting neurotoxin at physiological concentrations (10-100μM) (Horning *et al.*, 2000). Upon exposure to iron or copper ions, lipid hydroperoxides decompose, resulting in hydrocarbon gases e.g. ethane and pentane, radicals that can abstract further hydrogen atoms from fatty acid side chains, and cytotoxic carbonyl compounds (Gutteridge and Halliwell, 1990).

Lipid peroxidation is often a late event, accompanying rather than causing final cell death (Halliwell and Gutteridge, 1984). Thus, "a failure to protect tissue damage by chain-breaking antioxidant inhibitors of lipid peroxidation does not rule out free radical damage as an injury mechanism" (Halliwell *et al.*, 1992).

Transition metals promote lipid peroxidation in two ways: (1) by catalyzing the formation of oxygen free radical species capable of initiating lipid peroxidation and (2) by catalyzing the decomposition of preformed lipid peroxides to propagate lipid peroxidation (Rikans and Hornbrook, 1997).

Following peroxidation of ω-6 and ω-3, PUFA's relatively unstable fatty acid hydroperoxides are converted by rearrangement, consecutive scission and oxidation reactions into more stable carbonyls, which include: malondialdehyde (MDA), *n*-alkenals, unsaturated ketones, alkanes and alkenes (De Zwart *et al.*, 1999).

The most widely used index of lipid peroxidation is MDA formation, which is assayed with the thiobarbituric acid (TBA) assay. The lipid material is heated with TBA at low

pH (with trichloroacetic acid [TCA]). One molecule of MDA reacts with two molecules of TBA, resulting in the formation of a MDA-TBA complex. This complex is a pink chromogen, which is measured at 532 nm. The acid hydrolysis with TCA, results in the formation of the complex and the release of the protein-bound MDA (Gutteridge and Halliwell, 1990).

The liver is the major organ of copper deposition, but other organs such as the kidney, brain and heart also accumulate copper (Lal and Sourkes, 1971). These authors studied the effect of copper loading in various tissues in rats by injecting copper *i.p.*, with monitoring over 18 weeks. The authors reported that all tissues except hair and nails accumulated copper with liver and kidney accumulating the highest levels. The percentage of injected copper retained in the liver or kidney varied with the dose and duration of loading.

Melatonin, a chain-breaking antioxidant, functions as a free radical scavenger and repairs damaged macromolecules [restores membrane lipids, but not membrane proteins] (Poeggeler *et al.*, 1994). Melatonin is a potent hydroxyl radical scavenger. Hydroxyl radicals are considered the most toxic and damaging radical of all the free radicals produced (Reiter *et al.*, 1993).

There is conflicting data regarding melatonin's ability to act as a peroxyl radical scavenger. Some authors have reported that melatonin is a potent peroxyl radical scavenger, indicating that it is a chainbreaking antioxidant (Pieri *et al.*, 1994, 1995; Marshall *et al.*, 1996). Other authors (Antunes *et al.*, 1999) have reported that melatonin is not a peroxyl radical-trapping, chainbreaking antioxidant, but it is a preventative antioxidant of the metal ion deactivating subclass. These authors suggest that melatonin is unlikely to be very effective in metal ion deactivation as it does not have the multidentate character possessed by all strong transition metal chelators (e.g. α-tocopherol). These authors also propose that *in vivo*, due to melatonin's weak antioxidant properties and its relatively low concentration, it is unable to inhibit lipid peroxidation (Antunes *et al.*, 1999).

2.2. Materials and methods

2.2.1.1. Animals

The Rhodes University Ethical Standards Committee approved all the experiments involving the use of animals. The animals used were male Wistar rats weighing 250-300g. The animals were housed in opaque plastic cages with metal grid floors and covers, under a diurnal lighting cycle 12 hours light: 12 hours dark, with food and water *ad libitum*. The intensity of the light during the 12-hour light phase was approximately 300 :Watts/cm^2. The temperature of the animal room was maintained between 20°C and 25°C, and the cages were cleaned daily. The food given was a commercial brand of dog food, which contained 5-8 mg of copper/kg of dog food. The approximate copper and zinc concentrations in the tap water, was 1 ppm and 82 ppm, respectively.

2.2.1.2. Sacrificing and dissection of the animals

The rats were killed swiftly by cervical dislocation and rapidly decapitated. The top of the skull was removed by making an incision through the bone on either side of the head. The skull was lifted with the use of a pair of forceps, exposing the brain and pineal gland. The brain and pineal gland were removed for either immediate use or stored at –70°C until needed.

A mid-vertical incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver and kidneys, which were removed carefully. The livers and kidneys were either used immediately or stored at –70°C until needed.

2.2.2. Chemicals and reagents

1.1.3.3-Tetramethoxypropane (MDA) was purchased from Fluka AG, Switzerland. Butylated hydroxytoluene (BHT), melatonin, bovine serum albumin (BSA) and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. TCA, Folin & Ciocalteu's reagent and copper chloride ($CuCl_2$) were purchased from Saarchem (Pty) Ltd, Krugersdorp, South Africa. All other reagents were obtained from local sources and were of the highest purity available.

2.2.3. Methods

2.2.3.1. Preparation of tissue homogenate

The livers, brains and kidneys were removed and either used immediately or stored at -70°C until used. Prior to homogenization, tissues that were stored at -70°C were thawed and weighed at room temperature. The tissue samples were homogenized in a Teflon® coated glass homogenizer for 60 seconds on ice. A 10% w/v homogenate was prepared with 0.1M PBS, pH 7.4.

2.2.3.2. Lipid peroxidation assay

The lipid peroxiadtion assay was determined according to the modified method of Anoopkumar-Dukie et al. (2000). Aliquots of 1ml of the homogenate were incubated at 37°C for 60 minutes. 0.5 ml BHT (0.5 mg/ml in methanol) and 1 ml TCA (15% w/v in aqua) was added to the mixture. The tubes were sealed and heated for 10 minutes in a boiling water bath to release protein-bound MDA. The samples were then cooled to avoid adsorption of MDA onto insoluble proteins and then centrifuged at 2000*g* for 15 minutes. Following centrifugation, 1 ml of this protein free supernatant was removed from each tube and a 1 ml aliquot of TBA (0.33% w/v in aqua) was added to this fraction. The tubes were sealed and incubated in a boiling water bath for 60 minutes.

After incubation, the mixture was cooled for 10 minutes on ice. The TBA-MDA complex was separated from other possible interfering thiobarbituric acid-reactive substances (TBARS) using an Isolute™ C_{18} solid phase extraction (SPE) column. The SPE column was pre-washed with 2 ml of methanol followed by 2 ml of distilled water. 1 ml of the sample was loaded onto the column, which was subsequently washed with 1 ml of distilled water. The TBA-MDA complex was eluted with 1 ml methanol. The absorbance was measured at 532nm with an UV-visible spectrophotometer. Methanol was used as a blank. A MDA standard curve was generated using 1.1.3.3-tetramethoxypropane in the same way as described above. Standards were dissolved in 1 ml of distilled water, and a series (0-20 nmoles/ml) were prepared (Appendix 2). Final results were expressed as nmoles/mg protein.

2.2.3.3. Protein assay

Protein estimation in the liver, brain and kidney homogenates were determined using the method described by Lowry *et al.* (1951). Briefly, to 1 ml homogenate, 5 ml of alkaline copper reagent (1 ml 1% copper sulphate, 1 ml 2% sodium tartrate, and 98 ml solution of 4% sodium carbonate and 0.1M sodium hydroxide) was added in a set of clean test tubes. The tubes were mixed and allowed to stand for 5 minutes at room temperature. Following this, 0.3 ml Folin-Ciocalteu's reagent was added to the tubes. The tubes were mixed and allowed to stand in the dark for 20 minutes at room temperature. The absorbance was then read at 500nm using an UV-visible spectrophotometer. A standard curve (0-300 g/ml) was also generated in the same manner, using 1 ml BSA instead of homogenate (Appendix 1).

2.2.3.4. Statistical analysis

Statistical tests were performed using the Student-Newman-Keuls test. Groups compared were accepted to be statistically significant at $p<0.05$. Statistical analysis was performed on the AA and lipid peroxidation assay samples.

2.3. Experiment 1: *In vivo* copper 2mg/kg administration for 2-weeks and 6-weeks

2.3.1. Materials and methods

Male Wistar rats weighing 250-300g were used in the experiments. The rats were randomly assembled into five groups of 5 rats per cage, and were maintained as described in section 2.2.1.1. . The control group (n=10), consisted of 2 groups of 5 rats that received the drug vehicle, ethanol:0.9% saline (40:60), for 2-weeks and 6-weeks, respectively. The two copper treated group (n=5) received 2mg/kg Cu^{2+} (copper chloride-$CuCl_2$) for 2 weeks and 6 weeks, respectively. The copper/melatonin treated groups received Cu^{2+} 2mg/kg and melatonin 12mg/kg for 2 weeks and 6 weeks, respectively. The rats were injected daily, with the vehicle, Cu^{2+} and Cu^{2+}/melatonin. Copper/melatonin groups were injected on opposite intraperitoneal sites, to prevent any interaction of the copper with the melatonin. The rats were sacrificed and the brains, livers and kidneys were removed as discussed in section 2.2.1.2.

Male Wistar rats were used as described in Section 2.2.1.1. Lipid peroxidation assay was performed as outlined in Section 2.2.3.2.

2.3.2. Results

The 2-week and 6-week liver, brain and kidney samples were analysed for lipid peroxidation, and the results are depicted in Figures 14-19. In the 2-week brain samples (fig. 14), there was no statistical significance found between the control, copper and copper/melatonin samples. The level of lipid peroxidation was minimal in the brain. The 2-week liver samples (Figure 15) showed a no statistical difference between the control samples, and the copper and copper/melatonin samples. There was no statistical difference found among the three groups in the kidney, although the level of lipid peroxidation was high (Figure 16).

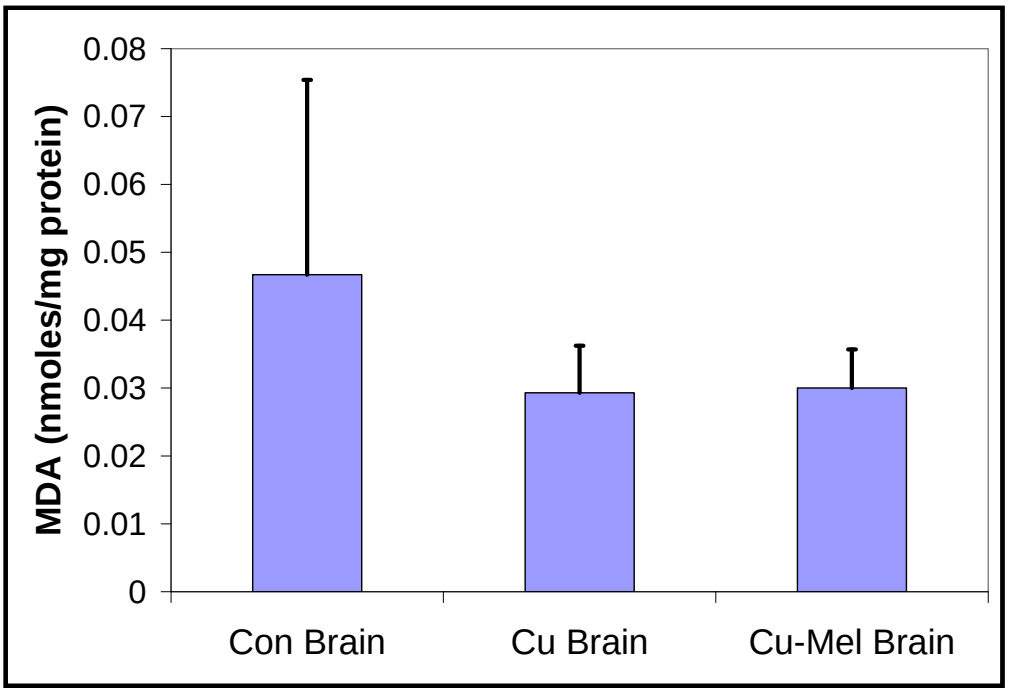

Figure 14. Lipid peroxidation of 2-week brain in vivo samples (n=5, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel).

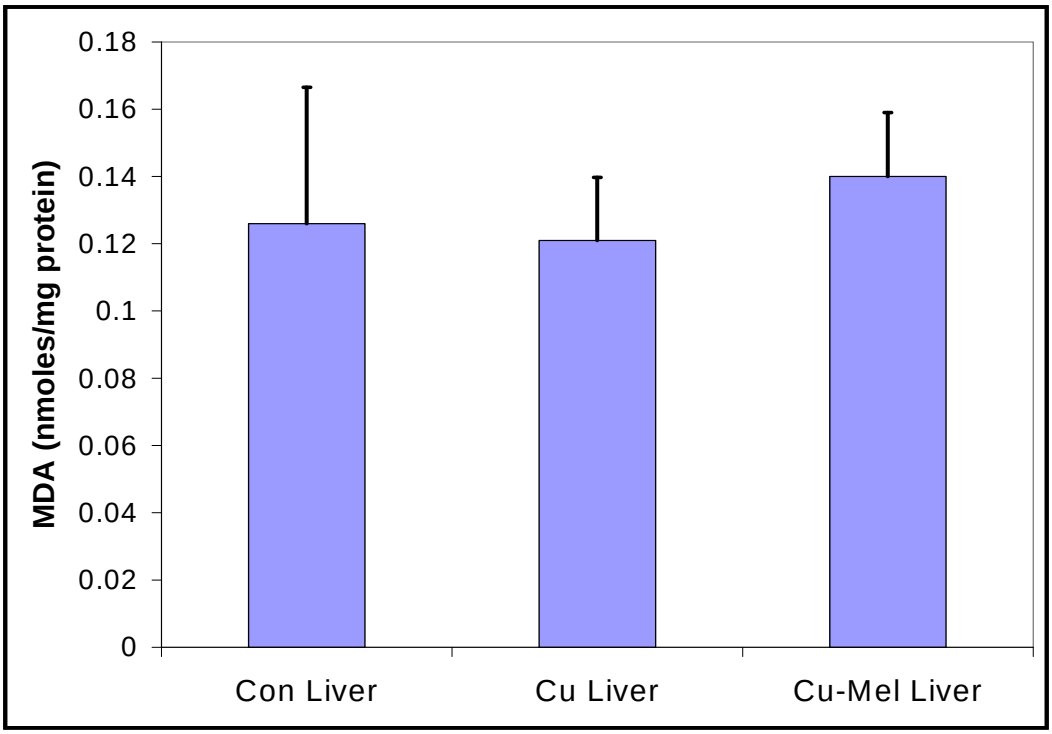

Figure 15. Lipid peroxidation of 2-week liver sample (n=5, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel)

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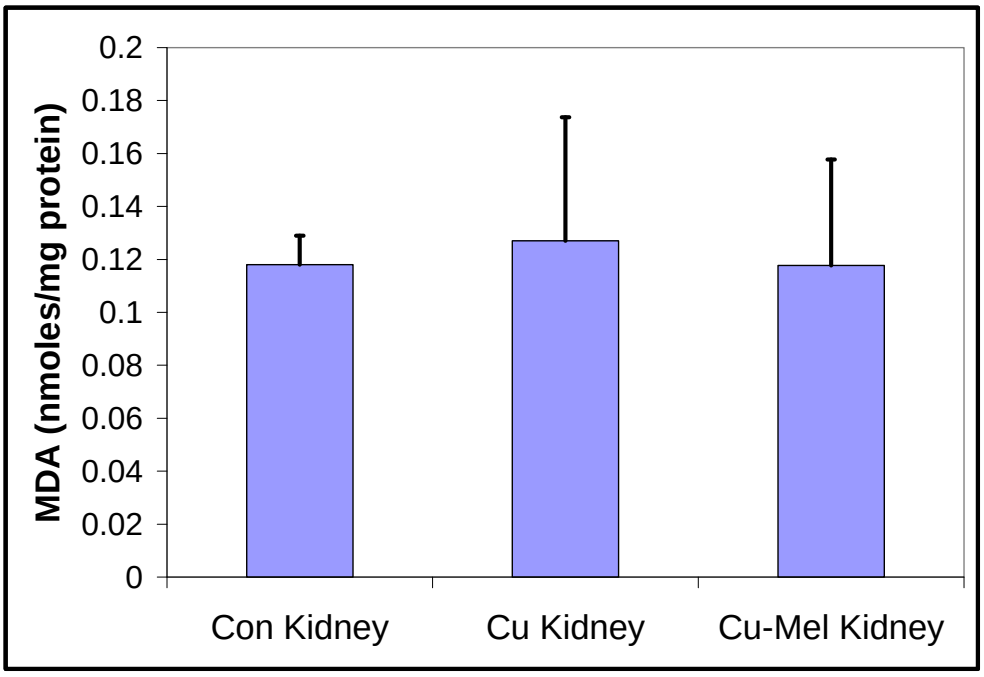


Figure 16. Lipid peroxidation of 2-week kidney sample (n=5, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel).

There was no statistical significance in the three 6-week brain groups (Figure 17). Within the 6-week liver samples (Figure 18) there was an increase in lipid peroxidation, with the copper/melatonin group showing the highest level of lipid peroxidation as compared to the control and copper groups (also the degree of lipid peroxidation is higher that the 2-week groups). There was a statistical difference between the control liver group, the copper and copper/melatonin liver groups at $p<0.001$. The copper/melatonin group had a higher degree of lipid peroxidation than the copper group, indicating that melatonin increases the incidence of lipid peroxidation, when in the presence of copper or that the melatonin is degraded before its antioxidant ability is utilized. The kidney samples had a higher level of lipid peroxidation (Figure 19) compared to the 2-week samples. The control, copper and copper/melatonin kidney samples showed no statistical difference among them.

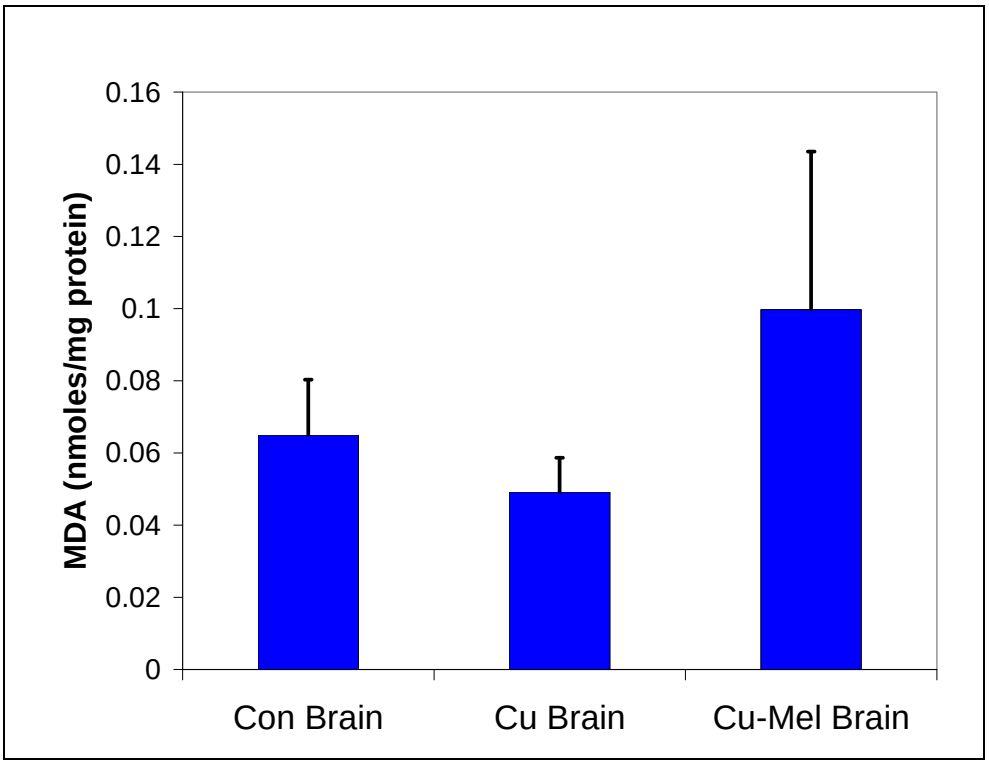


Figure 17. Lipid peroxidation of 6-week brain sample (n=3, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel).

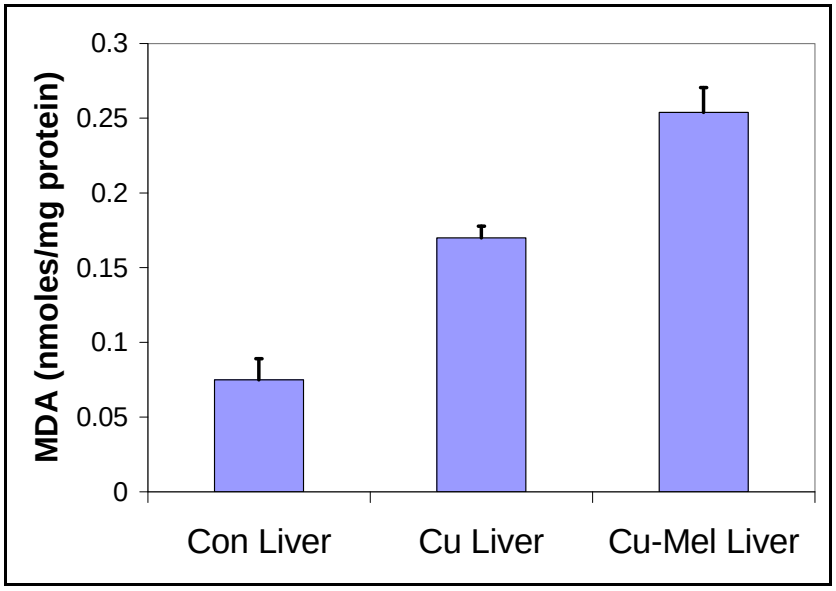


Figure 18. Lipid peroxidation of 6-week liver sample (n=3, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel). The Con liver vs Cu liver $p<0.001$; Con liver vs copper/melatonin $p<0.001$.

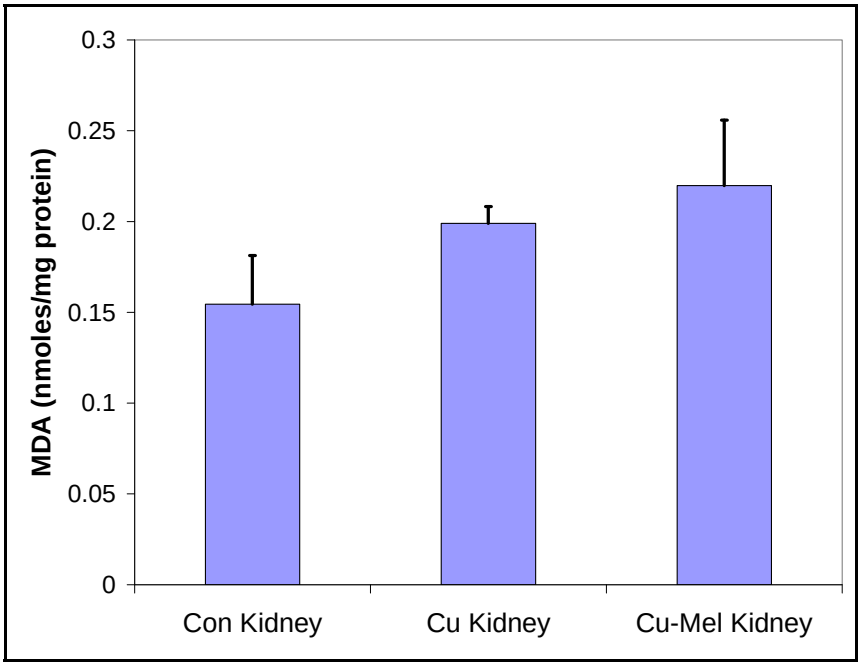


Figure 19. Lipid peroxidation of 6-week kidney sample (n=3, mean ± SEM). Control (Con), copper (Cu) and copper/melatonin (Cu-Mel).

2.4. Experiment 2: *In vitro* copper 1mM, 5mM and 10mM experiments

2.4.1. Materials and methods

Male Wistar rats weighing 250-300g were used in the experiments and sacrificed as mentioned in section 2.2.1.2. The brain and liver were removed and a 10% w/v homogenate was made with 0.1M phosphate buffered saline (PBS) pH 7.4. The experiment consisted of three groups, viz. a control group, a copper group and a copper/melatonin group. The control group consisted of 0.9 ml homogenate and 0.1 ml of drug vehicle (50:50 ethanol: water). Various stock solutions of copper chloride (2mM, 10mM and 20mM) were used. In the copper group, 0.1 ml of the copper stock solution was added, plus 0.1 ml distilled water to 0.8 ml homogenate to give a final copper concentration of 1mM, 5mM and 10mM, respectively. The copper/melatonin group consisted of 0.8 ml homogenate, plus 0.1 ml copper of the various copper stock solutions and 0.1 ml melatonin (10mM). This gave a final melatonin concentration of 5mM. Melatonin was dissolved in 50:50 ethanol:water.

Male Wistar rats were used as described in Section 2.2.1.1. Lipid peroxidation assay was performed as outlined in Section 2.2.3.2. All experiments were performed in duplicate.

2.4.2. Results

As shown in Figure 20, copper chloride 1mM produces an increase in lipid peroxidation in the liver, which is statistically significant at $p<0.001$, as compared to the control liver group. The copper and copper/melatonin liver groups are signifcantly different at $p<0.001$, indicating the melatonin reduces the degree of lipid peroxidation. In both the 5mM and 10mM liver copper experiments (Figure 21 and 22, respectively), there is no statistical difference between the copper and copper/melatonin groups, but there is a statistical difference between the control and copper 5mM ($p<0.01$) and the control and copper 10mM ($p<0.01$) groups.

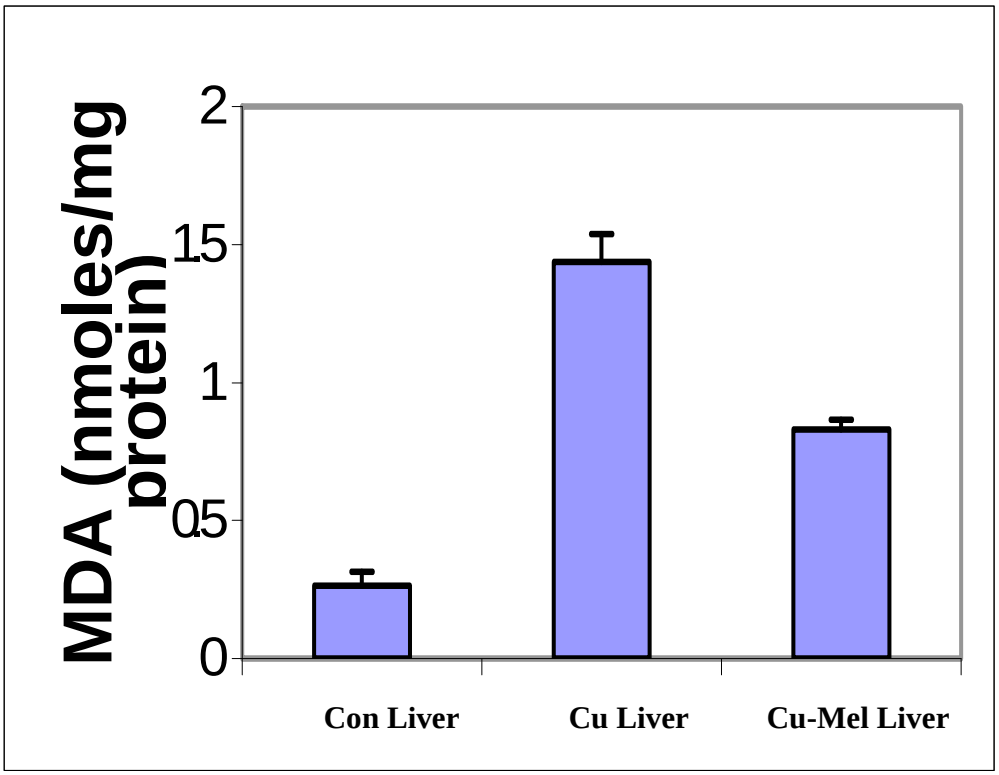


Figure 20. In vitro liver copper 1mM and melatonin 5mM experiments. The control (Con) vs copper (Cu) is statistically significant at $p<0.001$. The Cu 1mM group is significantly different compared to the Copper/melatonin (Cu-Mel) group at $p<0.001$.

The degree of lipid peroxidation in the liver is greatest in the copper 1mM experiment compared to the copper 5mM and 10mM liver experimental groups.

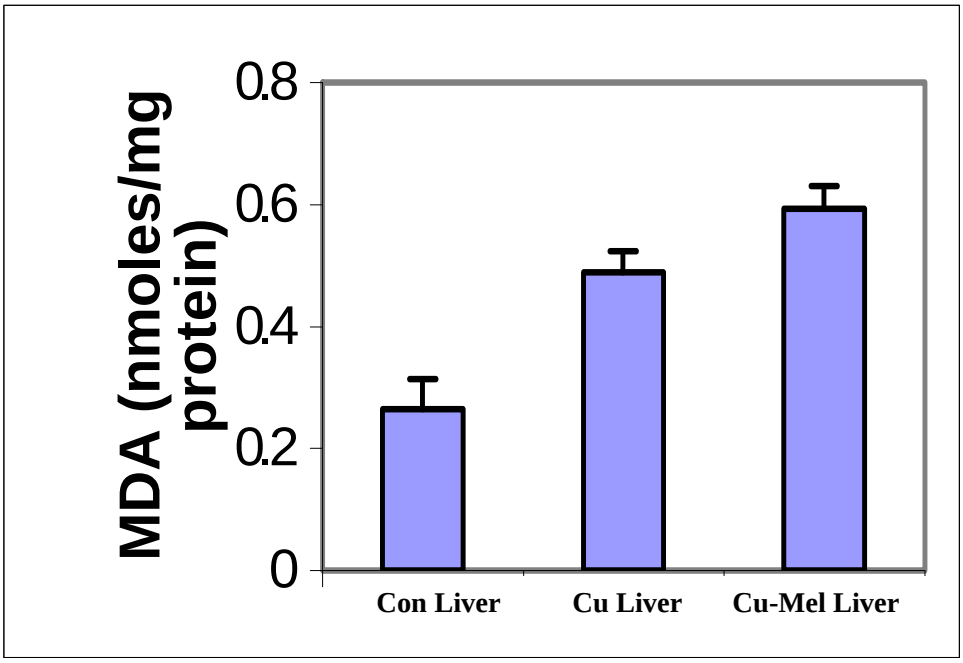


Figure 21. In vitro liver copper 5mM and melatonin 5mM experiments. The con group is statistically significant compared to the Cu 5mM group at $p<0.01$.

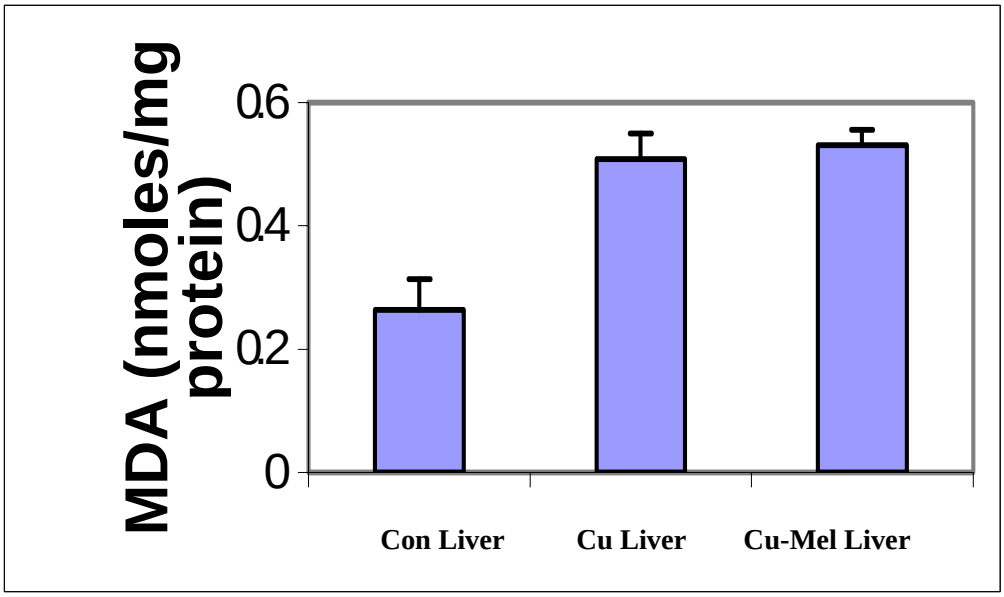


Figure 22. In vitro liver copper 10mM and melatonin 5mM experiments. The con group is statistically significant compared to the Cu 10mM group at $p<0.01$.

For the *in vitro* brain experiments, 1mM copper induced (Figure 23) the greatest degree of lipid peroxidation, as compared to the copper 5mM and 10mM brain experiments. The copper 1mM experiments showed a statistical significance between the control and copper groups ($p<0.05$), and the control and copper/melatonin groups ($p<0.01$). The copper and copper/melatonin groups had a greater level of lipid peroxidation as compared to the control group. The brain copper 5mM (Figure 24) shows the same trend as the copper 1mM experiment. There is no statistical difference between the control and the copper 5mM groups ($p>0.05$). The copper 10mM experiment (Figure 25) shows an increase in lipid peroxidation in the copper group. There is no statistical difference between the control group and the copper 10mM group ($p>0.05$).

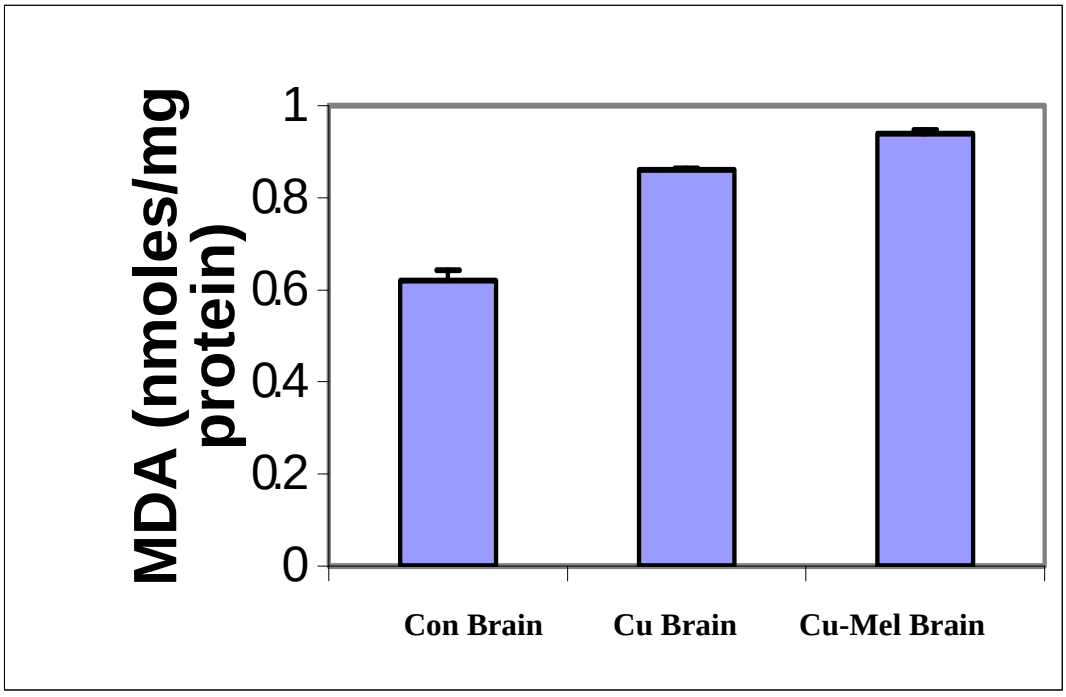


Figure 23. In vitro brain copper 1mM and melatonin 5mM experiments. The con group is statistically significant compared to the Cu 1mM group at $p<0.01$.

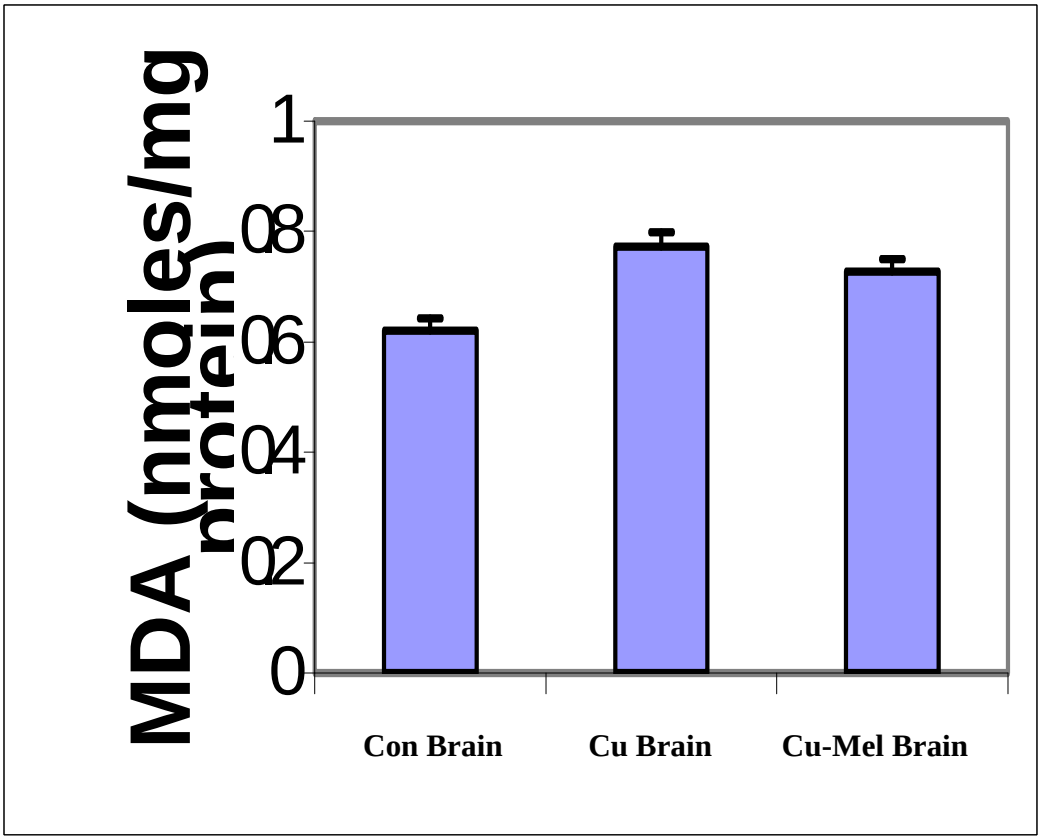


Figure 24. In vitro brain copper 5mM and melatonin 5mM experiments.

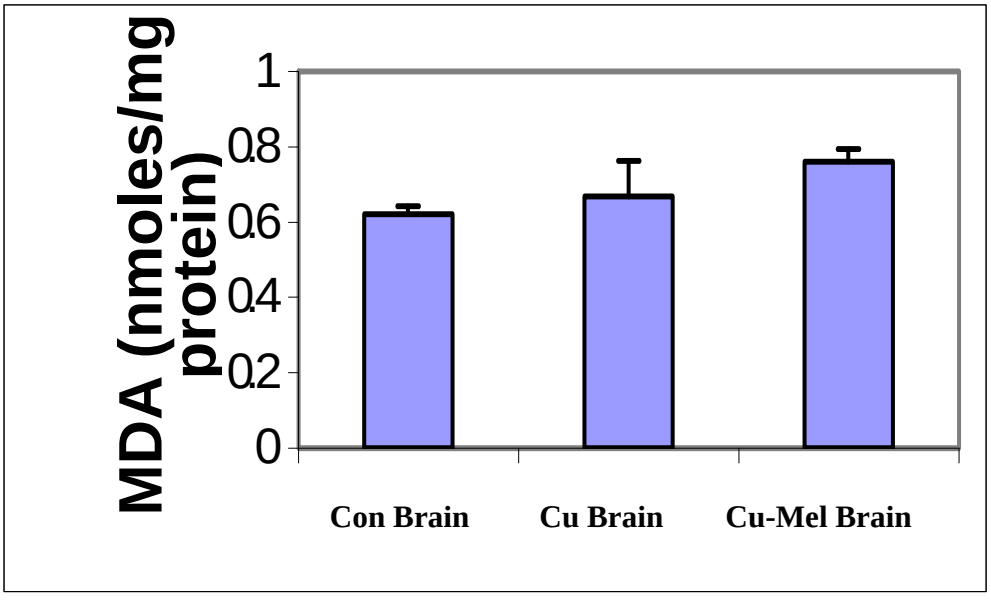


Figure 25. In vitro brain copper 10mM and melatonin 5mM experiments.

2.5. Experiment 3: Atomic absorption spectroscopy

2.5.1. Materials and methods

Total copper concentrations were measured in tissue samples of liver, kidney and brain. The tissues were weighed and placed in test tubes containing 1ml 65% nitric acid and 2ml of distilled water. The test tubes were placed on a boiling waterbath in a fume-hood. The tissue samples were allowed to digest, and then cooled. The solution was then filtered through a Osmonics® nylon, plain 0.22**:**m filter to remove any undigested matter. The samples were read on a GBC 909AA atomic absorption spectrophotometer. Total copper was measured at 324.7 nm and expressed as **:**g of copper/g of wet tissue. The 2-week samples (n=5) of each of the three experimental groups were read in triplicate and expressed as mean ± SEM. The 6-week samples (n=3) of each of the three experimental groups were read in triplicate and expressed as mean ± SEM.

2.5.2. Results

The copper concentrations in the livers, brains and kidneys of male Wistar rats, were measured. As shown in Table 3, there was no significant difference in the 2-week brain samples. However, in the 2-week liver samples, a significant difference was noted between the control, copper and copper/melatonin samples ($p < 0.001$). The total copper concentration in the 2-week brain and liver copper/melatonin groups were lower than in the copper administered samples of the brain and liver. The copper/melatonin kidney samples have a higher copper concentration than the copper treated samples. The three kidney 2-week groups showed no statistical significance among them.

Table 3. Total copper concentration in organs of Wistar rats given 2mg/kg copper for 2-weeks. Control liver vs copper liver, p< 0.001 and control liver vs copper/melatonin liver, p< 0.001, (mean ± SEM). Assays were performed in triplicate.

Organ	Control (n = 5) total copper (:g/g wet tissue)	Copper (n = 5) total copper (:g/g wet tissue)	Copper/melatonin (n = 5) total copper (:g/g wet tissue)
Brain (mean ± SEM)	2894.07 ± 594.32	3217.41 ± 251.33	2567.64 ± 80.64
Liver (mean ± SEM)	4702.65 ± 288.88 *	517688.06 ± 38844*	483259.41 ± 43855*
Kidney (mean ± SEM)	5382.33 ± 481.88	36408.95 ± 11449	37327 ± 13934

*p< 0.001

The total copper concentrations in the 6-week experiments were measured (Table 4). The highest copper concentrations were found in the copper and copper/melatonin treated tissue samples of the liver and kidney. The brain had a lower total copper concentration than the 6-week liver and kidney samples. The control liver copper concentration is statistically significantly different to the copper liver (p<0.001) and copper/melatonin liver samples (p< 0.01). The liver and kidney copper/melatonin treated samples had a lower total copper concentration than the livers and kidneys exposed to copper alone. All the tissue copper concentrations are greater than that of the 2-week samples.

Table 4. Total copper concentration in organs of Wistar rats given 2mg/kg copper for 6 weeks. Control liver vs copper liver, p< 0.001; control liver vs copper/melatonin liver, p< 0.01 and copper/melatonin liver vs copper liver, p< 0.001 (mean ± SEM). Assays were performed in triplicate.

Organ	Control (n = 3) total copper (:g/g wet tissue)	Copper (n = 3) total copper (:g/g wet tissue)	Copper/melatonin (n = 3) total copper (:g/g wet tissue)
Brain (mean ± SEM)	6948.21 ± 79.36	5115.78 ± 297.55	7766.94 ± 1902.80
Liver (mean ± SEM)	8745.40 ± 3232 *	688095.25 ± 89161*	320226.69 ± 139759 **
Kidney (mean ± SEM)	7056.84 ± 904.19	127259.07 ± 4044.4	88420.78 ± 38652

*p< 0.001, **p< 0.01

2.6. Discussion

2.6.1 Experiments 1 and 3: *In vivo* and atomic absorption spectroscopy experiments

Copper is active in oxidation-reduction reactions, where copper ions are able to catalyze the formation of hydroxyl radicals via the Haber-Weiss reaction. A common consequence of copper-induced production of reactive oxygen species is increased lipid peroxidation. Lipid peroxidation results in damage to cellular membranes, especially to mitochondrial membrane lipids (Bremner, 1998) (refer to section 3.3.2.).

In the *in vivo* experiments, copper 2mg/kg was injected daily for 2-weeks and 6-weeks *i.p.* The rats showed initially signs of diarrhoea, lethargy and weight loss, which was also reported by other researchers (Wolff, 1960; Haywood, 1985; Haywood and Loughran, 1985). The rats were injected *i.p.*, as it delivers an accurate dose and the drug rapidly accumulates in the various organs (Barka *et al.*, 1964). The only drawback was that if the rats were injected on the same site too often, the rats developed sores that caused irritation and pain. To overcome this, the rats were not injected on the same site. Many researchers fed rats chow supplemented with copper sulphate (Haywood, 1985; Haywood and Loughran, 1985; Fuentealba *et al.*, 1989; 1993) in high doses (1500mg/kg-6000mg/kg). These authors also reported acute toxicity signs in the rats. The drawback of feeding copper to the rats is that the researcher can not ensure that the rats will feed on all the copper in a day. *I.p.* injections are able to curtail this problem.

The 2-week *in vivo* brain experiments, the level of lipid peroxidation is low (0.03-0.07 nmoles/mg protein). The brain has an effective barrier (BBB), that prevents charged and large molecules from entering the brain (Rapoport *et al.*, 1979; Ganong, 1995). Free copper is charged and if complexed to chelating species (e.g. MT, Cp), it would not be able to traverse the BBB. In copper loading, copper is able to "spill-over" into the brain, due to higher concentration in the blood and with prolonged exposure to the copper loading. Figure 14 shows the control, copper and copper/melatonin groups. There is no

statistical difference between these groups. As compared to the 6-week brain *in vivo* experiment (Figure 17), where 2mg/kg copper was administered for 6 weeks, the level of lipid peroxidation is approximately the same, with no significant difference noted among the three groups. Even with an increase in exposure of copper to the brain, there was no increase in lipid peroxidation. It is interesting to note that the 6-week brain copper/melatonin lipid peroxidation level was increased, compared to the 6-week brain control and 6-week brain copper samples. This may be due to the lipophilicity of melatonin and its ability to easily traverse the BBB, thus possibly carrying copper across the BBB (refer to chapter 3) (Reiter, 1997). Section 2.5.2. displays AA of brain copper concentrations for the 2-week and 6-week experiments. The brain copper concentration is approximately the same, and appears to correspond to the level of lipid peroxidation depicted in Figures 14 & 17.

The level of lipid peroxidation in the control liver samples is statistically significant, compared to the copper and copper/melatonin groups, at $p<0.001$ (Figures 18), for the 6-week experiments. Since the majority of the copper is rapidly accumulated in the liver, approximately 60-90% of the administered copper is deposited within hours in the liver (Sternlieb, 1980). The liver samples of both the 2-week and 6-week experiments exhibited the greatest level of lipid peroxidation and accumulation of copper (Tables 3 and 4). The increase in lipid peroxidation in the 6-week liver copper/melatonin experiments, as compared to the 6-week copper liver samples, could possibly be due to the fact that melatonin is lipophilic, and when bound to copper, it is able to carry the copper into the cell. Within the cell, the melatonin is degraded, and the copper remains. From Table 4, the total copper concentration of the copper/melatonin group, is approximately half the level of the copper group. This indicates that less copper is found within the cells, but taken with the lipid peroxidation results, it could possibly indicate, that the copper within the cells, is free and able to interact with the lipid membranes (Figure 18). The copper concentration of the 6-week copper group was approximately double that of the copper/melatonin group, but the corresponding MDA concentration is less that of the copper/melatonin group. This could possibly be due to the copper being

tightly bound to proteins within the cells by MT and Cp, and thus less able to induce lipid peroxidation.

Copper is tranported to the kidney via the blood stream. It is bound to albumin and other copper transporting proteins, and thus unavailable to cause any cellular damage. When an organism is exposed to acute copper poisoning, the biliary excretory route is saturated. The copper concentration exceeds the chelating ability of albumin and other copper-binding proteins. Copper is then able to exert its cytotoxic effect on cells, resulting in necrosis of the proximal convoluted tubular epithelium (Vogel, 1960; Haywood *et al.*, 1985). The kidney is the major excretion organ for copper, thus it is expected, that the kidney would have a high lipid peroxidation level. The 2-week and 6-week kidney samples indicate similar levels in lipid peroxidation to the liver samples. Total copper concentrations of the 2-week kidney samples are similar for all three groups, and are not statistically significant (Table 3). The 6-week kidney total copper concentration is statistically not significant among all three groups. The 6-week copper and copper/melatonin kidney samples have approximately three times as much accumulated copper as compared to the 2-week copper and copper/melatonin kidney samples. This is due to the longer duration of copper administration and the kidney's ability to excrete the copper. The 6-week kidney MDA concentration (Figure 19) is greater than that of the 2-week kidney samples (Figure 16). The increase in copper exposure, results in an accumulative level of lipid peroxiadtion.

2.6.2. Experiment 2: *In vitro* experiments

The *in vitro* experiments were performed with the addition of copper and copper/melatonin to brain and liver homogenate. The copper 1mM liver experiment (fig. 20) shows an increase in lipid peroxidation in the copper and copper/melatonin groups. There is a statistical difference between the copper and copper/melatonin groups ($p < 0.001$) and this indicates that melatonin affords a degree of protection against copper-induced lipid peroxidation, at a copper concentration of 1mM and a melatonin concentration of 5mM. The 5mM and 10mM copper liver experiments (Figures 21 & 22)

indicate a decrease in MDA concentration for the copper and copper/melatonin groups, as compared to the copper 1mM experiment. Melatonin at 5mM, did not have any effect at reducing the level of lipid peroxidation, albeit insignificantly. On the contrary, when melatonin was added to the copper, it increased the level of lipid peroxidation. The copper 5mM and 10mM groups exhibited approximately a third of the level of lipid peroxidation (MDA 0.5 nmoles/mg protein), as compared to the copper 1mM group (MDA 1.5 nmoles/mg protein).

The *in vitro* brain experiments showed a similar trend at the various copper concentrations (Figures 23, 24 & 25). Copper increased the MDA concentration, as compared to the control, at the various copper concentrations, and this was statistically significant at $p<0.01$. It appears that melatonin did not have any effect on protecting the lipids from copper-induced lipid peroxidation. In the MDA assay, metal ions, antioxidants and chelating agents can influence not only peroxidation in the incubation medium, but also peroxide decomposition during the assay itself (Gutteridge and Halliwell, 1990).

The results of the *in vivo* 2-week and 6-week experiments, indicate that melatonin does not have a significant antioxidant role in copper-induced lipid peroxidation. *In vivo* experiments by Antunes *et al.* (1999) indicate that due to melatonin's weak antioxidant properties and its relatively low concentration, it was unable to inhibit lipid peroxidation induced by copper.

The *in vitro* liver copper 1mM experiments, indicate that melatonin at 5mM, exert a protective effect on copper-induced lipid peroxidation ($p< 0.001$). Further studies need to be carried out on determining the free radical associated with copper-induced lipid peroxidation. Superoxide radical assays need to be performed to determine the extent of copper-induce lipid peroxidation by the superoxide radical. The concentration of melatonin was possibly low (5mM for the in vitro experiments), and further studies need

to be carried out on the optimum concentration of melatonin needed to prevent copper-induced lipid peroxidation.

CHAPTER 3

HISTOCHEMICAL INVESTIGATIONS

3.1. INTRODUCTION

Copper is an essential trace element that is extremely toxic in excess. The liver maintains a copper reserve that is protein-bound, and is sufficient only for metabolic requirements. Copper toxicoses may be of genetic origin e.g. Wilsons disease in man and copper toxicosis in the Bedlington terrier, or acquired as in sheep (Haywood, 1985).

The histochemical demonstration of copper in organs plays an important role in the understanding of the pathogenesis and etiology of some metabolic and toxic diseases. Copper accumulation in organs may result from abnormal excretion mechanisms, decreased protein-binding substrates and increased copper consumption.

The liver is the major organ of copper deposition, but other organs such as the kidney, brain and heart also accumulate copper. Lal and Sourkes (1971) studied the effect of copper loading in various tissues in rats. These authors injected copper *i.p.* for 18 weeks and monitored the rats. The results show that all tissues, except hair and nails, accumulated copper, with liver and kidney accumulating the highest levels. The percentage of injected copper retained in the liver or kidney varied with the dose and duration of loading.

Rats develop tolerance very rapidly to copper, showing only mild signs of chronic hepatitis. Rats initially accumulate copper in the liver and kidneys in toxic concentrations, with a subsequent fall in hepatic copper levels. Regeneration of tissue occurs and the animals become tolerant to continued dosing (Haywood, 1985). Tolerance has been defined as " the ability to endure the continued or increased administration of a toxic substance and the capacity subsequently to exhibit a lower tissue damage response to a challenge" (Luckey and Venugopal, 1977).

Haywood (1985) showed that rats fed high quantities of copper demonstrate a dose-related response with regard to the onset of the toxic effects and the subsequent tissue changes. This study also shows that above the upper limit of copper intake, adaptation does not occur. Changes in copper storage in the liver and copper excretion by the kidneys contribute to the development of tolerance.

Elevated copper levels induce many changes in tissue (Figure 26). In the liver, copper overload results in hypertrophy of hepatocytes, hepatitis, hepatocellular necrosis and eventually hepatocellular death (Haywood and Loughran, 1985). In the kidney, copper overload induces necrosis of the proximal convoluted tubule epithelium, which leads to death. Epithelial regeneration occurs in copper tolerant animals with residual hyperplasia and calcification (Vogel, 1960).

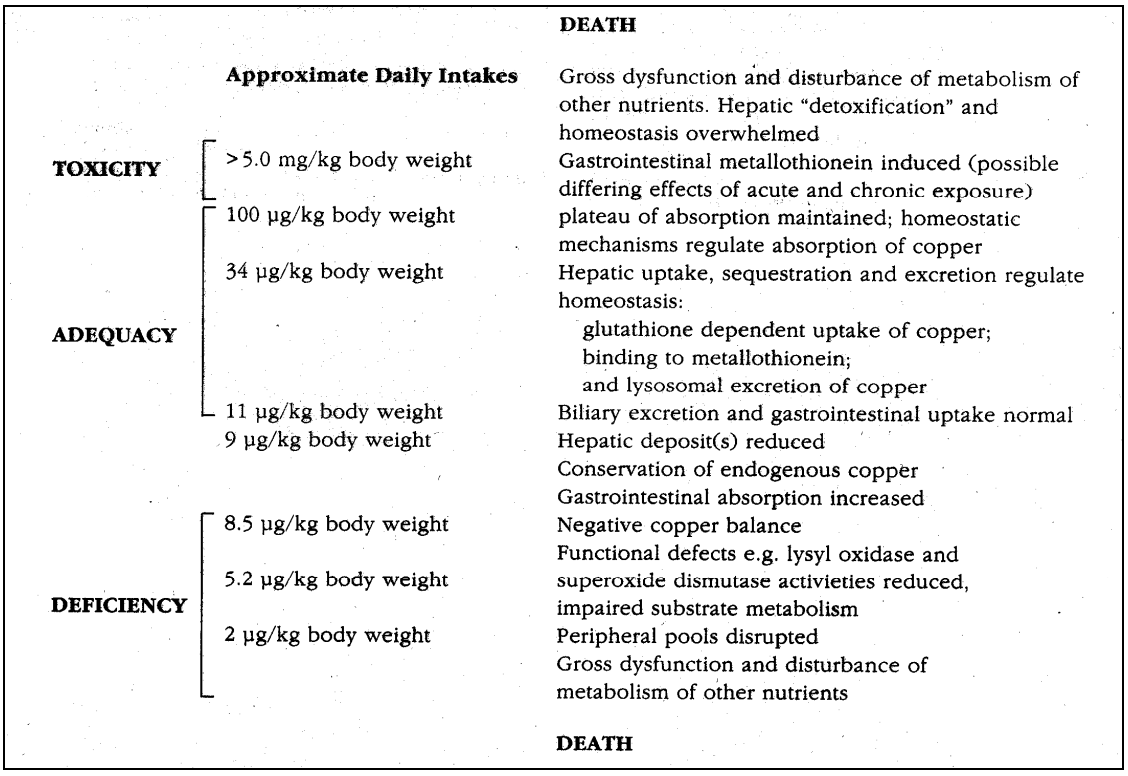


Figure 26. A postulated spectrum of copper metabolism with various copper intakes (mg/kg body weight) and the corresponding toxic/deficient effects (Aggett, 1999).

In the brain, copper toxicity leads to Parkinsonian-like symptoms with localized tremors or involuntary movements, dystonia, mental deterioration, and psychosis (Bickel *et al.*, 1957; Lal and Sourkes, 1971). There is little data available on copper-induced brain pathology, but it appears that neuronal damage occurs, resulting in striatal degeneration. Damage to the presynaptic terminals of the nigral dopaminergic neurons has also been observed. Copper also causes depolarization of neurons, blocks K^+ and Na^+ channels in axons, inhibits synaptosomal uptake of monoamine transmitters, which may secondarily increase transmitter efflux. It stimulates leutenizing hormone-releasing hormone release from median eminence explants (Wang, 1999). Wang (1999) has demonstrated that copper caused a massive calcium-dependent release of neurotransmitters from isolated CNS dopaminergic and noradrenergic nerve terminals, which may contribute to the neuropathological processes associated with copper toxicity.

Three copper stains (rubeanic acid, rhodanine and silver sulphide) were used and compared for sensitivity. Rubeanic acid (dithiooxiamide) forms a black-green precipitate with as little as 0.006:g of copper. Rubeanic acid binds to copper to form a linear polymer copper rubeanate. Rubeanic acid is a sensitive and fairly specific cytochemical reagent for copper, and the black-green precipitates formed are fine and discrete. P-Dimethylaminobenzylidene rhodanine reacts with Cu^{1+} and Cu^{2+}, to form a red-brown precipitate. It is sensitive to as little as 0.04:g of copper, and compares to the sensitivity of rubeanic acid in tissue sections (Lindquist, 1969). The silver sulphide method (modified Timm's method) depends on the conversion of tissue copper to copper sulphide by hydrogen sulphide. The copper ion is then displaced by silver ion to form silver sulphide. In the presence of a reducing agent, the ionic silver in silver sulphide is reduced to metallic silver. Gum arabic is used to enhance the stability of the silver ions and TCA is used in the pretreatment as it effectively removes zinc and iron, leaving copper as the major metal in the tissues (Fujii *et al.*, 1993).

Limson *et al.* (1998) reported that melatonin binds/complexes with heavy metals: copper, aluminium, zinc, cadmium, iron and lead. These experiments were performed in vitro utilizing adsorptive cathodic stripping voltammetry. The results show that melatonin

shows binding affinity for copper and aluminium. This implies that melatonin, besides acting as an antioxidant, may bind these metals and prevent them from partaking in free radical generation. It is also important to note that any free metal ions in the brain are toxic, and may participate in free radical production, especially copper and iron. Thus, melatonin may function as a metal ion binder, and thus remove "free" metal ions from participating in the generation of free radicals.

In this study, liver, kidney and brain were investigated for copper loading. Electron microscopy and light microscopy techniques were employed to investigate copper accumulation in these tissues and the possible protective effect of melatonin in copper loading.

3.2. Materials and methods

3.2.1. Animals

Male Wistar rats weighing 250-300g were used in the experiments. The rats were randomly assembled into five groups of 5 rats per cage, and were maintained as described in Appendix 1. The two control group (n=5) received the drug vehicle, ethanol:0.9% saline (40:60) for 2-weeks and 6-weeks, respectively. The copper treated group (n=5) received 2mg/kg Cu^{2+} (copper chloride-$CuCl_2$) for 2-weeks and 6-weeks, respectively. The copper/melatonin treated groups received Cu^{2+} 2mg/kg and melatonin 12mg/kg for 2-weeks and 6-weeks, respectively. The rats were injected daily, with the vehicle, Cu^{2+} and Cu^{2+}/melatonin. Copper/melatonin groups were injected on opposite intraperitoneal sites, to prevent any interaction of the copper with the melatonin.
In another experiment, larger doses of 4mg/kg, 10mg/kg and 500mg/kg Cu^{2+} were injected to the copper and copper/melatonin groups (12mg/kg melatonin). The livers, brains and kidneys were removed as described in Section 2.2.1.2.

3.2.2. Chemicals and reagents

All chemicals and reagents required for the electron microscopy were supplied by the Electron Microscopy Unit, Rhodes University, Grahamstown.
Melatonin was purchased from Sigma Chemical Co., St. Louis, MO, USA. 5-4-dimethylaminobenzylidene rhodanine and dithiooximide (rubeanic acid) were purchased from Merck, Darmstadt, Germany. Silver nitrate was purchased from BDH LTD., Poole, England. TCA, copper chloride ($CuCl_2$), sodium sulphide, gum arabic, citric acid, hydroquinone and sodium acetate were purchased from Saarchem (Pty) Ltd, Krugersdorp, South Africa. All other reagents were obtained from local sources and were of the highest purity available.

3.2.3. Transmission electron microscopy

Tissue samples of liver, brain and kidney, were placed in liquid nitrogen, and then stored in a -70°C freezer until used. The tissue pieces were cut using a scalpel into pieces approximately 2 mm^3. The tissue samples were placed in tubes containing buffered glutaraldehyde (2.5% glutaraldehyde in 0.1M sodium phosphate buffer) for 12- 24 hours at 4°C. The glutaraldehyde was replaced with cold buffer and allowed to wash for 10 min. This was repeated twice. The final buffer wash was followed by fixation in a 1% solution of osmium tetroxide in 0.1M sodium phosphate buffer. After 60-90 minutes, the tissue pieces were washed again in two changes of buffer (10 minutes each). The buffer washes were followed by dehydration carried out by immersing the tissue pieces in ascending concentrations of ethanol (30%, 50%, 70%, 80%, 90% and 100% ethanol) for 3-5 minutes in each solution. A second change of 100% ethanol was replaced by propylene oxide, a transitional solvent, and allowed to infiltrate for 15 minutes, after which the propylene oxide was changed. After a further 15 minutes, resin infiltration was carried out, by replacing the propylene oxide by solutions containing increasing concentrations of resin (TAAB 812 and Araldite mixture) in propylene oxide. Three mixtures were used (25:75, 50:50 and 75:25) and the infiltration carried out for 60-90

minutes in each mixture. The final mixture was replaced by pure resin and further infiltration allowed to take place overnight. The tissue pieces were then transferred to moulds containing pure resin. This was allowed to polymerize at 60°C for 36 hours. The solidified samples were removed and placed in labeled tubes until use (Rhodes University, Electron Microscopy Unit Handbook).

The sample blocks were trimmed and sectioned with an RMC MT-7 ultramicrotome for examination. The sections were viewed using a Joel JEM 1210 transmission electron microscope

3.2.4. Light microscopy

Tissue samples were placed in liquid nitrogen, and transferred to -70^{o}C freezer until used. Pieces of tissue ($3mm^3$) were cut and placed in Polytop tubes containing 10% buffered formalin for 24 hours. The formalin was decanted and replaced with 50% ethanol and allowed to infiltrate for 60 minutes. The 50% ethanol was decanted and replaced with 70% ethanol and allowed to infiltrate for 60 minutes. This procedure was repeated for 70% ethanol, 80% ethanol, 90% ethanol and absolute ethanol. The absolute ethanol step was repeated twice. The absolute ethanol was decanted and replaced with xylene, which was allowed to infiltrate for 60 minutes, and was repeated twice. The xylene was decanted, and melted wax was added and allowed to infiltrate for 60 minutes. The wax was then replaced with fresh wax. The samples were placed in an oven at 57°C overnight. The wax samples were then removed and allowed to cool. The wax samples were trimmed and mounted on mounting blocks. The tissue samples were sectioned at 5μm thickness. Sample sections were fixed onto the slides with Houpts solution, and placed in an oven at 37°C in a formalin atmosphere, until stained.

The slides were stained with rhodanine, rubeanic acid and silver sulphide (according to the modified Timm's method). The staining protocols appear as Appendix 3.

3.3. Results

3.3.1. Clinical findings

Intraperitoneal administration of copper results in an accurate control of the dose and rapid accumulation of the copper in the various organs (Barka *et al.*, 1964). There was some peritoneal irritation at the injection site, but this subsided after a few days. The rats that were constantly injected on the same site, experienced irritation and pain, and developed sores. The injection sites were then rotated as to minimize the development of these sores (similar in appearance to insulin-tumors that diabetics experience due to constantly injecting themselves at the same site).

Toxicity data for cuprous chloride was not available, so a range of copper concentrations was tried to induce copper toxicity, but not to kill the rats. 2mg/kg, 4mg/kg, 10mg/kg, and 500mg/kg copper concentrations were administered. The 4mg/kg, 10mg/kg and 500mg/kg copper proved too toxic and lethal. The rats that received 4mg/kg Cu^{2+} (the copper and copper/melatonin groups) all had severe diarrhoea. These rats when injected felt limp and had distended abdomens. The animals died within 3 days after the initial copper injection. Similar reports were made by Wolff (1960), with 4mg/kg copper *i.p.* injections.

In the 10mg/kg Cu^{2+} experimental group, 5 rats in the copper group died within 1 hour of the copper administration, compared to 3 rats in the copper/melatonin group. The 10mg/kg dose of copper was toxic and lethal.

The 500mg/kg Cu^{2+} experiment proved to be lethal, as all the copper and copper/melatonin administered rats died within 30 minutes of the copper injection. Within minutes, the rats went limp and showed signs of toxicity.

Animals were treated with 2mg/kg copper for 2-weeks and 6-weeks, respectively. The copper and copper/melatonin treated groups, in both the 2-week and 6-week experiments,

experienced slight diarrhoea initially. The rats ate well and drank water. But as time progressed, these two groups failed to gain weight and appeared lethargic, despite eating well. The rats exhibited distended abdomens. The copper and copper/melatonin groups were smaller in size compared to the control group. The livers of these animals were pale and small, when dissected

3.3.2. Electron microscopy experiments

Electron micrographs of liver, kidney and brain were prepared as described in Section 3.1.3. A 2-week brain copper treated sample (Figure 27), shows disrupted mitochondria, with dilated cristae. The mitochondria are swollen and appear “exploded” from the inside. A 2-week brain copper/melatonin treated sample, presents a similar picture to Figure 28, with swollen, “exploded” mitochondria with dilated cristae.

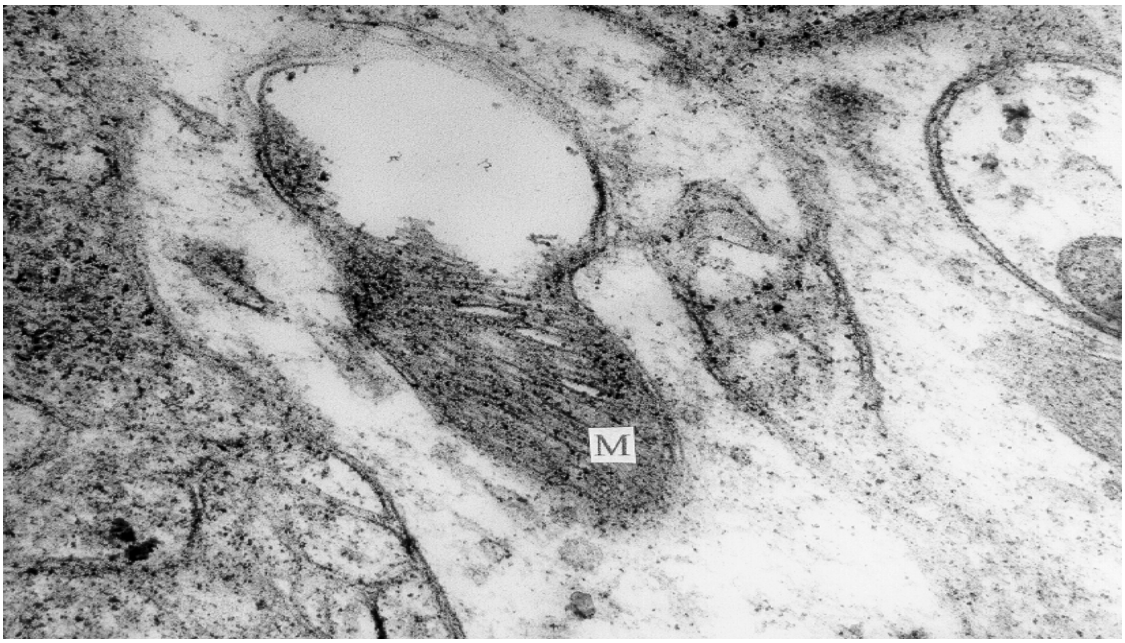


Figure 27. “Exploded” mitochondria from a rat brain treated with 2mg/kg copper for 2-weeks. The mitochondria (M) are swollen with dilated cristae, X 40 000.

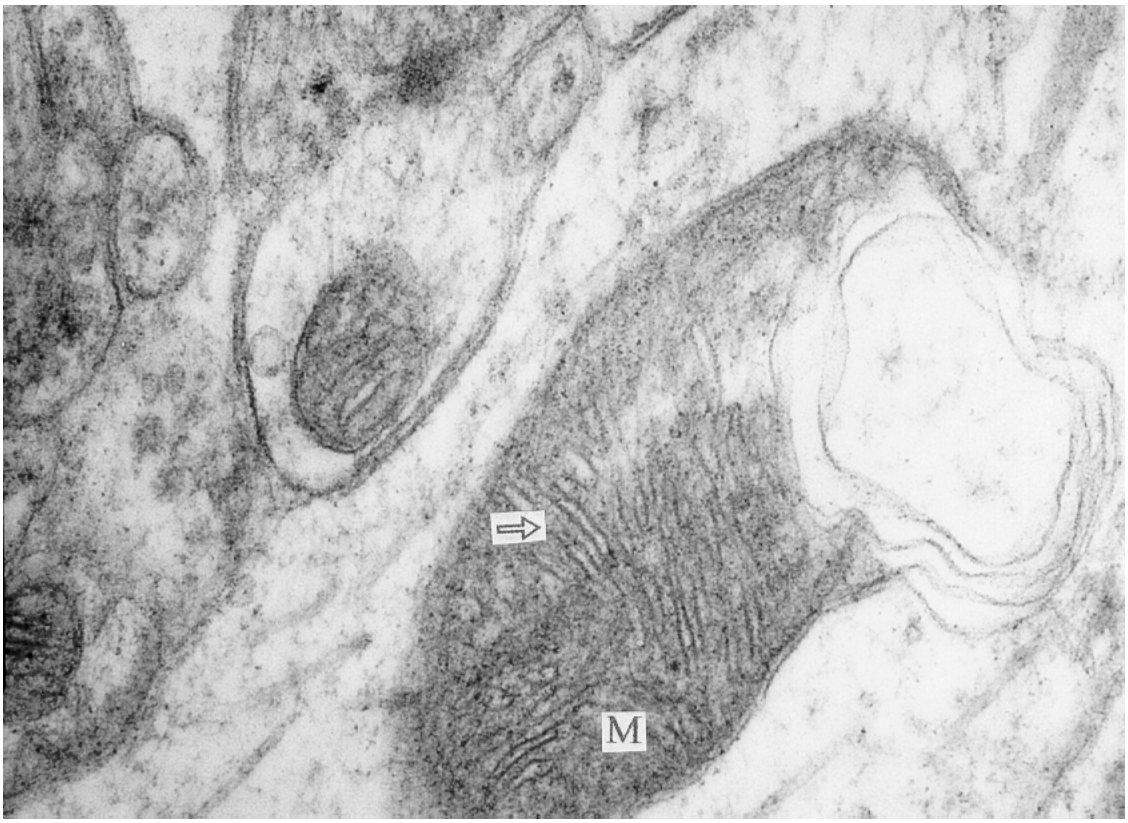


Fig 28. Brain mitochondria from a rat treated with 2mg/kg copper and 12mg/kg melatonin for 2-weeks. The mitochondria (M) are swollen and disrupted, showing extensive damage. The cristae (') are dilated, X 40 000.

The rat brain mitochondria from a 6-week copper treated sample, are disrupted and swollen. There was internal damage, with dilated cristae (Figure 29). A similar pattern is seen in the 6-week copper/melatonin treated brain mitochondria (Figure 30), but the internal structure of the mitochondria is not as extensively damaged. The mitochondria are swollen and the cristae are dilated. Glycogen is abundant throughout, as in Figure 29.

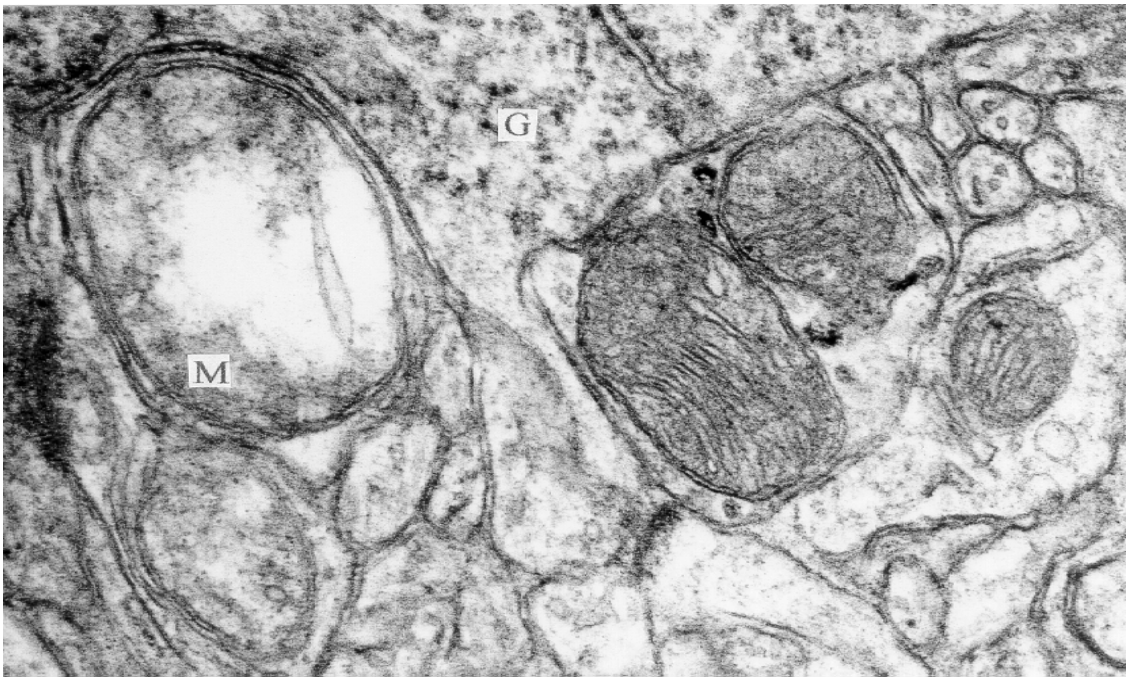


Figure 29. Brain mitochondria from a rat which received 2mg/kg copper for 6-weeks. The cristae are dilated and the mitochondria (M) are swollen. The internal damage is not as extensive as the 2-week copper brain sample. Glycogen (G) is present, X 40 000.

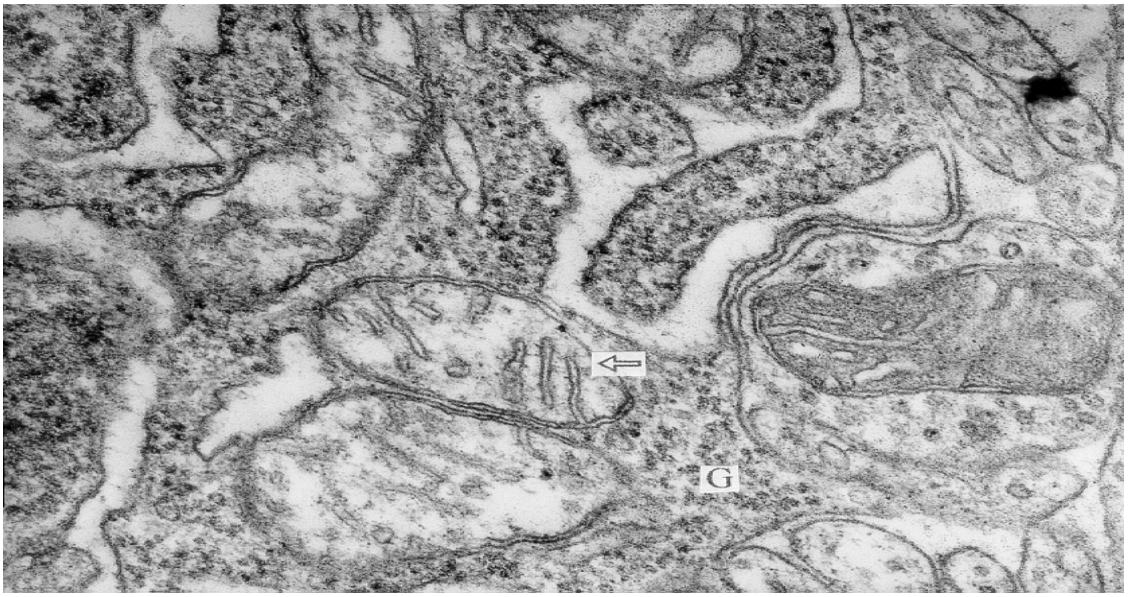


Figure 30. Brain mitochondria from a rat treated with 2mg/kg copper and 12mg/kg melatonin for 6-weeks. The mitochondria are slightly swollen and the cristae (ʾ) are dilated. Glycogen (G) is present throughout the cell, X 40 000.

The 2-week experiment with 2mg/kg Cu, yielded extensive hepatocyte damage in the copper group. Figures 31, 32 and 33 illustrate the control, copper and copper/melatonin samples. In Figure 31, the nucleus is spherical, with normal mitochondria and extensive glycogen. The copper treated sample (Figure 32) exhibits damaged mitochondria and a cretinated nucleus. The chromatin in the nucleus is condensed, and there is less noticeable glycogen. Figure 33 shows a copper/melatonin treated sample. Here less damaged mitochondria are noticeable when compared with the copper sample (Figure 32). The nucleus is spherical and is similar in appearance to the control nucleus (Figure 31).

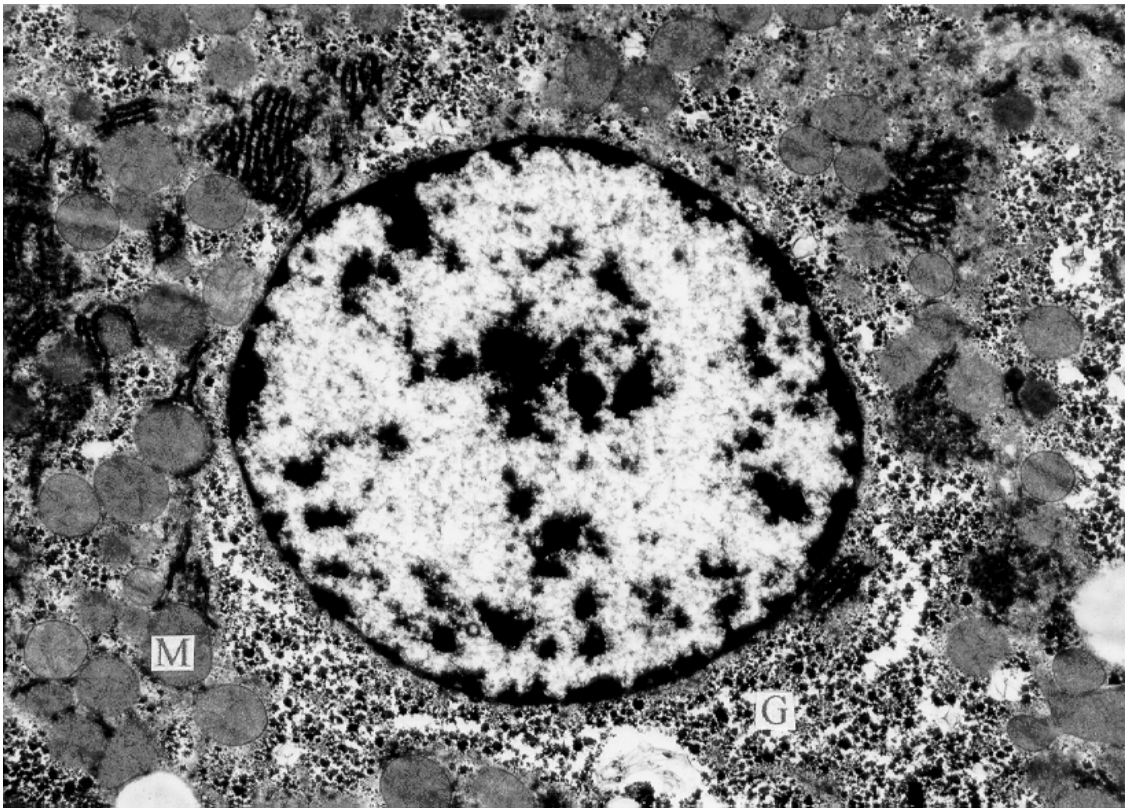


Figure 31. A control rat hepatocyte 2-week sample. The nucleus is spherical and appears normal. There is extensive glycogen (G) present. The mitochondria (M) appear normal, X 5000.

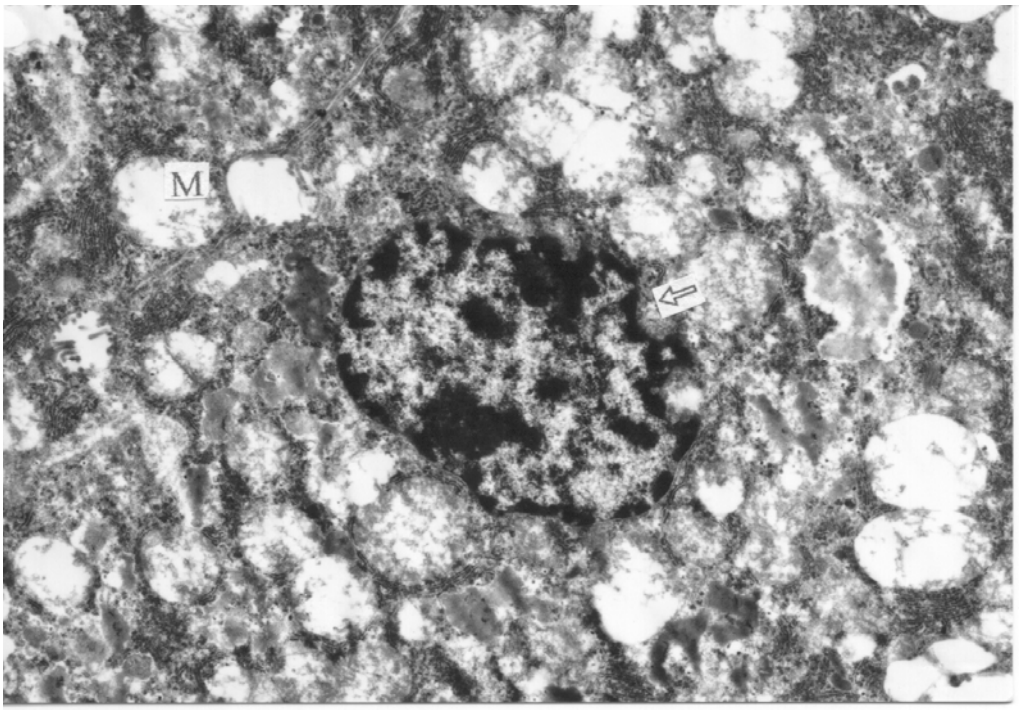


Figure 32. A 2-week copper treated rat hepatocyte, which received 2mg/kg copper. Extensive damage is seen in the mitochondria (M) and the nucleus is cretinated (’), X 5000.

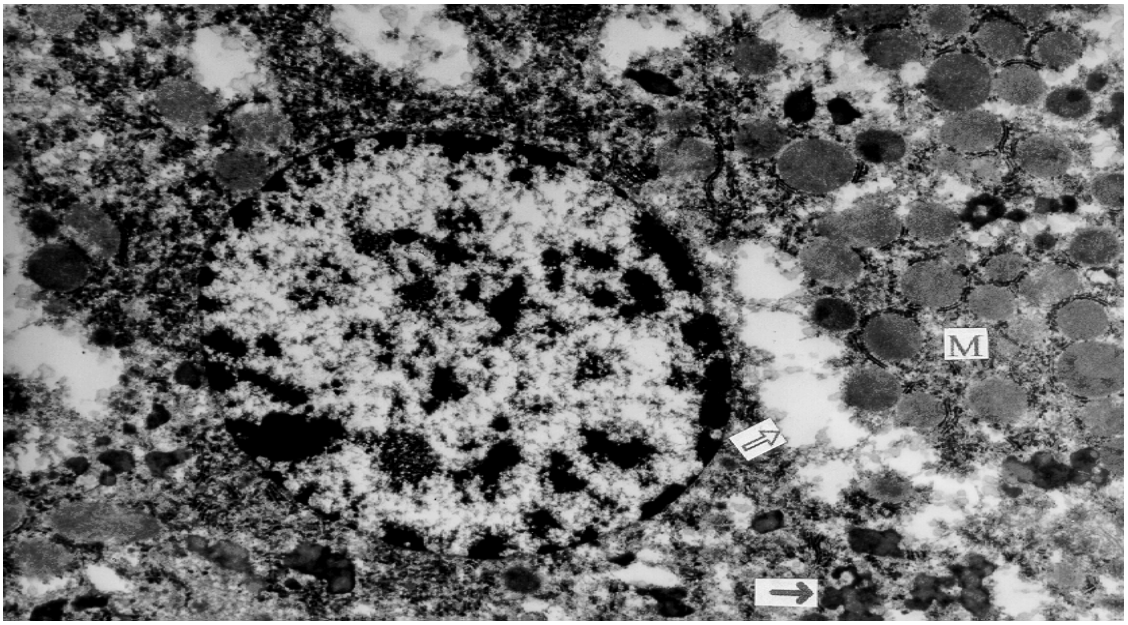


Figure 33. A copper/melatonin treated rat hepatocyte in the 2-week experiment. There are some damaged (’) pleomorphic mitochondria (M) present, with a normal appearing nucleus. Lysosomes (⊥) are irregularly shaped and dense. There is glycogen present, X 5000).

The 6-week copper treated liver samples (Figure 34) have extensive mitochondrial damage and a large number of damaged mitochondria. The nucleus is cretinated and the mitochondrial cristae are dilated. The copper/melatonin treated 6-week liver sample (Figure 35) shows less mitochondrial damage. The large nucleus is normal. Glycogen is present throughout, but in greater density around the mitochondria. Many dense, irregularly shaped lysosomes are present.

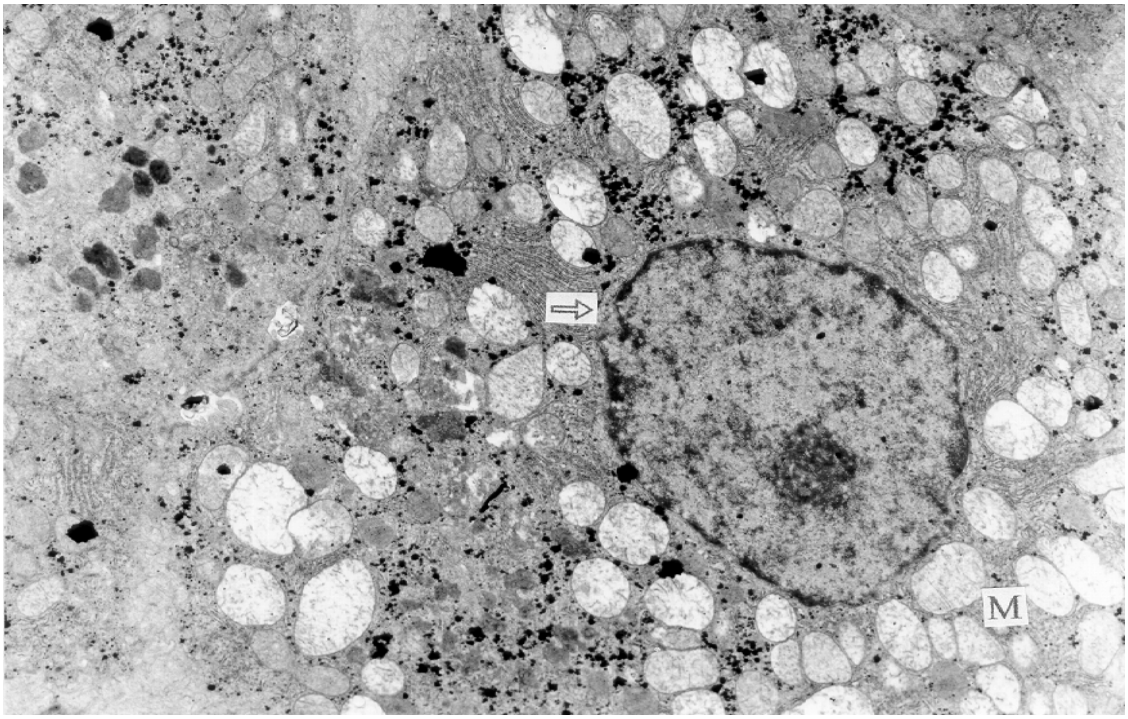


Figure 34. A 6-week copper treated sample of a rat liver. The nucleus is cretinated (ʾ) and extensively damaged mitochondria are present throughout. Mitochondria (M) are swollen, with cristae lying peripherally. Glycogen is sparse, X 6700.

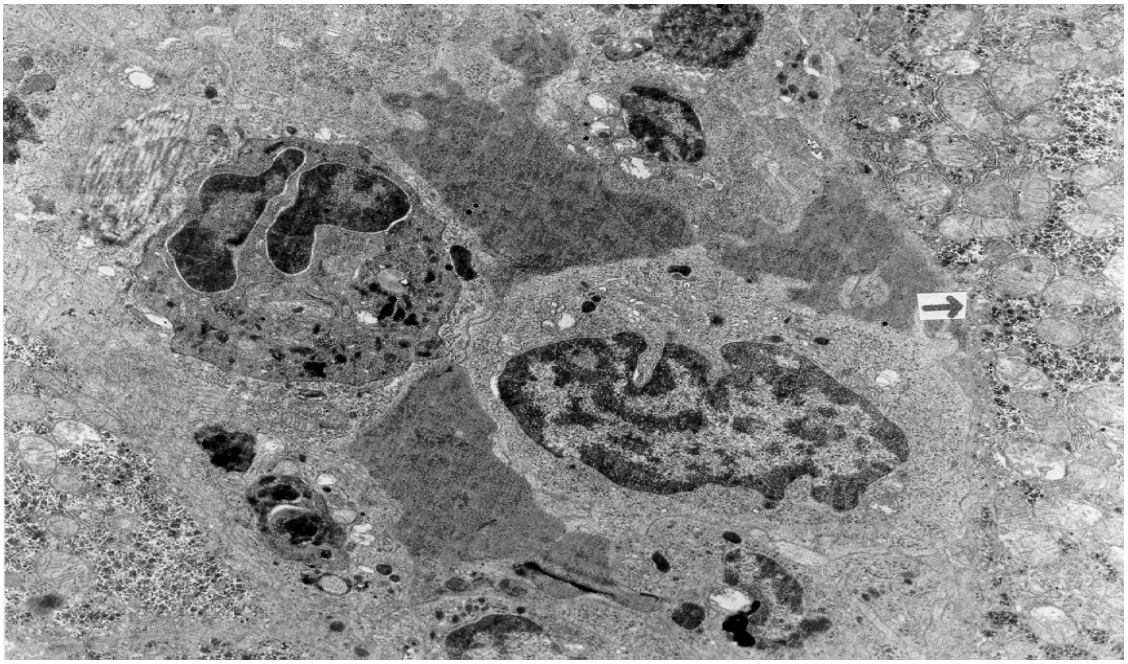


Figure 35. A copper/melatonin treated sample of a 6-week rat liver. There is glycogen present throughout, but concentrated around the mitochondria (⊥). There are less damaged mitochondria present. The mitochondria are pleomorphic, and irregularly shaped, X 6700.

The kidney is involved with copper excretion. The 2-week rat copper treated kidney sample (Figure 36) indicates large lysosomes containing dense material, possibly accumulated copper.

The 2-week copper/melatonin treated group displayed increased number of small mitochondria, which indicates an increase in mitochondrial respiration. The nuclei appear slightly cretinated. A prominent belt of black precipitate is evident around the border of the cell, and could possibly be precipitated copper (Figure 37).

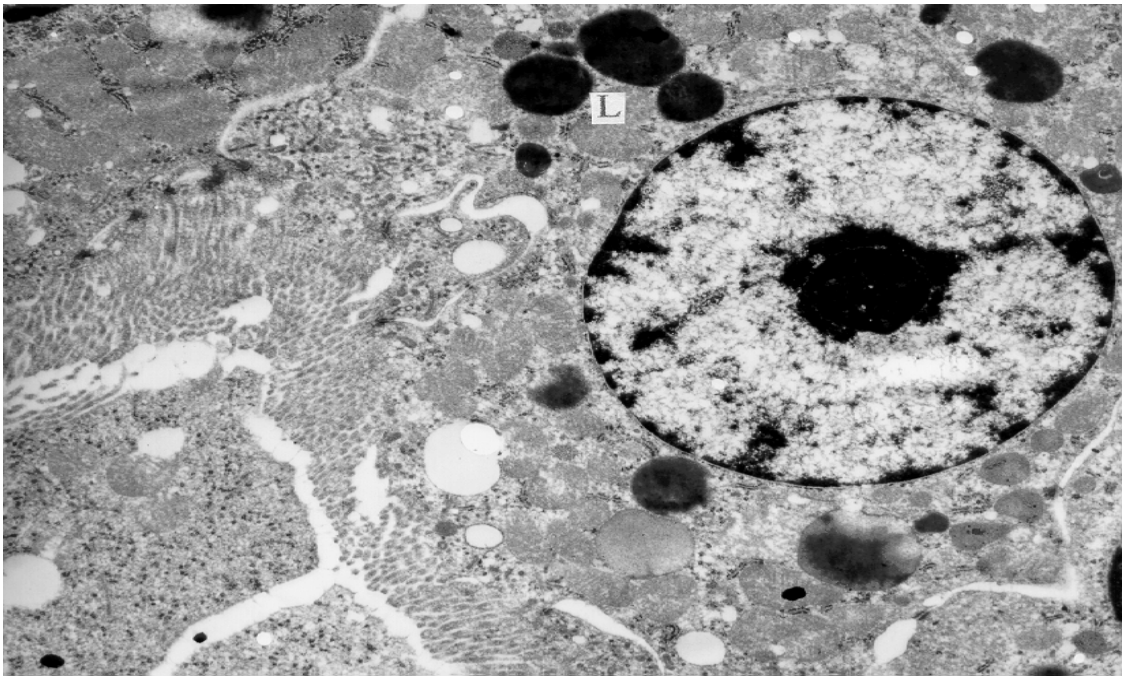


Figure 36. Rat kidney of a 2-week treated copper sample. A large nucleolus is present, with lysosomes (L) packed with dense matter, X 5000.

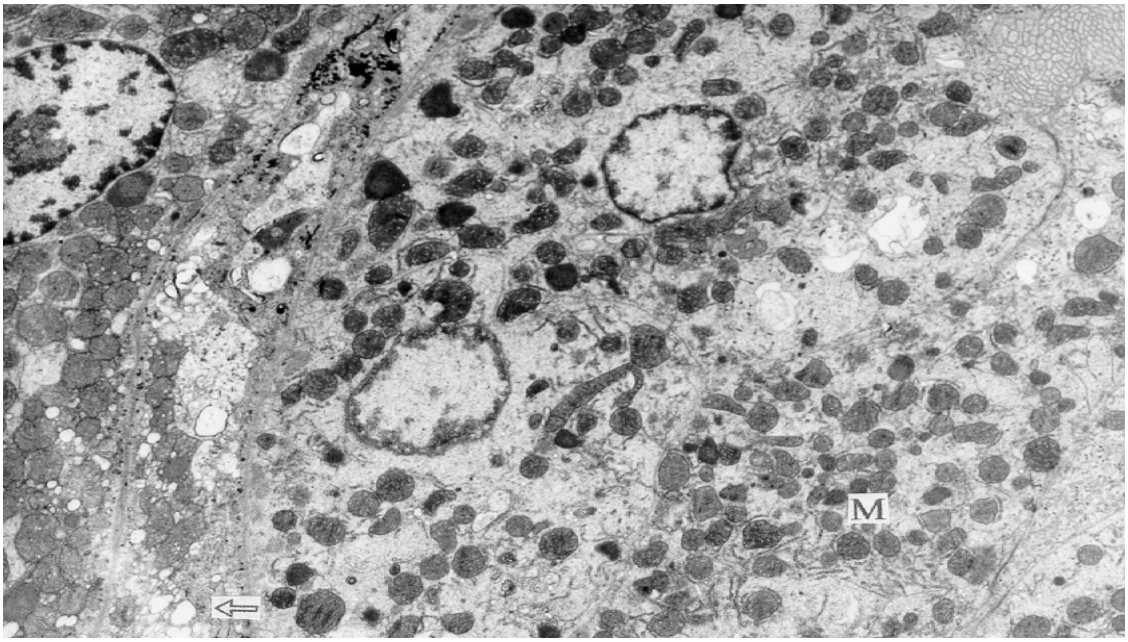


Figure 37. Rat kidney of a 2-week treated copper/melatonin sample. A large number of pleomorphic mitochondria (M) are present, indicating increased respiration. Some damaged mitochondria (') are evident, X 3000.

The 6-week kidney control sample indicates normal mitochondria, both in size and shape (Figure 38). The 6-week copper treated kidney sample, displays a large number of mitochondria, that appear to be swollen. A large number of lysosomes are present with densely packed black precipitate (Figure 39). The copper/melatonin treated 6-week sample has a large number of lysosomes present. The mitochondria are numerous and small (Figure 40).

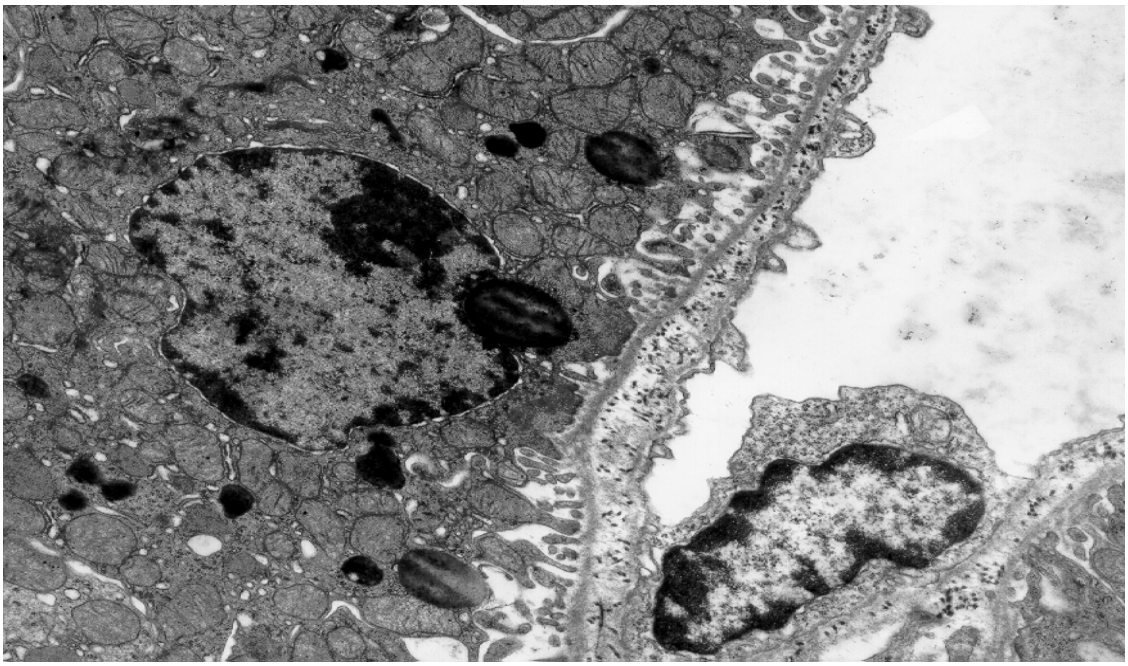


Figure 38. A 6-week control kidney sample. The mitochondria and nuclei are normal, X 10000.

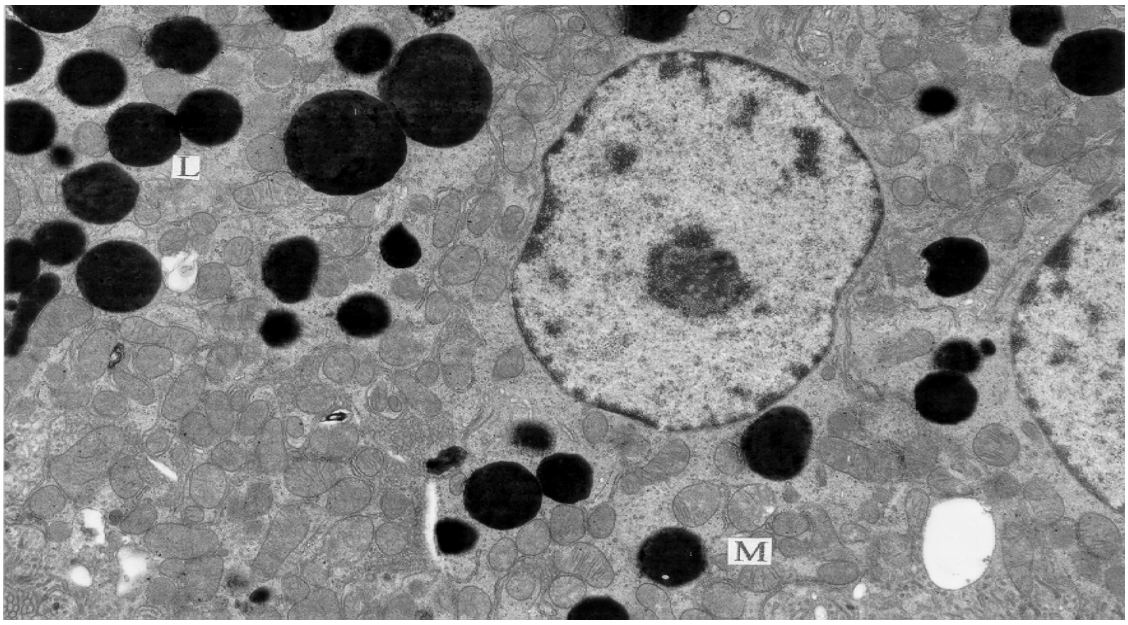


Figure 39. A 6-week copper treated kidney sample. A large number of swollen mitochondria (M) and lysosomes (L) are present, X 8000.

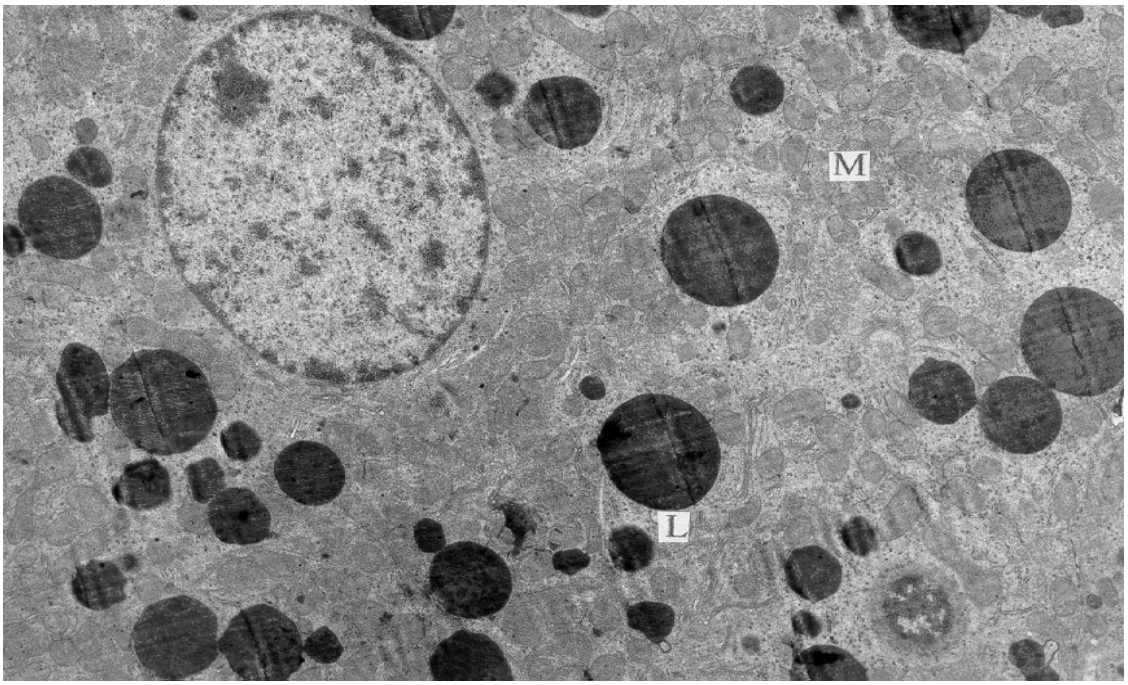


Figure 40. A 6-week copper/melatonin administered kidney sample. A large number of small mitochondria (M) and large lysosomes (L) are present, X 6700

3.3.3. Light microscopy experiments

Three copper stains were investigated, viz. rhodanine, rubeanic acid and silver sulphide (modified Timm's method). The rubeanic acid stain did not prove to be effective at all, as no copper was seen in any of the samples tested, though copper was evident from atomic absorption spectrophotometric analysis (refer to Section 2.5). Thus, the comparative study was limited to the rhodanine and silver sulphide stains.

In the 2-week copper treated samples, copper was visualized in the liver and kidney, but not in the brain. The rhodanine stain for the 2-week control liver sample (Figure 41) indicates no copper, which was expected. The rhodanine (Figures 42 and 43) and silver sulphide (Figure 44) stains, were both effective, showing a diffused pattern of copper distribution in the liver. Both stains were ineffective in visualizing the copper in the 6-week samples.

The kidney displayed a slight copper-rhodanine stain in the 2-week copper treated sample (Figure 45). The silver sulphide stain was not effective in the 6-week samples.

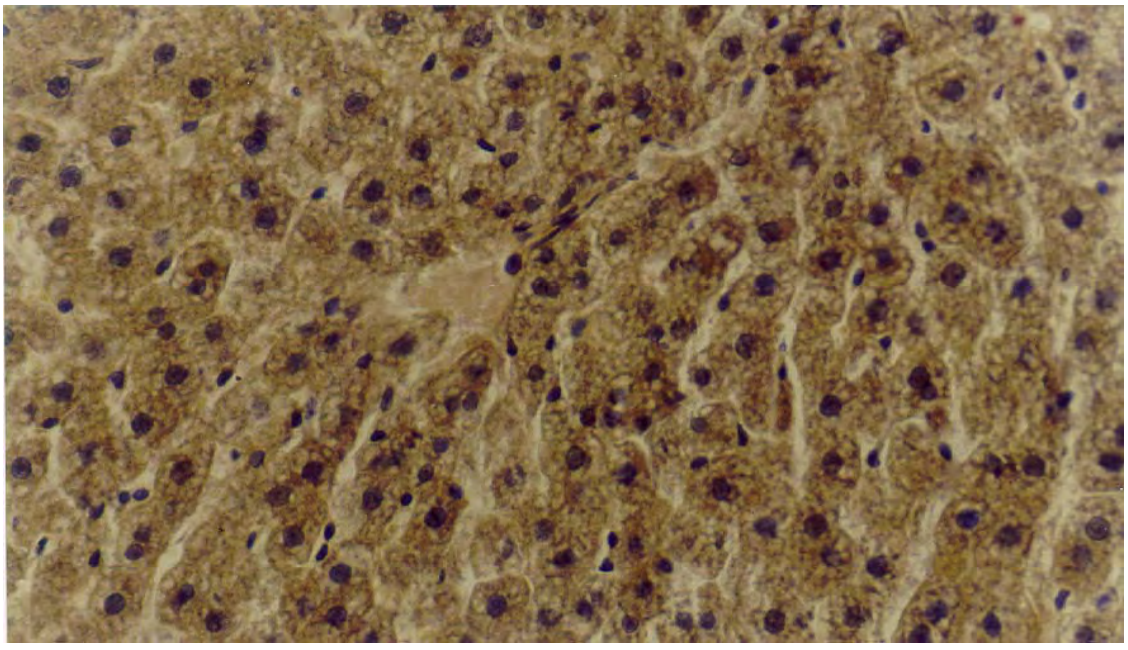


Figure 41. A 2-week control liver sample stained with rhodanine. No copper is visble.

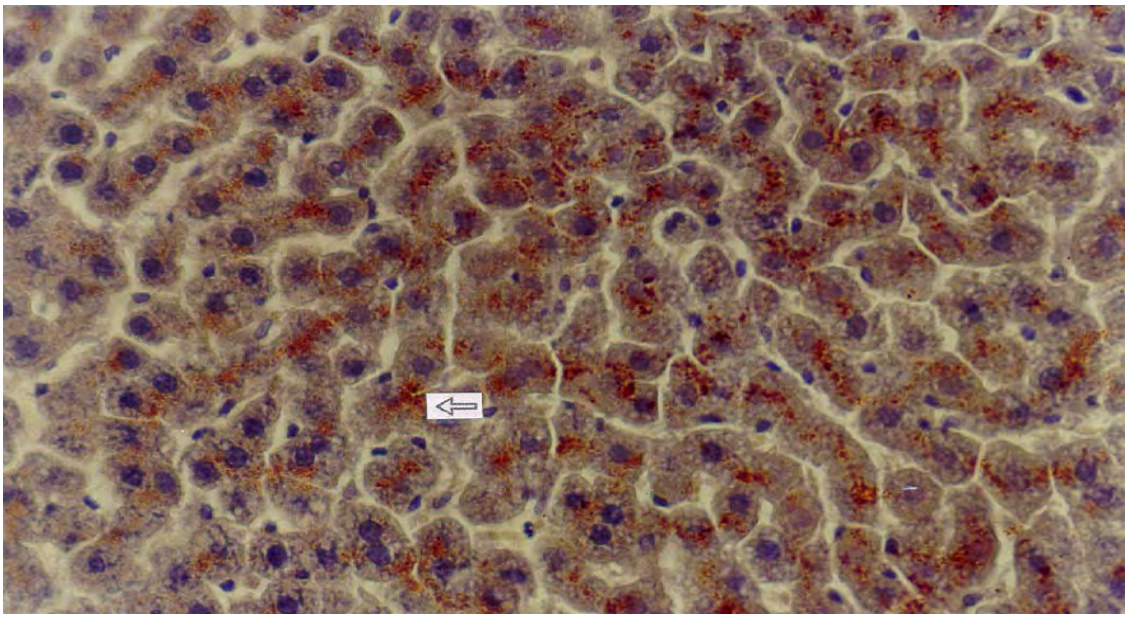


Figure 42. A 2-week copper treated rat liver sample receiving 2mg/kg copper. A diffuse pattern of copper is seen with the rhodanine stain, as red specks ('), within the cell, X 400.

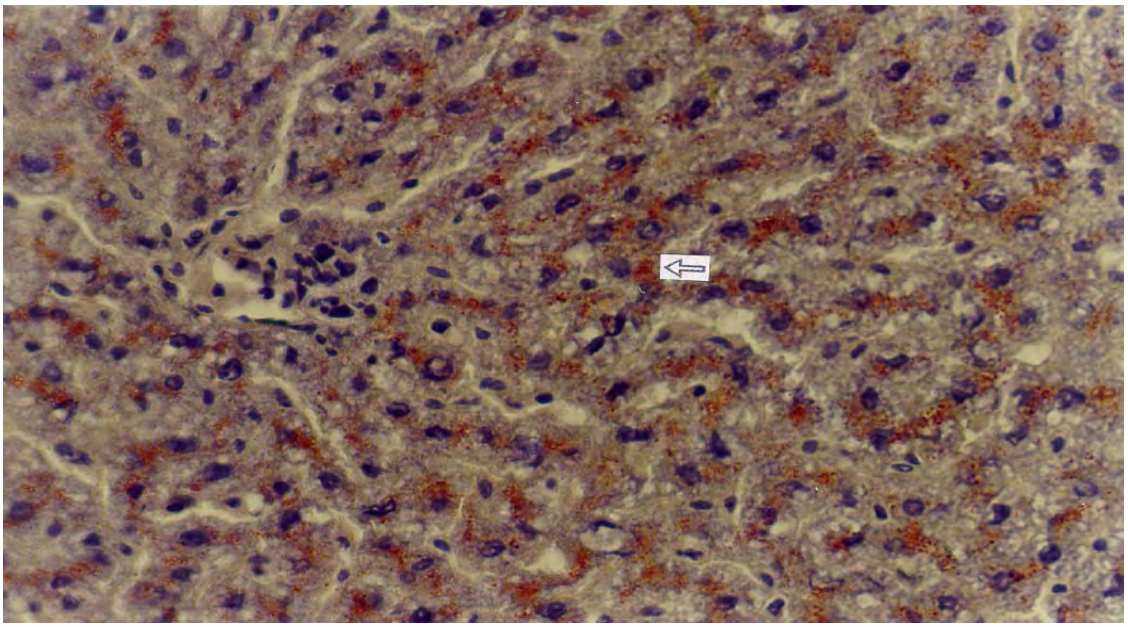


Figure 43. A copper/melatonin rat liver sample receiving copper and melatonin for 2-weeks. The rhodanine stain displays copper as red specks ('), which are localized within the cells. The cells are counterstained with haematoxylin, X 400.

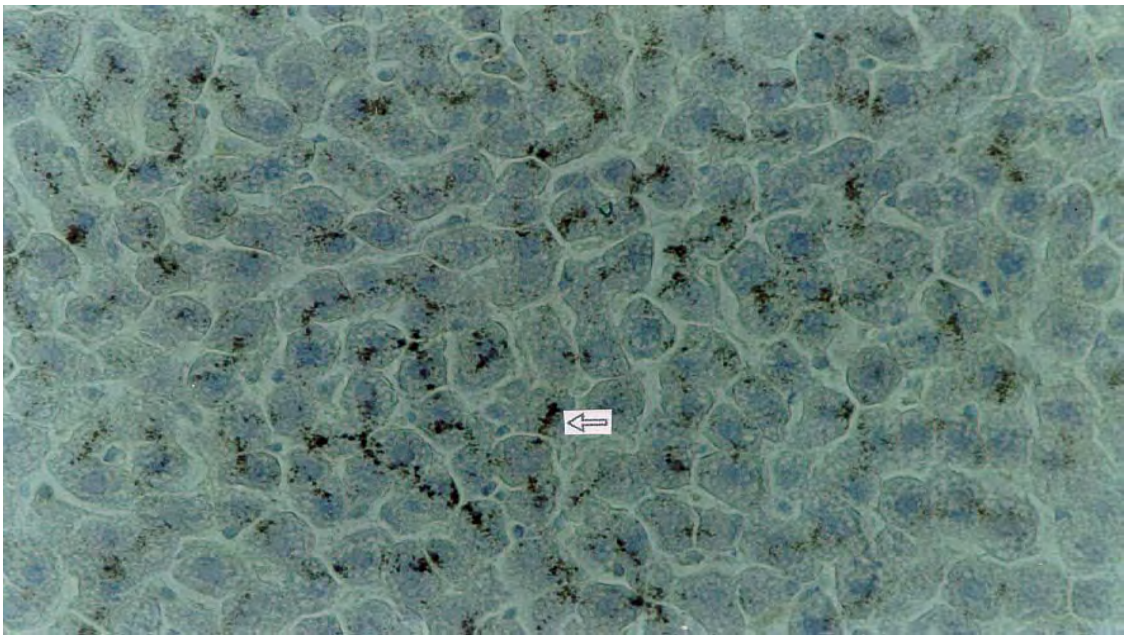


Figure 44. Silver sulphide stain of a rat liver copper sample treated with 2mg/kg copper for 2-weeks. The copper appears as a black precipitate (') within the cells. The cells are counterstained with haematoxylin and the nuclei appear blue, X 400.

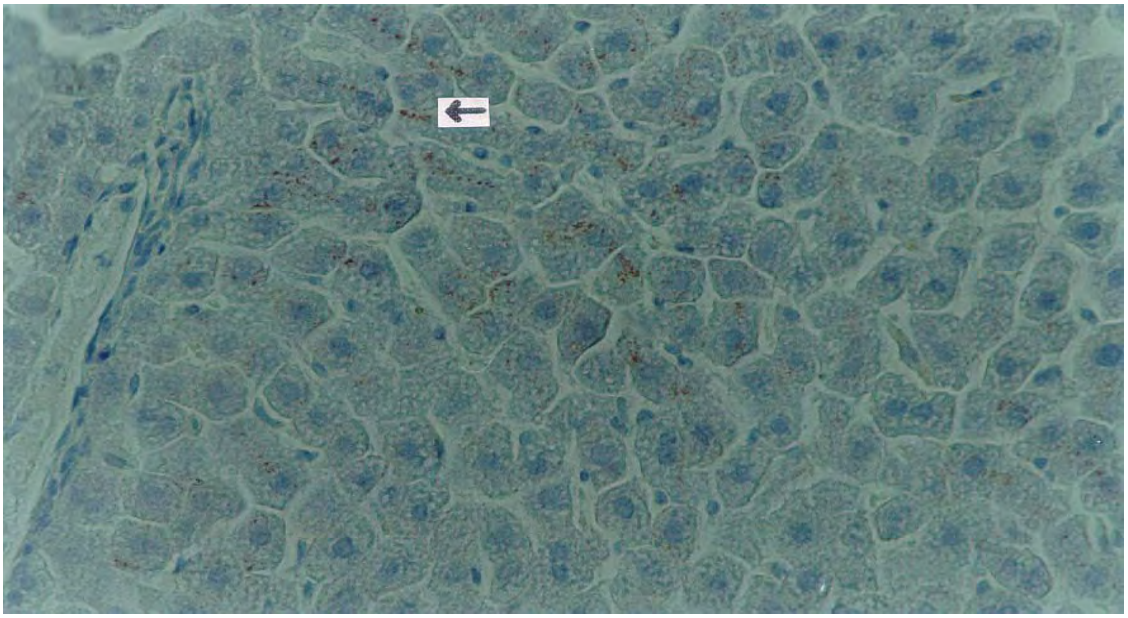


Figure 45. Rhodanine stain (') of a 2-week copper treated rat kidney, X 400.

3.4. Discussion

Various doses of copper (2mg/kg, 4mg/kg, 10mg/kg and 500mg/kg) were used. Copper-induced toxicity for the length of the experiment was attemped. Toxicity and lethality was apparent at the 500mg/kg, 10mg/kg and 4mg/kg copper dose. In the 2mg/kg copper experiments for 2-weeks and 6-weeks, the rats appeared to tolerate the copper dose. The 2mg/kg dose did induce copper toxicity, as the copper and copper/melatonin treated rats failed to grow and thrive, as compared to the control rats.

The copper is bound in lysosomes within the cell to limit the damage caused by free copper ions within the cell. Sequestration of copper into lysosomes is a cytoplasmic adaptive process, that protects the cell from free, toxic copper (Fuentealba *et al.*, 1993). In Figures 33, 34, 36, 37 and 39, lysosomes are abundantly present.
Fuentealba *et al.* (1989) demonstrated that Type 1 lysosomes appeared early in copper loading, and included simple copper complexes. Type 2 lysosomes appear later and represent a more enduring form of copper sequestering lysosome. Type 3 lysosomes, are late occurring and persists throughout the phase of recovery. These contain low amounts of copper and represent lysosomes that have released copper as part of the unloading process.

Copper within the nucleus interacts with DNA and results in cellular damage (Agarwal *et al.*, 1989). Cretinated nuclei are a direct result of copper loading (Figures 32, 34, 37).
Chromatin condensation occurs as early as Week 2 of copper loading. Cretinated nuclei appear as early as Week 3 of copper loading (Fuentealba *et al.*, 1989). Chromatin condensation is evident in copper and copper/melatonin 2-week and 6-week samples (Figures 32, 33, 34, 36, 37). In Figure 32, a cretinated nucleus is observed in the 2-week experiment.

The rubeanic acid copper stain did not stain any copper in any of the samples. The reason for this is unknown. In the copper and copper/melatonin treated samples, the rubeanic acid stain was unreactive, and no copper was detected, although AA demonstrated copper

present (refer to Section 2.5). The rhodanine and silver sulphide (modified Timm's method) proved to be equally sensitive to copper for the 2-week copper and copper/melatonin treated samples. In the 6-week copper and copper/melatonin treated samples, both stains were equally unreactive. None of the three stains were sensitive to any copper in the brain, for the 2-week and 6-week, copper and copper/melatonin treated samples. Pilloni *et al.*, (1998) and Fujii *et al.*, (1993) demonstrated that the modified Timm's staining method was more sensitive than the rhodanine stain. Both the modified Timm's and rhodanine stains were more sensitive than rubeanic acid. Copper concentrations were determined using AA for all the 2-week and 6-week samples (refer to Section 2.5.).

The light microscope photographs do not seem to correlate with the staining by the copper stains, to the concentration of copper in the tissues. Haywood (1985) observed that the stainable copper content did not always correlate well with the copper concentration measured by chemical analysis (refer to Section 2.5.). This was most evident when liver copper concentrations were high and yet the stains demonstrated little copper. Similar findings have been reported in Wilsons disease, where copper concentrations are high in the initial phase of the disease, yet copper stains failed to demonstrate the metal (Jain *et al.*, 1978; Pilloni *et al.*, 1998). The 6-week kidney copper treated samples exhibited higher copper concentration compared to the 6-week kidney copper/melatonin treated samples (refer to Section 2.5.). Thus, it appears that melatonin could have a beneficial effect on the kidney in excreting the copper.

Rats are rather tolerant to copper loading. Initially, the rats receiving copper exhibit all the signs of acute copper toxicity, but as time progresses, the rats induce MT and Cp production. This aids in intercellular copper chelation, and decreases the potential of further copper toxicity. Liver, kidney and intestines are able to induce MT production when faced with initial copper loading (Haywood, 1985; Fuentealba *et al.*, 1993). On continuous copper exposure, additional protective mechanisms are activated. The copper-MT complexes are incorporated into lysosomes, which then release the complex into biliary ducts for excretion. Other chelating molecules e.g. GSH, can remove the copper

and prevent its destructive cellular effects (Fuentealba *et al.*, 1993). Melatonin, which has been shown to interact with Cu^{2+} *in vitro* (Limson *et al.*, 1998), possibly interacts with copper in vivo, as the copper/melatonin 2-week and 6-week copper liver and kidney concentrations, are lower than the corresponding copper treated samples.

Further studies need to be conducted on copper-specific stains for electron microscopy, so that the copper can be visually correlated to the cellular damage. Energy dispersive X-ray microanalysis, need to be performed to determine the copper content in the lysosomes.

CHAPTER 4

IN VITRO INVESTIGATIONS OF THE INTERACTION BETWEEN COPPER AND MELATONIN

4.1. INTRODUCTION

Copper is located in Group IB on the periodic table, between nickel and zinc. The chemical properties of copper allow it to exist in three stable oxidation states: Cu^0, Cu^{1+} and Cu^{2+}. The cuprous ion (Cu^{1+}) rapidly disproportionates in aqueous solutions to cupric (Cu^{2+}) and metallic copper (Cu^0). Cupric compounds are often blue or green in colour (Barceloux, 1999).

Copper is an essential trace and transition metal. It is the third most abundant trace element found in the body, after iron and zinc. Transition metal elements are characterized by having partially filled *d* orbitals in many of their compounds. Copper has the ability to mediate electron transfer by valence-shuttle mechanisms. The biological functions of copper are intimately related to its properties as a transition metal (Malmstr`m and Leckner, 1998).

Copper has been found to be involved in the neuropathology of neurodegenerative disorders e.g. Wilsons disease, Menkes disease, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Prion disease (Sayre *et al.*, 1999; Waggoner *et al.*, 1999).

Copper overload results in many pathologic conditions that are consistent with oxidative damage to membranes or molecules. Copper ions are active in oxidation-reduction reactions. Cu^{1+} is also known to generate free radicals by a Fenton type reaction. Copper ions are able to catalyze the formation of hydroxyl radicals via the Haber-Weiss reaction (in vitro):

$$O_2^{\cdot-} + Cu^{2+} \rightarrow O_2 + Cu^{1+} \quad \text{… Equation 1.1}$$

$$Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^{\cdot} \quad \text{… Equation 1.2}$$

Cu^{2+} can also react with hydrogen peroxide to yield Cu^{1+} and superoxide radical. The Cu^{1+} can then partake in the reaction (Equation 1.2).

The cupric state (Cu^{2+}) is found most often in biological systems and copper plays a vital role as a cofactor and as an allosteric component of several cuproenzymes (Uauy, 1998; Frieden, 1985). The cuproenzymes operate by means of a redox cycle, the 'valence shuttle' hypothesis, which involves the oxidation of the substrate by Cu^{2+} (with its own reduction to Cu^{1+}). Cu^{2+} is subsequently regenerated from Cu^{1+}, using molecular oxygen as the oxidizing agent (Phipps, 1976). Copper also functions in different redox enzymes and is thus essential for normal physiologic function such as cellular respiration, free radical defense, synthesis of melanin pigment, connective tissue biosynthesis and cellular iron metabolism (Uauy, 1998).

Glutathione (GSH), an important biological peptide, plays an important role in postabsorptive copper transport. It has been postulated that GSH forms an intermediary complex with copper in the erythrocyte, before copper is transferred to other proteins e.g. superoxide dismutase and metallothionein (Freedman, 1989; Harris, 1991).

GSH reduces Cu^{2+} to Cu^{1+}, which can then result in copper binding to DNA. GSH is found in high concentrations within the nuclei and has been shown to actually inhibit the free radical formation caused by Cu^{1+}. GSH stabilizes the copper in the Cu^{1+} state, and prevents it from participating in free radical generation. Thus, GSH may protect against copper-induced DNA damage (Milne *et al.*, 1993). GSH is required for biliary excretion of copper in adult rats (Houwens *et al.*, 1990). However, the GSH system may become saturated in copper-overloading, thus resulting in free Cu^{1+} interacting with intracellular organelles, resulting in peroxidation and DNA damage.

It has been suggested that the majority of cytoplasmic copper is complexed to GSH as Cu^{1+} (Freedman, 1989). Copper can then be donated from the Cu^{1+}-GSH complex to various other intracellular proteins, like metallothionein (MT) (Ciriolo, 1990; Ferreira, 1993; Musci, 1996).

Amino acids form complexes with the copper, and facilitate copper absorption (Kirchgessner, 1970). Cysteine is an effective chelating agent for copper, but it produces a reduction in bioavailabity, probably due to the reduction of copper from a divalent to a monovalent state (Baker, 1987).

Copper has a filled-shell d^{10} configuration that allows it to more likely form covalent bonds. Copper compounds in this oxidation state are diamagnetic. The formation of the Cu^{2+} ion is due to the disturbing of its d^{10} configuration and its stability depends largely on its greater hydration energy of the +2 cation. Due to its higher charge and lower ionic radius, Cu^{2+} is less easily distorted than Cu^{1+}. It forms a wide variety of stable complexes with sulphur, nitrogen and oxygen donor ligands. Its tendency to bind B-bonding ligands, is small. The d^9 electronic configuration of Cu^{2+} allows it to be arranged in the octahedral splitting pattern. This configuration leads to a Jahn-Teller distortion, which impacts on the stereochemistry of Cu^{2+} complexes (Phipps, 1976).

Kroneck *et al.* (1980) reported that copper coordination in biological systems depended on the deprotonation of the NHCO of amino acids. Various amide ligands were used to study the Cu^{2+}-NHCO interactions. The copper ion must be coordinated to a primary site of sufficient unidentate Cu^{2+} affinity e.g. the imidazole nitrogen of a histidine sidechain, mercaptide sulphur or an amino nitrogen.

Sugiura (1977) studied the coordination of Cu^{1+} with mercaptide sulphur. Mercaptide sulphur is the most potently relevant biological ligand for Cu^{1+} complexes. It was reported that sulphydryl and imidazole groups are adequate ligands for Cu^{2+} and Cu^{1+}.
Miller *et al.* (1990) report that chelators with nitrogen atoms that primarily bind the metal (like melatonin), prefer the reduced forms of iron or copper, and tend to increase the redox potential of the metal.

Limson *et al.* (1998) reported that melatonin binds/complexes with heavy metals: copper (Cu^{2+}), aluminium, zinc, cadmium, iron and lead. These experiments were performed *in vitro* utilizing adsorptive cathodic stripping voltammetry. The results indicate that melatonin shows binding

affinity for copper and aluminium. This may indicate that melatonin, besides acting as an antioxidant, may bind these metals and prevent them from partaking in free radical production. It is also important to note that any free metal ions in the brain are toxic, and may participate in free radical production, especially copper and iron. Thus, melatonin may function as a metal ion binder, and thus remove "free" metal ions from participating in the generation of free radicals. Antunes *et al.* (1999) state that melatonin is only likely to form a single coordinated bond through its ring nitrogen atom, with the metal ion. This would result in melatonin's decreased ability to act as a preventative antioxidant, unlike other multidentate metal ion chelators (e.g. α-tocopherol).

Melatonin's ability to bind/chelate Cu^{2+} and Cu^{1+} ions are further explored, through the use of electrochemical, nuclear magnetic resonance (NMR) and infrared (IR) spectroscopic methods. The electrochemical studies were performed in collaboration with Dr. J. Limson.

4.2. Materials and methods

4.2.1. Chemicals and reagents

Melatonin, hexafluorophosphoric acid (HPF_6) and d^3-actonitrile were purchased from Sigma Chemical Co., St. Louis, MO, USA. Cupric chloride (Cu^{2+}) and cuprous oxide (Cu^{1+}) were purchased from Saarchem (Pty) Ltd, Krugersdorp, South Africa. Tetrakis (acetonitrile) copper (I) hexafluorophosphate $[Cu(CH_3CN)_4]PF_6$ was manufactured in the laboratory with Cu_2O and HPF_6 (section 4.2.2.). Acetonitrile-d_3 was purchased from Aldrich Chemical Company, Inc., Milwaukee, USA. All other reagents were obtained from local sources and were of the highest purity available.

4.2.2. Synthesis of $[Cu(CH_3CN)_4]PF_6$

The synthesis by Kubas (1979), of tetrakis (acetonitrile) copper (I) hexafluorophosphate was used. This procedure was carried out in a well-ventilated fumehood, due to the toxicity of the acetonitrile and HF fumes evolved from the HPF_6. 4.0g (28 mmoles) of cuprous oxide was reacted with 80 ml of acetonitrile in a 125 ml Erlenmeyer flask, under magnetic stirring. 10 ml of 60-65% HPF_6 (about 113 mmoles of HPF_6) was added slowly to the stirring suspension in 2 ml aliquots. The reaction is exothermic, which aids in the dissolving of the product. After the addition of the final aliquot of HPF_6, the solution was stirred for 3 minutes. The hot solution was then filtered through a medium-porosity frit, to remove small amounts of undissolved black solid. To the pale-blue solution, equal volume of dried diethyl ether was added, and the solution is cooled at 0°C for several hours, whereupon, the blue-tinged white microcrystalline precipitate of $[Cu(CH_3CN)_4]PF_6$ formed. The solid was collected by filtration, washed with diethyl ether and immediately redissolved in 100 ml of acetonitrile. A small amount of blue material (possibly Cu^{2+}) remains undissolved and was removed by filtration. 100 ml diethyl ether was added to the filtrate, and the mixture was allowed to stand at 0°C for several hours. A second recrystallization was carried out, if the precipitate retains a bluish cast, using 80 ml each of acetonitrile and diethyl ether. The filtered product ($[Cu(CH_3CN)_4]PF_6$) is pure white and was dried under *in vacuo* for 30 minutes. The yield was 12.5g (60%). The product was stored under N_2 atmosphere, as slight surface oxidation occurs if exposed to air for longer than 1 hour.

4.2.3. NMR investigations

NMR analysis determines the relative position of protons in a compound. When a complex is formed, chemical signal shifts are noted. Cu^{2+} is paramagnetic, and distorts the magnetic field, resulting in broadening of the signals, and therefore, unsuitable for NMR analysis. Cu^{1+} is diamagnetic and does not distort the signals. Standards of melatonin in acetonitrile-d_3 were run. The Cu^{1+} and Cu^{2+} complexes were then compared against the melatonin standards.

Cu^{1+} and Cu^{2+} were reacted with melatonin. The copper and melatonin were dissolved in 0.5 ml acetonitrile-d_3. 0.5 ml Cu^{1+} and Cu^{2+} (0.025M) was reacted with 0.5 ml melatonin (0.1M) in a 1:4 ratio. The melatonin was added in excess. The Cu^{1+} and Cu^{2+} were dissolved in 0.5ml acetonitrile-d_3 and then added to melatonin in a NMR tube. The resulting mixture was analysed within 2 days by NMR.

4.2.4. Infrared spectroscopy

IR analysis simplistically determines the functional group chemistry of the compound. The samples were scanned from 1000 cm^{-1} to 4000cm^{-1}. The IR was blanked with acetonitrile-d_3. The NMR samples were analysed by IR using a calcium fluoride (CaF) disk. A small aliquot of sample was drawn from the NMR tube, and placed onto the CaF disk. Care was taken to ensure that no air was trapped between the discs, before the sample was scanned. Standards of melatonin in acetonitrile-d_3 were run, against which, the complexes were compared.

4.2.5. Electrochemistry

Cyclic Voltammetry is used to characterise species in solution. Electroactive analytes in solution produce characteristic redox patterns when a potential window is scanned. Any change in the potentials or the intensity of current response are strong indications of new species in solution. Cyclic Voltammograms (CV) were recorded in a C2 cell stand using a glassy carbon electrode (GCE). Appropriate concentrations of Cu^{1+} and melatonin in Tris-HCl buffer were introduced into the glass cell, and degassed for five minutes with nitrogen before scanning a potential window.

Adsorptive Stripping Voltammetry has been used with success to examine metal-ligand complex formation at an electrode. This technique relies on the natural tendency of analytes to preconcentrate at the surface of a working electrode. Theoretically, the introduction of a successful ligand to a metal solution will increase preconcentration of the metal at the electrode if it forms a metal-ligand complex. The increase in preconcentration of the metal, as a metal-

ligand complex, results in an increase in the current response due to the metal reduction. A shift in the reduction potential indicates that a new species is being reduced and may also be an indication of the strength of the metal-ligand complex. Acetate buffer (pH = 4.4) and appropriate concentrations of Cu^{1+} and of the ligands (melatonin or glutathione), were introduced into an electrochemical cell. The solution was then deaerated with nitrogen for 5 min, after which a flow of nitrogen was maintained over the solution throughout the measurement. The metal complexes of melatonin, or glutathione are expected to have formed at this stage. An optimum deposition potential of 150 mV *vs* Ag/AgCl was applied for 60 seconds to effect the formation and adsorption of the metal complex onto the hanging mercury drop electrode (HMDE). The voltammograms were then scanned in the negative direction from 150 mV *vs* Ag/AgCl to –300 mV *vs* Ag/AgCl at the scan rate of 100 mV s^{-1} to strip the adsorbed metal-ligand complex from the electrode. During the stripping step, current response due to the reduction of the metal complexes of melatonin or glutathione, were measured as a function of potential.

4.3. Results

4.3.1. NMR analysis

NMR analysis of the Cu^{1+} and Cu^{2+}-melatonin complexes, are depicted in tables 5 and 6. Melatonin's structural formula and a numbering scheme adopted for the NMR assignment, are depicted in Figure 46.

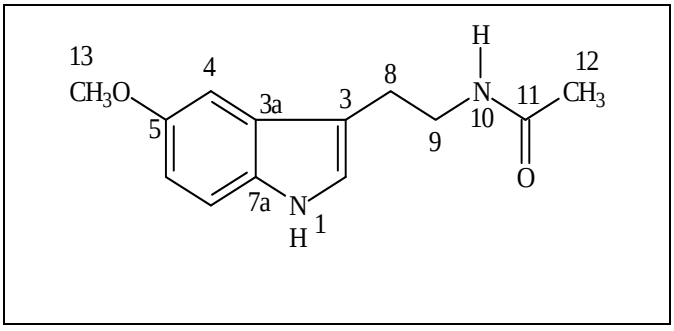


Figure 46. Melatonin's structure and numbering scheme

Table 5. NMR data of proton chemical shifts of melatonin, Cu^{1+} -melatonin 1:4 complex

and Cu^{2+} -melatonin 1:4 complex

Atom number	**Melatonin (ppm)**	**Cu^{1+} -melatonin 1:4 complex (ppm)**	**Cu^{2+} -melatonin 1:4 complex (ppm)**
1	8.95 s, NH	8.95 s, NH	8.95 s, NH
2	7.05 d, CH	7.05 d, CH	7.07 d, CH
4	7.08 d, CH	7.07 d, CH	7.07 d, CH
6	6.77 dd, CH	6.78 dd, CH	6.78 dd, CH
7	7.28 d, CH	7.28 d, CH	7.28 d, CH
8	2.85 t, CH_2	2.85 t, CH_2	2.88 t, CH_2
9	3.40 q, CH_2	3.40 q, CH_2	3.46 q, CH_2
10	6.38 s, NH	6.39 s, NH	6.92 s, NH
12	1.81 s, CO- CH_3	1.80 s, CO- CH_3	1.80 s, CO- CH_3
13	3.81 s, OCH_3	3.84 s, OCH_3	3.81 s, OCH_3

Table 6. NMR data of ^{13}C chemical shifts of melatonin, Cu^{1+} -melatonin 1:4 complex and Cu^{2+} -melatonin 1:4 complex

Atom Number	**Melatonin (ppm)**	**Cu^{1+} -melatonin 1:4 complex (ppm)**	**Cu^{2+} -melatonin 1:4 complex (ppm)**
2	124.1 HN-**C**=C	124.1 HN-**C**=C	124.3 HN-**C**=C
3	112.8 -C=**C**	112.8 -C=**C**	112.8 -C=**C**
3a	132.6 -**C**=C	132.6 -**C**=C	132.6 -**C**=C
4	113.3 =**C**-C	113.3 =**C**-C	112.9 =**C**-C
5	154.6 **C**–O CH_3	154.6 **C**–O CH_3	154.6 **C**–O CH_3
6	101.2 C=**C**-C	101.3 C=**C**-C	101.3 C=**C**-C
7	112.3 C-**C**=C	112.3 C-**C**=C	112.4 C-**C**=C
7a	128.9 C=**C**-C	128.9 C=**C**-C	128.7 C=**C**-C
8	22.98 **C**H_2-Ar	23.0 **C**H_2-Ar	22.3 **C**H_2-Ar
9	40.4 **C**H_2-N	40.4 **C**H_2-N	41.1 **C**H_2-N
11	170.4 –**C**=O	170.4 –**C**=O	172.3 –**C**=O
12	25.9 –C=O- **C**H_3	25.9 –C=O- **C**H_3	25.6 –C=O- **C**H_3
13	56.1 O-**C**H_3	56.1 O-**C** H_3	56.1 O-**C** H_3

NMR proton analysis (Table 5) indicates a chemical shift occurring at H_{10} for the Cu^{2+}-melatonin complex, which is the amide in the side chain. ^{13}C NMR analysis indicates (Table 6) chemical signal shifts at H_9 (**C**H_2 adjacent to the amide H_{10}) and atom 11 (carbonyl atom). At the carbonyl atom, the chemical shift is 1.9 ppm between the Cu^{2+}-melatonin complex and melatonin. The CH_2 next to the carbonyl atom has a chemical shift of 0.7 ppm. This indicates coordination occurring at the carbonyl of the melatonin structure with Cu^{2+}.

4.3.2. Infrared spectroscopy

The IR spectroscopic graphs of melatonin, Cu^{1+}-melatonin and Cu^{2+}-melatonin (Figures 47, 48 and 49, respectively) indicate a signal shift at 1671.8 cm^{-1}. The 1671.8 cm^{-1} represents the carbonyl. In the Cu^{1+}-melatonin IR spectrum, the carbonyl signal appears at 1671.8 cm^{-1}, and is split and shifted to 1633 cm^{-1}. This is also seen in the Cu^{2+}-melatonin complex, where the carbonyl has split and shifted to 1625 cm^{-1}. This indicates coordination taking place at the carbonyl. The 3392.4 cm^{-1} represents the NH in melatonin (Figure 47). There is no change in this signal in both the Cu^{1+}-melatonin (Figure 48) and Cu^{2+}-melatonin complexes (Figure 49), indicating that it is uncoordinated.

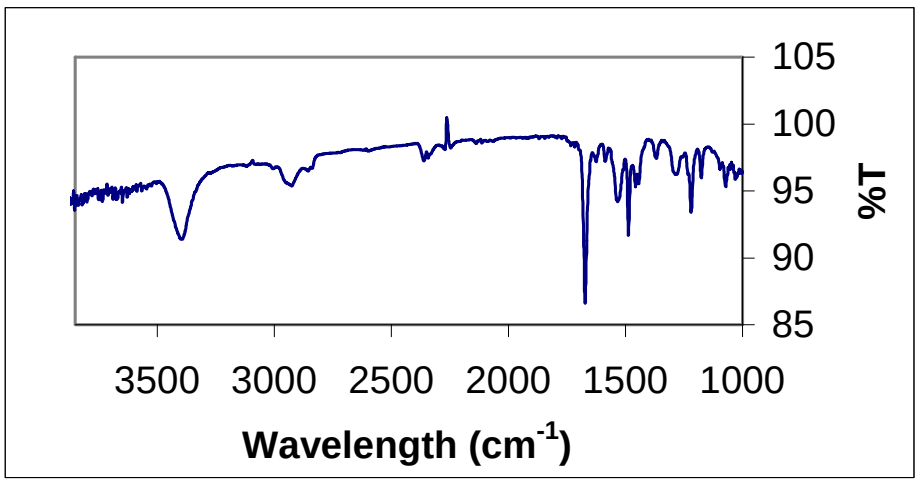


Figure 47. Infrared spectrum of melatonin

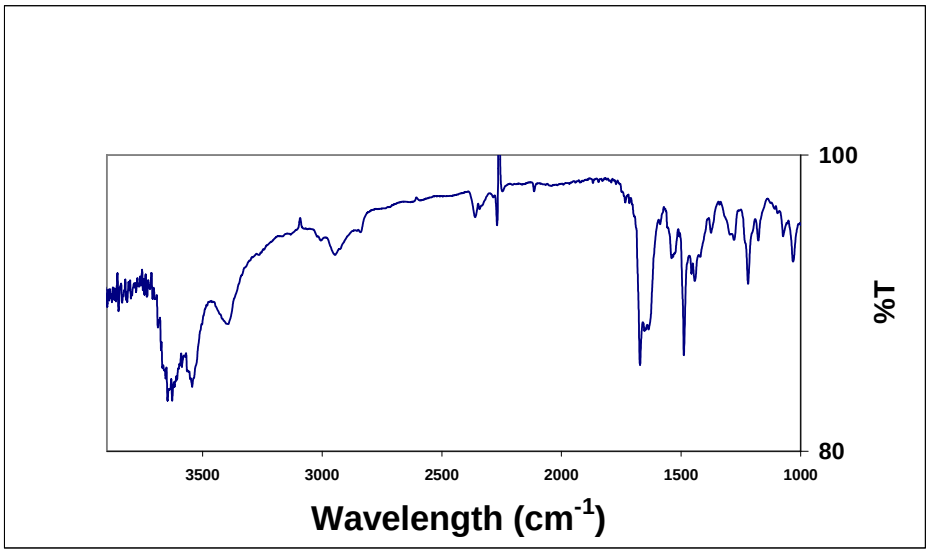


Figure 48. Infrared spectrum of Cu^{1+}-melatonin complex. The carbonyl peak at 1671.8 cm^{-1} is split and shifted to 1633 cm^{-1}.

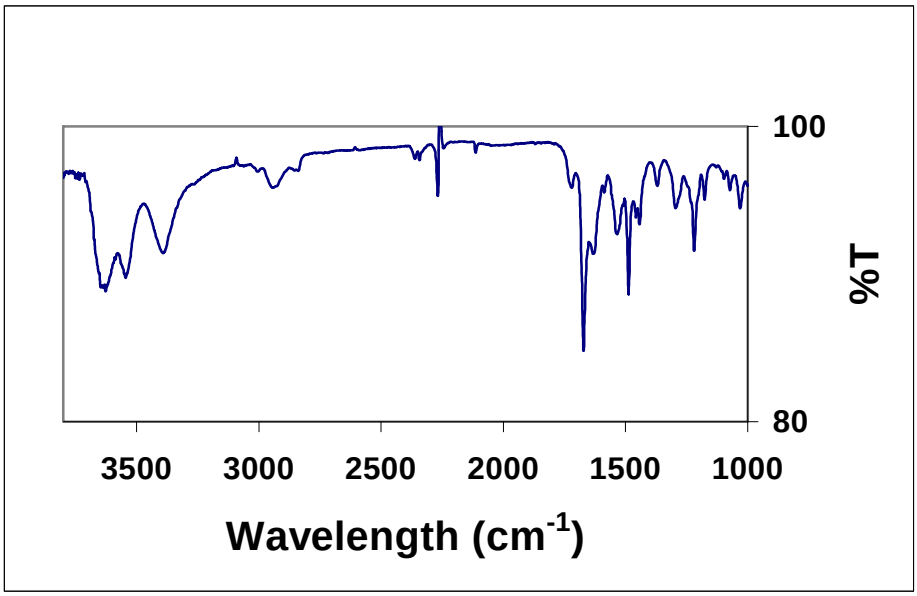


Figure 49. Infrared spectroscum of Cu^{2+}-melatonin complex. The carbonyl peak at 1671.8 cm^{-1} is split and shifted to 1625 cm^{-1}.

4.3.3. Electrochemistry

4.3.3.1. Cyclic Voltammetry (CV)

Figure 50, represents the CV of melatonin in the absence of Cu^{1+}. A peak due to the oxidation of melatonin is observed at 0.73 V *vs* Ag/AgCl. A very weak peak is observed on the return scan, corresponding to the reduction of melatonin. Upon several successive scans, a quasireversible couple with $E_{1/2}$ at 0.06 V *vs* Ag/AgCl appears, which may be related to the adsorption of melatonin at the electrode surface, as shown in Figure 51.

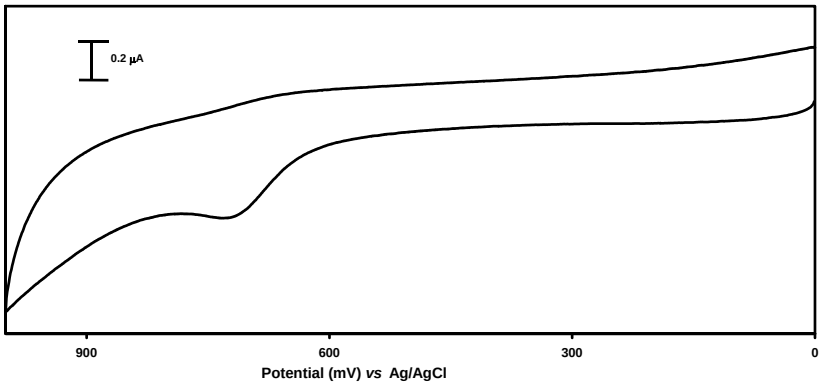


Figure 50. A cyclic voltammogram of melatonin (1×10^{-5} M) in the absence of Cu^{1+}

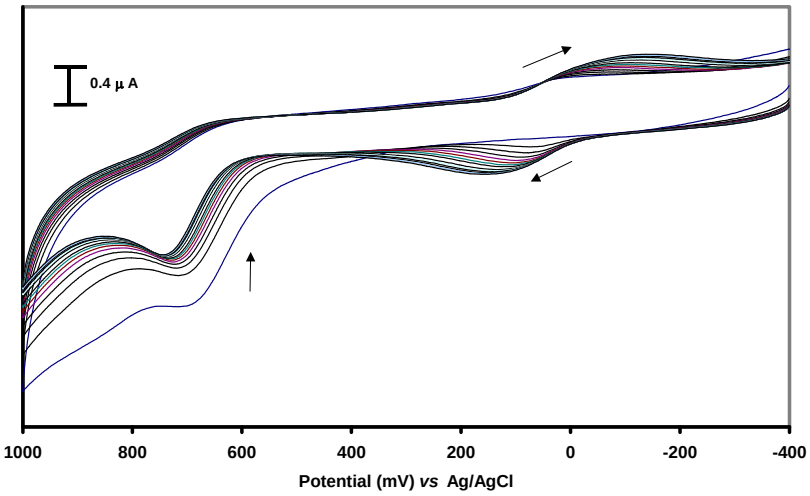


Figure 51. Successive cyclic voltammograms of melatonin (4×10^{-5} M), indicating a quasi-reversible couple at 0.06 V *vs* Ag/AgCl

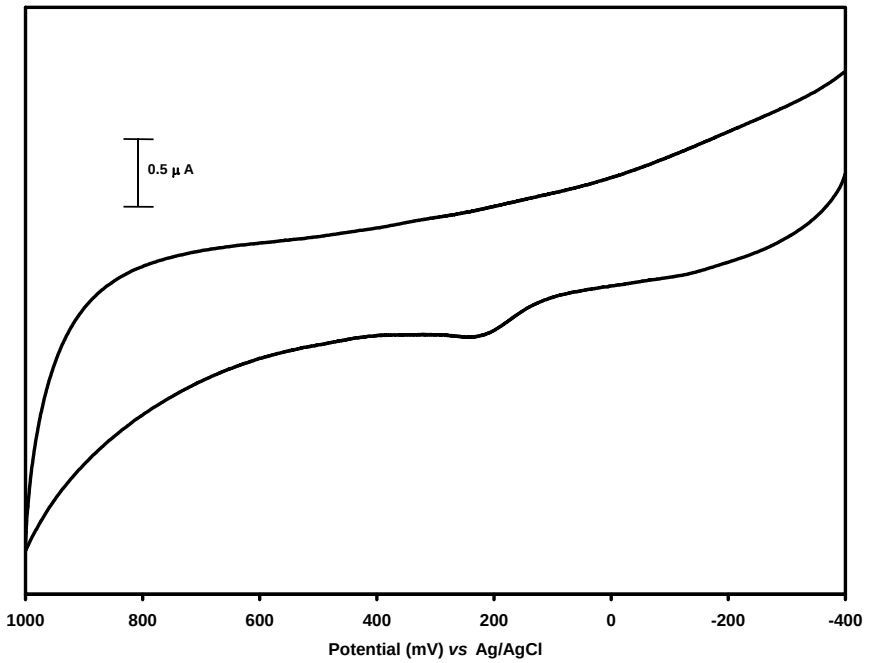


Figure 52. A cyclic voltammogram of Cu^{1+} (4×10^{-5} M) in the absence of melatonin

Figure 52 shows the CV of Cu^{1+} in the absence of melatonin. An irreversible oxidation peak is observed at 0.24 V *vs* Ag/AgCl. Upon several successive scans this peak does not show any significant increases, indicating that it is not being electrodeposited at the electrode.

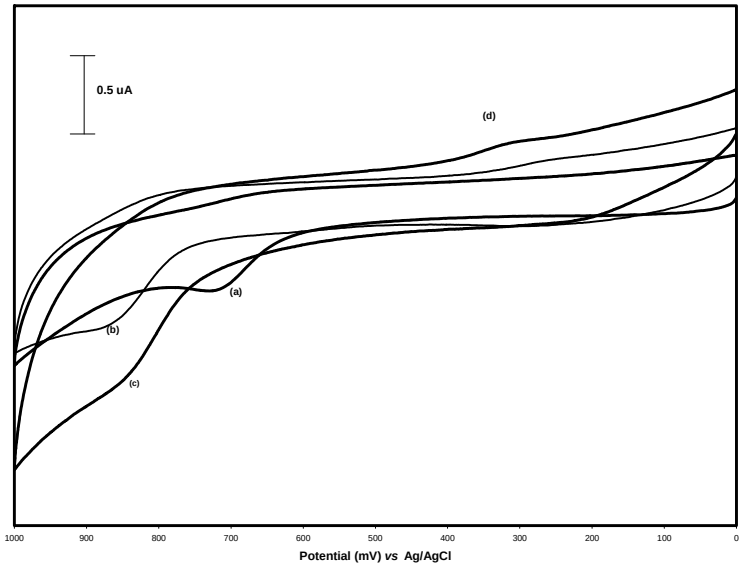


Figure 53. Cyclic voltammograms of (a) melatonin solution (1×10^{-5} M); (b) solution of melatonin (1×10^{-5} M) and Cu^{1+} (1×10^{-5} M), decrease in current strength from 0.73 V *vs* Ag/AgCl to 0.87 V Ag/AgCl in the presence of Cu^{1+}; (c) further increases in Cu^{1+} (3×10^{-5} M) results in a decrease in current strength, and disappears in the presence of melatonin; (d) a new peak is observed at 0.31 V Ag/AgCl.

Figure 53(a), shows the CV obtained for a solution of melatonin (1×10^{-5} M). Figure 53(b), shows the CV obtained for melatonin (1×10^{-5} M) and Cu^{1+} (1×10^{-5} M) in solution. The peak due to the oxidation of melatonin decreases in current strength and shifts from 0.73 V *vs* Ag/AgCl to 0.87 V *vs* Ag/AgCl in the presence of Cu^{1+}. Further increases in Cu^{1+} concentration (3×10^{-5} M) led to further decrease in the current response due to melatonin, Figure 53(c). The weak peak observed for Cu^{1+} in solution decreases in current strength and disappears in the presence of melatonin. In Figure 53(d), a new, but weak peak is observed on the return scan at 0.31V *vs* Ag/AgCl.

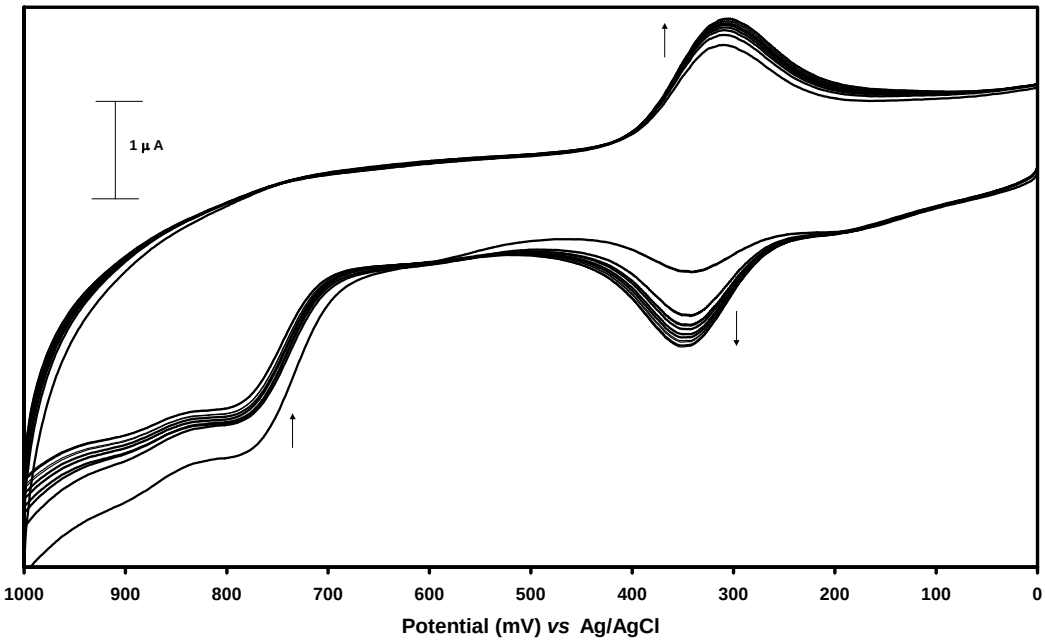


Figure 54. Cyclic voltammogram of a solution of Cu^{1+} (1×10^{-3} M) and melatonin (4×10^{-3} M). A new quasi-reversible couple is noticed at 0.34 V *vs* Ag/AgCl

Upon several successive scans of a solution of melatonin (4×10^{-3} M) and Cu^{1+} (1×10^{-3} M) we observe the appearance of a new quasi-reversible couple (Figure 54) at 0.34 V *vs* Ag/AgCl. With increasing scan number these peaks increase in strength, while the peak due to melatonin oxidation at 0.79 V *vs* Ag/AgCl decreases. This indicates the formation of a new compound electrodeposited at the electrode. The decrease in melatonin current response and disappearance of the peak observed for Cu^{1+} alone in Figure 52(c), and the increase in current strength of the couple at 0.34 V *vs* Ag/AgCl, indicates that both melatonin and Cu^{1+} are being consumed in the synthesis of the couple. After several scans, this electrode was rinsed in Tris-HCl buffer and placed in a fresh Tris-HCl buffer. Figure 55(b), shows the CV obtained. No peak due to the oxidation of free melatonin in solution is observed at 0.87 V *vs* Ag/AgCl. The redox couple observed 0.071 V *vs* Ag/AgCl is stable upon multiple scans of the electrode as shown in Figure 55(c). The electrode was allowed to dry and a blue-purple film was observed at the electrode.

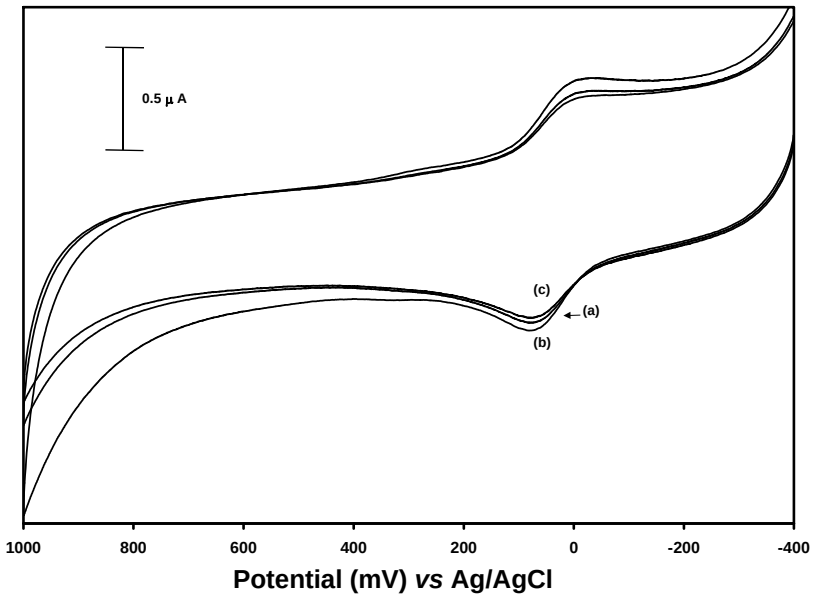


Figure 55. Cyclic voltammograms of melatonin (4×10^{-3} M) and Cu^{1+} (1×10^{-3} M) in Tris-HCL solution pH 7.3.

4.3.3.2. Adsorptive Stripping Voltammetry

Figure 56(a), shows the cathodic adsorptive stripping voltammogram (CSV) of Cu^{1+} (4×10^{-5} M) in the absence of melatonin. A peak due to the reduction of Cu^{1+} is observed at 0 V *vs* Ag/AgCl. Figure 56(b), shows the CSV obtained for Cu^{1+} (4×10^{-5} M) and melatonin (1×10^{-5} M). In the presence of melatonin, an increase in the Cu^{1+} reduction peak and a negative potential shift is observed. Increases in melatonin concentration (1×10^{-5} M to 4×10^{-5} M) increased the current response due to Cu^{1+} as shown in Figure 56(b), (c), (d), (e), respectively. For 0.04mM melatonin, a shift to –0.078 V *vs* Ag/AgCl is observed. The increase in current strength as well as the potential shift indicates the formation of a melatonin- Cu^{1+} complex.

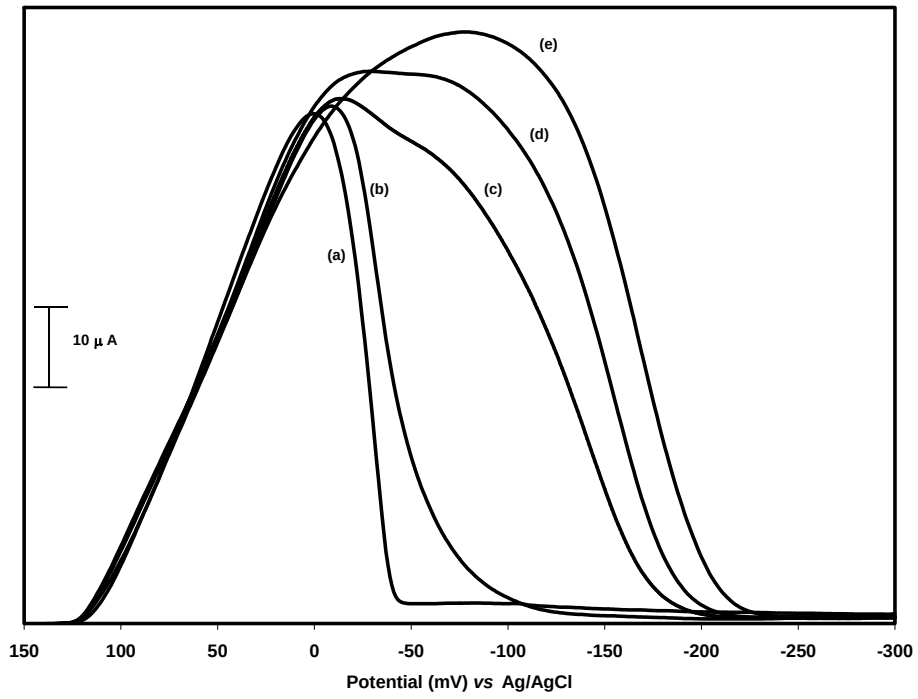


Figure 56. Cathodic adsorptive stripping voltammograms of Cu^{1+} and melatonin. (a) Cu^{1+} (4×10^{-5} M)in the absence of melatonin; (b) Cu^{1+} (4×10^{-5} M) in the presence of melatonin (1×10^{-5} M); (c), (d), (e) cathodic adsorptive stripping voltammograms of increasing melatonin concentrations (2×10^{-5} M to 4×10^{-5} M, respectively) in the presence of Cu^{1+}

As glutathione is known to bind Cu^{1+} we ran a similar set of experiments for Cu^{1+} with glutathione. Figure 57(a), shows the CSV for Cu^{1+} (4×10^{-5} M) in the absence of glutathione. As before for melatonin, the presence of glutathione (2×10^{-5} M), resulted in an increase in current strength for Cu^{1+} reduction accompanied by a strong negative potential shift. Figure 57(b), shows the current response observed and the strong negative potential shift to –0.091 V *vs* Ag/AgCl. Increase in glutathione concentration also increased the current response observed.

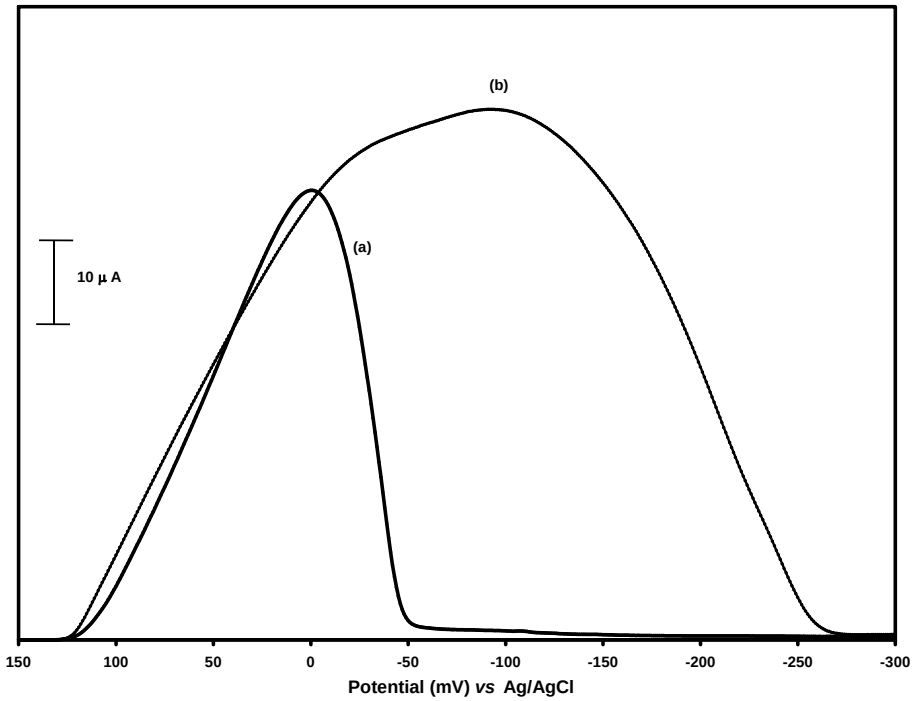


Figure 57. (a) Cathodic adsorptive stripping voltammogram of Cu^{1+} (4×10^{-5} M) in the absence of glutathione; (b) and of Cu^{1+} (4×10^{-5} M) in the presence of glutathione (2×10^{-5} M), which results in an increase in current strength for Cu^{1+} reduction

4.4. Discussion

The proton NMR indicates that melatonin interacts with Cu^{2+} at H_9 and H_{10} of the melatonin molecule. The chemical shift for the Cu^{2+}-melatonin complex at atom 10 is 6.92 ppm compared to 6.38 ppm for melatonin. The amide nitrogen (H_{10}) is deprotonated by the Cu^{2+}. The ^{13}C NMR, further elucidates the copper coordination to melatonin. The Cu^{2+} coordinates at the carbonyl, as the chemical shift of the Cu^{2+}-melatonin complex is 172.3 ppm as compared to 170.4 ppm for melatonin. The CH_2 chemical shift (H_9) is also changed for the Cu^{2+}-melatonin complex (41.1 ppm) as compared to melatonin (40.4 ppm). From the NMR data, it appears that melatonin coordinates preferably with Cu^{2+}, rather than with Cu^{1+}. There was no chemical shifts noted between the melatonin and Cu^{1+}-melatonin complex sample, indicating that no interaction

occurred. Cu^{2+} prefers binding to oxygen, and that is clearly seen in the NMR and IR. Cu^{1+} prefers binding to nitrogen and then oxygen, and this is seen in the electrochemistry, as Cu^{1+} is forced to bind through one of the nitrogens on the melatonin.

The IR analysis of melatonin, Cu^{2+}-melatonin and Cu^{1+}-melatonin complexes, indicate that no chemical shifts are noted for the amide nitrogen. In the Cu^{2+}-melatonin and Cu^{1+}-melatonin complexes, the carbonyl chemical signal is unchanged (1671.8 cm^{-1}), but the signal is split and shifted (1625 cm^{-1} and 1633 cm^{-1}, respectively). This indicates that the copper (Cu^{2+} and Cu^{1+}) coordinates at the carbonyl, and not at the amide nitrogen (H_{10}).

It has been shown previously that Cu^{2+} forms a complex with melatonin (Limson *et al.*, 1998). In these experiments, cyclic voltammetric and adsorptive stripping voltammetric experiments indicate strongly that, that melatonin forms a complex with Cu^{1+}. From these experiments we suggest that the bond is fairly strong but it is not possible to say whether it is simple ligation or whether it is chelation. In a separate set of adsorptive stripping voltammetric experiments, it was observed that at low melatonin and high Cu^{1+} concentrations, a sharp decrease in Cu^{1+} response was observed in the presence of melatonin (Figure 58(a)–(d)). This decrease did not appear to be due to competition by melatonin for sites at the electrode as increased concentrations of Cu^{1+} increased the Cu^{1+} reduction current response of Figure 58(d). The sharp decrease may be indicative of chelation of Cu^{1+} by melatonin. Ethylenediamine tetraacetate (EDTA) which is known to chelate Cu^{1+} brings a sharp reduction in metal current responses during adsorptive stripping experiments (Freedman *et al.*, 1989). This happens because Cu^{1+} is not released by the EDTA during the stripping step. At low melatonin concentrations this may be the case, but the way in which melatonin binds Cu^{1+} can only be verified by other methods.

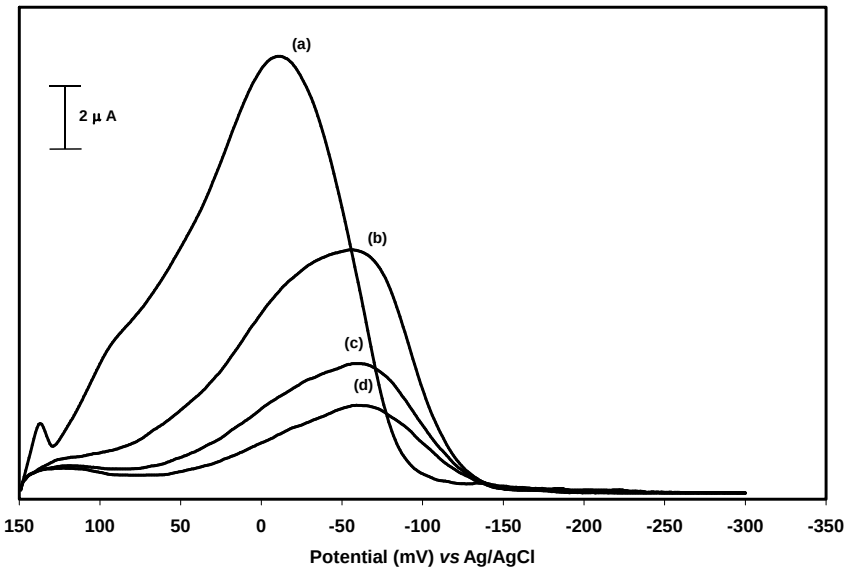


Figure 58. Adsorptive stripping voltammograms of high Cu^{1+} concentrations (6×10^{-5} M) in the presence of low melatonin concentrations ((a) 5.4×10^{-6} M, (b) 1.08×10^{-6} M, and (c) 2.16×10^{-6} M, melatonin). A sharp decrease in Cu^{1+} response was observed in the presence of melatonin.

These studies indicate that melatonin binds/chelates Cu^{1+}. Melatonin may have a potential benefit in copper accumulation diseases, where copper in excess partake in neurodegenerative processes. *In vitro*, it has been demonstrated that melatonin interacts with copper (Cu^{2+} and Cu^{1+}). Further studies need to be performed to investigate the coordination of melatonin with copper. *In vivo* studies need to be performed to determine if the copper-melatonin complex is formed.

CHAPTER 5

PINEAL ORGAN CULTURE STUDIES: THE EFFECT OF COPPER ADMINISTRATION ON RAT PINEAL INDOLE METABOLISM

5.1. Introduction

The pineal gland is responsible for the synthesis of indolamines (Reiter, 1989). Pineal indole synthesis and metabolism is discussed in section 1.2.4. The organ culture technique is able to quantify the pineal indoles and its metabolism, as it is sensitive and able to mimic normal physiological processes and conditions. This technique enables the neuroscientist to finely control experimental conditions and avoid complications of *in vivo* interactions.

Under optimum conditions, the pineal gland is able to remain viable in organ culture for as long as six days. The pineal gland is able to utilize radioactive (^{14}C)serotonin and (^{3}H)tryptophan to produce the various indoles, including melatonin and serotonin (Daya *et al.*, 1989). More than 95% of the synthesised indoles are secreted into the culture medium, which can then be isolated and quantified (Klein and Notides, 1969).

A bi-directional thin layer chromatograph system is employed to isolate the pineal indoles. This system utilizes two organic solvents. The first solvent, chloroform: methanol: glacial acetic acid (93:7:1), is used to separate melatonin (aMT) from N-acetylserotonin (aHT), and the 5-hydroxyindoles from the 5-methoxyindoles. The second solvent, ethyl acetate, is used to separate 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) from aMT, as well as 5-hydroxyindole acetic acid (HA) and 5-hydroxytryptophol (HL) from aHT. Tryptophan, serotonin (HT) and 5-hydroxytryptophan, and 5-methoxytryptamine are not affected by either of the solvents

and remains at the origin. The TLC technique is a rapid, simple and effective method of separating trace quantities of the pineal indoles.

The pineal gland contains high concentrations of copper, zinc and manganese, as compared to other anatomical brain structures (Wong and Fritze, 1969). PINA, a novel splice variant of the ATP7B gene disrupted in Wilsons disease, is expressed in pinealocytes. It is suggested that PINA may function as a copper transporter in rat pinealocytes, and studies by Borjigin *et al.* (1998; 1999), suggest a potential role of rhythmic copper metabolism in pineal and/or retinal circadian function. PINA expression patterns and regulation *in vitro* and *in vivo*, parallel that of NAT expression. LEC rat pineals (where PINA is deleted) display a defect in NAT protein and activity, but this defect is due to a germ-line mutation in the NAT gene, and is independent to the PINA mutation (Borjigin *et al.*, 1999). The high copper concentration in the pineal gland may be linked to the regulation of NAT and PINA genes. Brain copper concentrations in patients dying of neurological Wilsons disease is higher than that of similar patients with hepatic Wilsons disease (Walshe and Gibbs, 1987).

5.2. Materials and methods

5.2.1. Animals

Male Wistar rats weighing 250-300g were used in the experiments. The rats were randomly assembled into five groups of 5 rats per cage, and were maintained as described in section 2.2.1.1. The control group (n=10), consisted of 2 groups of 5 rats that received the drug vehicle, ethanol:0.9% saline (40:60), for 2-weeks and 6-weeks, respectively. The two copper treated group (n=5) received 2mg/kg Cu^{2+} (copper chloride-$CuCl_2$) for 2-weeks and 6-weeks, respectively. The copper/melatonin treated groups received Cu^{2+} 2mg/kg and melatonin 12mg/kg for 2-weeks and 6-weeks, respectively. The rats were injected daily, with the vehicle, Cu^{2+} and Cu^{2+}/melatonin. Copper/melatonin groups were injected on opposite intraperitoneal sites, to prevent any interaction of the copper with the

melatonin. The rats were sacrificed on day 15 (for the 2-week group) and day 43 (for the 6-week group), respectively. The pineal glands were removed as discussed in section 2.2.1.2.

5.2.2. Chemicals and reagents

(^{3}H) tryptophan (specific activity 55mCi/ml) was obtained from Amersham International, England. The culture medium, BGJb culture medium, was purchased from Gibco, Europe, and fortified with the antibiotics streptomycin and benzyl penicillin (Hoechst, South Africa). The aluminium TLC plates coated with silica gel 60, Type F254 (0.25mm), were purchased from Merck, Darmstadt, Germany. Beckman Ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd., Scotland. The indole standards, MT, HA, HL, MA, ML, aMT, and aHT were purchased from Sigma Chemicals Co, St Louis, MO, USA. All other reagents and chemicals were obtained locally and were of the highest purity available.

5.2.3. Organ culture of rat pineal gland

The pineal glands were placed individually into sterile 75 × 10mm Kimble tubes containing 52µl BGJb culture medium. 8µl of (^{3}H) tryptophan (specific activity 55mCi/ml) was added to each tube. The tubes were then saturated with carbogen and sealed. The tubes were then placed at 37°C in the dark for 24 hours in a Forma scientific model 3028 incubator. After the 24 hour incubation period, the reaction was terminated by the removal of the pineal glands from the solution.

5.2.4. Separation of the indoles using TLC

A modification of the technique employed by Klein and Notides (1969), was used to separate the radio-labelled indoles. 10 × 10cm TLC plates were activated by placing them

in an oven at 100 °C for 10 minutes. 5µl of the culture medium was spotted on a plate and, with the aid of a gentle stream of nitrogen, the spot was dried. 10µl of the standard solution was spotted onto the culture medium, and dried with a gentle stream of nitrogen. The standard solution was prepared as follows: 0.1mg of each standard indolamine was dissolved in a test tube containing 95% ethanol and 1% ascorbic acid. The ascorbic acid serves as an antioxidant. The plates were spotted under subdued light to prevent photo-oxidation of the indolamines.

The plates were then placed in a TLC tank containing chloroform: methanol: glacial acetic acid (97:7:1). The plates were developed twice in this direction, and allowed to develop until the solvent front had migrated approximately 9 cm. The plates were allowed to dry, and then placed at 90° (right angles) to the first direction of development, to develop once in ethyl acetate. Following this, the plates were allowed to dry, and then sprayed with Van Urks reagent, to allow for the visualization of the developed spots. Van Urks reagent is prepared by adding 1g 4-dimethylamino-benzaldehyde to 50ml 25% HCL, followed by the addition of 50ml 95% ethanol. The sprayed plates were subsequently dried in an oven at 60°C for 10 minutes to allow for the colour development of the spots. The spots were then cut and individually placed into scintillation vials. To the vials, 1ml absolute ethanol and 3mls of Beckmans Ready-Sol scintillation fluid was added. The vials were vortexed on a Vortex rotor mixer for 30 seconds. The radioactivity of each metabolite was then measured in a Beckman LS 2800 scintillation counter

5.3. Results

A typical bi-directional thin layer chromatogram of the pineal indole metabolites was obtained, as represented by Figure 59. Excellent separation of the indoles was achieved. The results (an average of three estimations) obtained are expressed as DPM/10µl/pineal gland (mean ± SEM) for each indole. The background counts were negligible. The data was statistically analysed and the difference between the control and copper groups, and between the copper and copper/melatonin groups, was determined using the Student-

Newman-Keuls test. Groups compared were accepted to be statistically significant at $p<0.05$. As shown in Table 7, copper/melatonin administration, significantly decreases ($p<0.05$) pineal (^3H) 5-methoxytryptophol synthesis in the 2-week copper treated rat pineals. It appears that melatonin administration decreases the ML synthesis, as compared to the control and copper pineals.

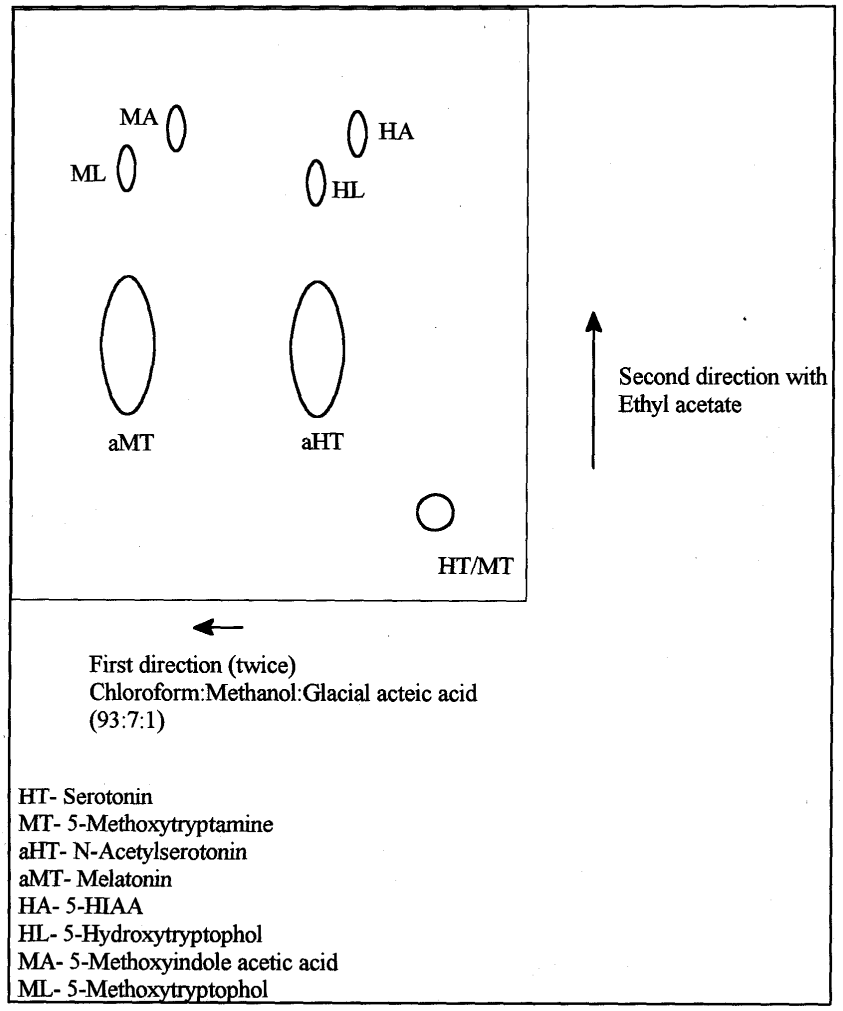


Figure 59. A trace of the TLC plate illustrating the direction in which the plate was developed and the location of the pineal indole metabolites (Klein and Notides, 1969)

The 6-week copper treated rat pineals (Table 8) show a statistical difference between the aHT control and copper pineals ($p<0.01$). A statistical difference between copper aHT and copper/melatonin aHT ($p<0.01$) is seen, which indicates that copper administration decreases the aHT pineal synthesis. The melatonin administration in the copper/melatonin experiments appears to reverse the effect of copper, as the values obtained are similar to the control. The copper and copper/melatonin administration appears to increase the ML synthesis, as compared to the control ($p<0.01$).

Table 7. The effects of 2-week copper treatment on rat pineal indole metabolism. The control ML is statistical significant between the copper/melatonin ML at $p<0.01*$. The copper/melatonin ML is statistical significant between the copper ML at $p<0.01*$.

Pineal Metabolites	**Control (DPM/10μl/pineal) Mean ± SEM (n=3)**	**Copper (DPM/10μl/pineal) Mean ± SEM (n=3)**	**Copper/Melatonin (DPM/10μl/pineal) Mean ± SEM (n=3)**
N-acetyl serotonin (aHT)	25190 ± 2661	34929 ± 8737	22804 ± 8154
5-hydroxyindole acetic acid (HA)	1275 ± 171	1612 ± 254	2067 ± 279
5- hydroxytryptophol (HL)	4311 ± 155	3432 ± 410	3151 ± 421
Melatonin (aMT)	5843 ± 593	6885 ± 31	5157 ± 768
5-methoxyindole acetic acid (MA)	10847 ± 954	12336 ± 1213	8856 ± 999
5-methoxytryptophol (ML)	36930 ± 7083	31390 ± 7018 *	13392 ± 2145*

Table 8. The effects of 6-week copper treatment on rat pineal indole metabolism. The control ML is statistical significant between the copper ML at $p<0.01*$. The control ML is statistical significant between the copper/melatonin ML at $p<0.01*$. The copper aHT is statistical significant between the copper/-melatonin aHT at $p<0.01*$. The control aHT is statistical significant between the copper aHT at $p<0.01*$.

Pineal Metabolites	**Control (DPM/10μl/pineal) Mean ± SEM (n=3)**	**Copper (DPM/10μl/pineal) Mean ± SEM (n=3)**	**Copper/Melatonin (DPM/10μl/pineal) Mean ± SEM (n=3)**
N-acetyl serotonin (aHT)	15779 ± 3391	9453 ± 947*	18609 ± 1861*
5-hydroxyindole acetic acid (HA)	2347 ± 275	3534 ± 495	5191 ± 592
5- hydroxytryptophol (HL)	2776 ± 163	2318 ± 188	4687 ± 1015
Melatonin (aMT)	1613 ± 96	1364 ± 159	2957 ± 1329
5-methoxyindole acetic acid (MA)	4640 ± 781	7974 ± 2257	5014 ± 2061
5-methoxytryptophol (ML)	8714 ± 1453	18023 ± 3022*	17644 ± 1885*

5.4. Discussion

Pineal indole metabolism occurs in the pinealocyte. Tryptophan is taken up from the blood stream into the pinealocytes, where it is utilized in the synthesis of pineal indoles. Most of the tryptophan is converted to 5-hydroxytryptophan via tryptophan hydroxylase in the mitochondria (Hori *et al.*, 1976). In the synthesis of serotonin from tryptophan, this appears to be the rate-limiting step. Trytophan hydroxylase requires the presence of oxygen, ferrous iron, and a reduced pteridine co-factor to function. A high concentration of the pteridine co-factor is found in the pineal gland (Loveberg *et al.*, 1967; Levine *et al.*, 1979). 5-hydroxytryptophan is converted via L-amino acid decarboxylase to serotonin (5HT). This enzyme is located in the cytosol and requires a pyridoxal phosphate to function (Snyder *et al.*, 1965). Serotonin can undergo three different metabolic pathways (figure 9):

(1) It can be acetylated to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (NAT), with acetyl coenzyme A, a cofactor, being the acetyl donor (Weissbach *et al.*, 1960). N-acetylserotonin is the precursor of melatonin (Klein *et al.*, 1971). N-acetylserotonin is then converted to melatonin by O-methylation in the 5-position by the enzyme hydroxyindole-O-methyltransferase (HIOMT).

(2) It may undergo deamination and oxidation reactions, where serotonin is oxidized by the enzyme monoamine oxidase to 5-hydroxyindole acetaldehyde. 5-hydroxyindole acetaldehyde is an unstable intermediate and undergoes further metabolism (Axelrod *et al.*, 1969). The acetaldehyde is converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968). A proportion of the 5-hydroxyindole acetaldehyde is converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is the methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman and Axelrod, 1967).

(3) It may be methoxylated by HIOMT to form 5-methoxytryptamine. S-adenosylmethionine donates the methyl to the serotonin.

The rat pineal gland in organ culture is able to metabolise (^{3}H)tryptophan to various pineal indoles. From Table 7, the 2-week administration of copper did not affect the ML synthesis, whereas there was a statistical difference between the copper ML and copper/melatonin ML synthesis level ($p<0.01$). It appears that melatonin administration decreased ML synthesis, by probably interacting with the HIOMT.

In the 6-week pineal experiments (Table 8), the aHT level was decreased by copper and this was statistically significant at $p<0.01$ between the copper and control pineals. It appears that copper affects the NAT enzyme, resulting in a decrease aHT production from serotonin. When melatonin is administered with copper, the aHT levels are similar to that of the control pineals. This possibly indicates that melatonin is interacting with the copper and preventing the inhibition of the NAT activity. Further investigations are needed to determine the NAT activity in the copper and copper/melatonin administered pineals. The copper and copper/melatonin pineals were statistically different for the aHT levels at $p<0.01$.

The ML synthesis for the 6-week pineals showed a different picture to that of the 2-week pineals. Both the copper and copper/melatonin pineals for ML, were statistically different ($p<0.01$) to that of the control pineals. It appears that after 6 weeks, copper and copper/melatonin administration increased ML synthesis. Further investigations are needed to determine the HIOMT activity in the 6-week experiments with copper and copper/melatonin administration.

The pineal gland has a high concentration of copper (Wang and Fritze, 1969). Since PINA is a copper transporter in the pineal gland, and it's expression parallels that of NAT, it appears that at night when PINA activity is at its maximum (NAT levels peak at night), copper is rapidly transported into the pinealocytes. From the 6-week pineal organ culture experiments, aHT levels are decreased when copper is administered, and the aHT level approximates that of the control, when copper/melatonin is administered.

Copper may interact with NAT, through non-specific binding, and thus decreases the NAT activity. Copper may "poison" the NAT enzyme, rendering it unavailable to convert

serotonin to N-acetylserotonin, and thus in effect, decrease the level of melatonin produced.

On the scale of electrode potential of metals, copper has an electrode potential of +0.34 V, and iron −0.44 V (Steele, 1966). NAT has a Fe-S core, and in an environment of high copper concentration, copper could not possibly displace the Fe-S, due to the varied electrode potential difference between the copper and iron.

NAT activity has been measured in Prof. Borjigin's laboratory, with or without various concentartions of copper, and it has been found that NAT activity appears to be insensitive to elevated levels of copper. Also, these authors showed that melatonin synthesis and release is normal in LEC rats, a Wilsons disease model. Thus, it appears that elevated copper does not affect melatonin synthesis (personal communication).

Further studies need to be carried out to measure the concentration of copper within the pineal, and to correlate this with the levels of NAT and PINA activity, and to investigate any possible interaction between NAT and copper binding.

CHAPTER 6
ELECTRON TRANSPORT CHAIN
MITOCHONDRIAL ALTERATIONS DUE TO COPPER OVERLOAD

6.1. INTRODUCTION

Disorders of copper metabolism, such as Wilsons disease, lead to the accumulation of excess copper in the liver and associated hepatic injury (Sokol *et al.*, 1990; Sternlieb, 1980). If not treated, copper toxicity results with a "spill over" effect into the bloodstream, resulting in hepatolenticular degeneration, and eventually, death (Linder, 1996). Although copper is undoubtedly involved in the pathogenesis of Wilsons disease, the mechanism by which excess copper-induced cellular toxicity results, has not been fully established experimentally (Sternlieb, 1980).

Copper is a component of cytochrome *c* oxidase (complex IV), which is an integral part of the mitochondrial respiration chain. It is known that copper may substitute for iron in redox reactions that generate free radicals. Thus, copper accumulation might be associated with increased oxidative stress within tissues where copper concentrations are high (Halliwell and Gutteridge, 1989).

Mitochondrial function is impaired in copper-loaded rat livers, with a decrease in state 3 respiration and the respiratory control ratios in the mitochondria when several electron donors were used (Sokol *et al*., 1993). Mitochondrial electron transport proteins are also affected as analysis of the oxidoreductase activities indicate a reduction in complex IV (CCO) activity. Such changes in mitochondrial function contribute to hepatocellular dysfunction by reducing cellular energy charge, increasing mitochondrial leakage of calcium into the cytosol, or exposure of the cell to increasing amounts of superoxide generated by the disruption of normal electron flow (Bremner, 1998). These changes in mitochondrial function are due to oxidative stress.

Oxidative stress is defined as the imbalance between biochemical processes leading to the production of reactive oxygen species (ROS) and the cellular antioxidant cascade, causing molecular damage that can lead to a critical failure of biological functions and ultimately cell death (Sayre *et al.*, 1999).

The result of impaired mitochondrial function is the increased generation of free radicals, e.g. superoxide and hydroxyl radicals. These radicals are normally produced as by-products of oxidative metabolism. Mitochondria are known to be the most important physiological source of superoxide radicals in animal cells. Impaired mitochondrial function also impairs intracellular Ca^{2+} buffering. An increase in intracellular Ca^{2+} leads to increased free radical generation, and to the activation of nitric oxide synthase (NOS) (Beal, 1996).

In Wilsons disease patients, mitochondrial changes have been identified in hepatocytes, which include morphological abnormalities. It has been reported that, in Wilsons disease patients, that mitochondrial respiratory-chain activity in the livers are affected, with an associated deficiency of aconitase (Gu *et al.*, 2000).

6.2. MATERIALS AND METHODS

6.2.1. Chemicals and reagents

Malic acid, NAD, 2,6-dichlorophenol-indophenol (DPI) and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. Copper chloride was purchased from Saarchem (PTY) Ltd, Krugersdorp, South Africa. Tetrakis (acetonitrile) copper (I) hexafluorophosphate $[Cu(CH_3CN)_4]PF_6$ was manufactured in the laboratory with Cu_2O and HPF_6 (refer to Section 4.2.2.). All other reagents used, were of the highest purity available.

6.2.2. Animals

Adult male rats of the Wistar strain, weighing between 250-300g were used. The animals were housed in a controlled environment with a 12 hour light:dark cycle, and were given access to standard laboratory food and water *ad libitum*. The protocols for the experiments were approved by the Rhodes University Ethical Standards Committee.

6.2.3. Tissue preparation

Rats were killed by cervical dislocation and the livers were rapidly excised and homogenised (2.5% w/v) with 50mM potassium phospate buffer, pH 7.4.

6.2.4. Biological oxidation

A modification of the spectrophotometric technique described by Plummer (1971) was used to determine the activity of the liver mitochondrial electron transport chain. This is determined by the rate of reduction of the synthetic electron acceptor dye, 2,6-dichlorophenol-indophenol (DPI), in the presence of L-malate. Potassium phosphate buffer (0.1M, pH 7.4) was used as the assay buffer. Homogenate (6 ml) containing copper (10mM) was incubated at 37°C in a water bath for varying preincubation times (0 and 60 minutes). Following incubation, 1 ml homogenate was removed and added to a tube containing NAD (5mM), L-malate (0.1M) and DPI (1.5mM) and buffer. In the copper/melatonin reaction vessels, 10mM melatonin was added, and preincubated with the homogenate and copper. The final volume in the reaction vessel was 3 ml. The decrease in absorbance over a 5 minute period was read at 600nm, on a UV-vis spectrophotometer. Data is expressed as $\in$Abs. versus time.

6.3. Results

Copper in both Cu^{1+} and Cu^{2+} oxidation states was used, to determine which oxidation state of copper resulted in changes in electron transport. Figure 60 shows that in the presence of Cu^{1+}, electron transport was decreased, whereas with the Cu^{1+}/melatonin, an increase in electron transport was observed, at t=0. Melatonin alone, caused a decrease in electron transport, indicating that melatonin alone might protect against an increase in electron transport.

Rat liver homogenate preincubated for 60 minutes with Cu^{1+} and Cu^{1+}/melatonin (Fig.61) indicate that copper causes an increase in electron transport, with Cu^{1+}/melatonin causing a decrease in electron transport. Melatonin alone, as at t=0 (Fig. 60) causes a decrease in electron transport.

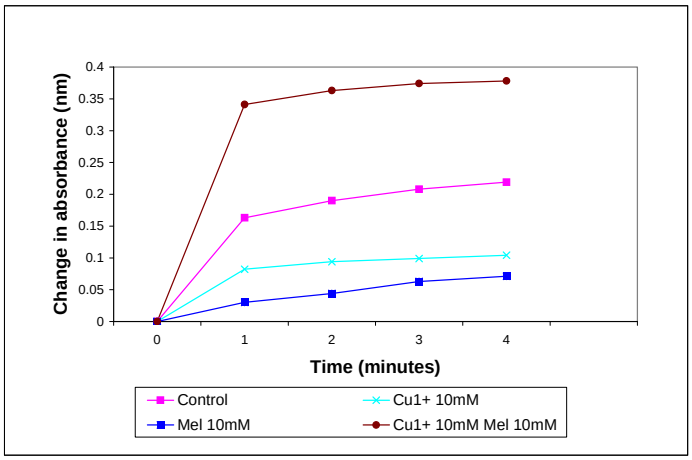


Figure 60. Rat liver homogenate preincubated with Cu^{1+} (10mM) and melatonin (10mM) at t=0.

The change in absorbance at t=60 is less than at t=0, indicating that electron transport is slower when the homogenate is preincubated for 60 minutes.

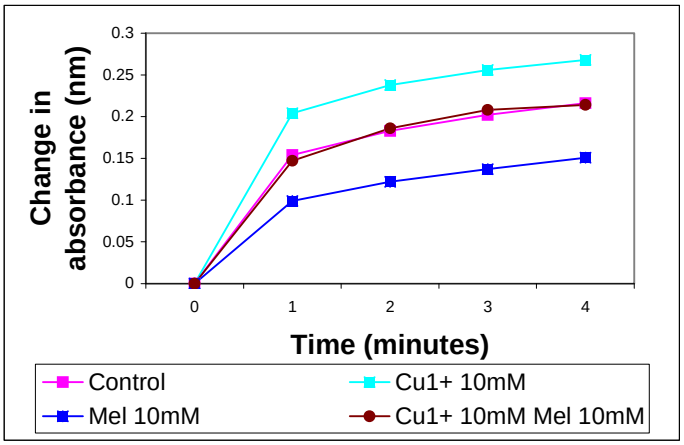


Figure 61. Rat liver homogenate preincubated with Cu^{1+} (10mM) and melatonin (10mM) at t=60.

Cu^{2+} decreases the electron transport, and Cu^{2+}/melatonin causes an even greater decrease in the electron transport (Fig. 62) at t=0. This indicates that melatonin when co-administered with Cu^{2+}, results in an interaction, resulting in a decrease in electron transport.

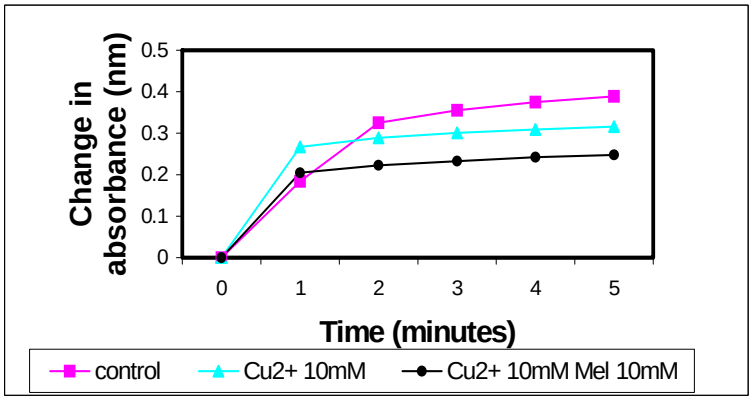


Figure 62. Rat liver homogenate preincubated with Cu^{2+} (10mM) and melatonin (10mM) at t=0.

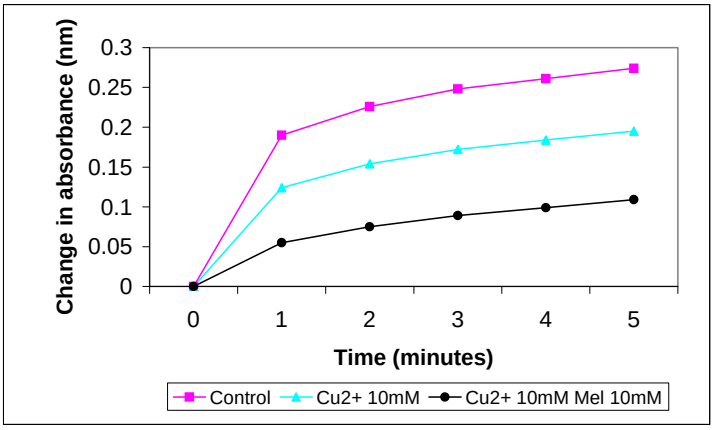


Figure 63. Rat liver homogenate preincubated with Cu^{2+} (10mM) and melatonin (10mM) at t=60.

Figure 63 shows the results of Cu^{2+} and Cu^{2+}/melatonin preincubated at t=60. The Cu^{2+} causes a decrease in electron transport, with a greater decrease observed with Cu^{2+}/melatonin. A similar trend is seen as in Fig. 62. Melatonin appears to further decrease electron transport, by interacting with copper.

6.4 Discussion

Malate was chosen as a substrate, as it was observed that succinate was reduced too quickly and this made it very difficult to measure. Heron *et al.* (in press) observed that in the presence of copper, mitochondrial electron transport were dose and time-dependently reduced, when succinate and malate was used as substrates.

Malate was used as a substrate to determine the status of the electron transport chain. Any change in malate utilization affects the electron transport chain.

Copper overload results in mitochondrial damage, leading to mitochondrial respiratory-chain defects. The results herein, show that copper affects electron transport. Cu^{1+} (10mM) inhibits the electron transport, at t=0 (Fig. 60) (ΔABS at 4 minutes is 0.1 nm), but at t=60 (Fig. 61), Cu^{1+} stimulates the electron transport (ΔABS at 4 minutes is 0.26 nm). It appears that Cu^{1+} preincubated with the homogenate, results in an increase in electron transport, which thus results in an increase in ATP production. The mitochondrial cells may be damaged, and the mitochondrial increases ATP production in an attempt to curtail/fix the damage. Melatonin co-administration results in an increase in electron transport at t=0 (ΔABS at 4 minutes is 0.38 nm). Melatonin, alone, inhibits the electron transport, which means that ATP production, is slowed down. At t=60, Cu^{1+} stimulates the electron transport and Cu^{1+} co-administered with melatonin, results in an inhibition of electron transport chain.

Cu^{2+} administration (Fig. 62) results in a decrease in the electron transport (t=0). Cu^{2+}/melatonin administration further inhibits the electron transport. A similar trend is seen at t=60 (Fig. 63). The inhibition of the electron transport chain, at t=60 is less for the Cu^{2+} and Cu^{2+}/melatonin administered samples, than compared to the Cu^{2+} and Cu^{2+}/melatonin administered samples at t=0.

At t=0, the ΔABS at 4 minutes for Cu^{2+} and Cu^{1+} is 0.30 nm and 0.1 nm, respectively. It thus appears that at t=0, Cu^{2+} is more potent at stimulating the electron transport, than Cu^{1+}. At t=60, the ΔABS at 4 minutes for Cu^{2+} and Cu^{1+} is 0.19 nm and 0.26 nm, respectively. It appears that Cu^{1+} stimulates the electron transport at t=60. In solution, Cu^{1+} is oxidized to Cu^{2+}, and the results of the Cu^{1+} stimulation of the electron transport chain, is similar to that of Cu^{2+}.

A trend is seen that, that melatonin administered with copper, results in the inhibition of the electron transport chain. This could be a protective mechanism, as an increase in ATP synthesis, could result in the mitochondria overproducing ATP, and "burning" itself out.

Verity *et al.* (1967) reported that copper administration (Cu^{2+}) caused a significant decrease in total malate dehydrogenase activity, although the free component was significantly increased above the control value. The authors reported that the findings were compatible with an increased 'leakiness' of the semipermeable mitochondrial membrane, with the loss of soluble enzymes into the cytoplasm.

Heron *et al.* (in press) reported that brain mitochondrial electron transport was dose- and time-dependently reduced by Cu^{2+}, when malate was used as a substrate. The copper concentrations used were between 10-500 μM.

The results presented here indicate that Cu^{1+} at t=60 appears to be oxidized to Cu^{2+} in the homogenate, and it is Cu^{2+} that causes an inhibition of the electron transport. Melatonin administration appears to further inhibit the electron transport, and further research needs to be carried out in determining if this would have any protective/beneficial effects in preventing copper-induced mitochondrial damage.

The aim of further research, would be to determine where in the electron transport chain, melatonin actually acts.

CHAPTER 7

Summary of Results:
Conclusions and Recommendations for Future Work

7.1. Summary of results

Chapter 2: Lipid Peroxidation: An *in vitro* and *in vivo* examination of copper-induced lipid peroxidation in rat organs

This study examined the effect of copper on lipid peroxidation, and the effect of melatonin co-administration in preventing copper-induced lipid peroxidation. *In vivo* and *in vitro* studies were conducted to study the effect of copper-induced lipid peroxidation. In the *in vivo* studies, male Wistar rats were injected with copper and copper/melatonin for 2-weeks and 6-weeks. The livers, kidneys and brains were examined for lipid peroxidation. The results show that the liver accumulates the highest concentration of copper, followed by the kidney and finally the brain. The level of copper-induced lipid peroxidation was highest in the liver and kidney, with no copper-induced lipid peroxidation observed in the brain. Melatonin does not have a significant antioxidant role in copper-induced lipid peroxidation.

The *in vitro* experiments with various concentrations of copper (1mM, 5mM and 10mM) and 5mM melatonin, indicate that melatonin (5mM) was able to exert a protective effect against copper-induced lipid peroxidation in the liver, at a copper concentration of 1mM.

The lipid peroxidation technique employed here is widely used, however, there are more reliable and sensitive methods available for determining the peroxidation of cellular membranes like HPLC analysis and ELISA techniques, which produce more accurate and reliable results. The disadvantage is that these methods are more costly.

Chapter 3: Histochemical investigations

Electron microscopy and light microscopy techniques were employed to examine the effects of copper-induced cellular damage. Electron micrographs indicate that copper-induced cellular damage was primarily at mitochondria, and that melatonin did afford some degree of protection as less damaged mitochondria were observed. Lysosomes were plentiful in the copper and copper/melatonin samples. The lysosomes were densely packed, with presumably copper. Three copper stains were used *viz.* rhodanine, silver sulphide and rubeanic acid stain. The rhodanine and silver sulphide stains were equally effective in staining copper, but the rubeanic acid stain was unreactive. Further studies need to be conducted on copper-specific stains for electron microscopy, so that the copper can be visually correlated to the cellular damage. Energy dispersive X-ray microanalysis, needs to be performed to determine the copper content in the lysosomes.

Chapter 4: *In vitro* investigations of the interaction between copper and melatonin

Melatonin's ability to bind/chelate Cu^{2+} and Cu^{1+} ions was examined using nuclear magnetic resonance (NMR), infrared spectroscopic (IR) and electrochemistry techniques. NMR data indicate that melatonin coordinates preferably with Cu^{2+}, rather than Cu^{1+}, through the carbonyl of the melatonin. The IR analysis indicates that melatonin coordinates with Cu^{2+} and Cu^{1+} through the carbonyl of the melatonin. These studies show that melatonin bind/chelate with Cu^{1+}. *In vivo* studies need to be performed to determine if the copper-melatonin complex is formed.

Chapter 5: Pineal organ culture studies: The effect of copper administration on rat pineal indole metabolism

This study employed the use of organ culture and thin layer chromatography (TLC). 2-week copper and copper/melatonin administration resulted in a decrease in the 5-methoxytryptophol level in the copper/melatonin pineals. It appears that melatonin administration decreases 5-methoxytryptophol synthesis, by probably interacting with HIOMT. The 6-week copper and copper/melatonin administered samples resulted in a decrease in the N-acetylserotonin levels in the copper administered pineals. It appears that melatonin interacts with the copper and prevents the inhibition of the NAT activity. Also, there was an increase in both the copper and copper/melatonin 5-methoxytryptophol levels. This indicates that copper and copper/melatonin interacts with the HIOMT enzyme. Further studies need to be carried out to measure the concentration of copper within the pineal, and to correlate this with the levels of NAT and PINA activity, and to investigate any possible interaction between NAT and copper binding.

Chapter 6: Electron transport chain: Mitochondrial alterations due to copper overload

This study was performed to determine the effect of copper on the activity of the liver and brain mitochondrial electron transport chain. The results presented here indicate that Cu^{1+} appears to be oxidized to Cu^{2+} in the homogenate, and it is Cu^{2+} that causes an inhibition of the electron transport. Melatonin administration appears to further inhibit the electron transport, and further research needs to be carried out in determining if this would have any protective/beneficial effects in preventing copper-induced mitochondrial damage.

7.2. Conclusions

Section 1.3. outlines the two main research objectives of this study. The first objective was to evaluate melatonin's ability to protect cells against copper-induced toxicity. Copper-induced free radical generation results in lipid peroxidation. Copper toxicity causes hepatic cirrhosis and necrosis, which ultimately leads to hepatic failure and death. The results obtained indicate that melatonin is unable to prevent copper-induced toxicity, and thus was unable to protect against copper-induced lipid peroxidation. The free radicals generated from copper exposure cannot be hydroxyl radicals alone, as melatonin was unable in protecting against copper-induced lipid peroxidation. The concentration of copper within the various tissues decreased with increased exposure to copper, indicating that the rats were becoming tolerant, and were able to increase the excretion of the metal. The brain is relatively impermeable to copper, due to the effective BBB. On continuous exposure to copper, the brain is able to limit copper-induced lipid peroxidation, by increasing the synthesis of copper-binding proteins. The liver, being the major organ of copper accumulation, is also able to induce copper-binding protein synthesis, thus limiting the effect of copper-induced lipid peroxidation.

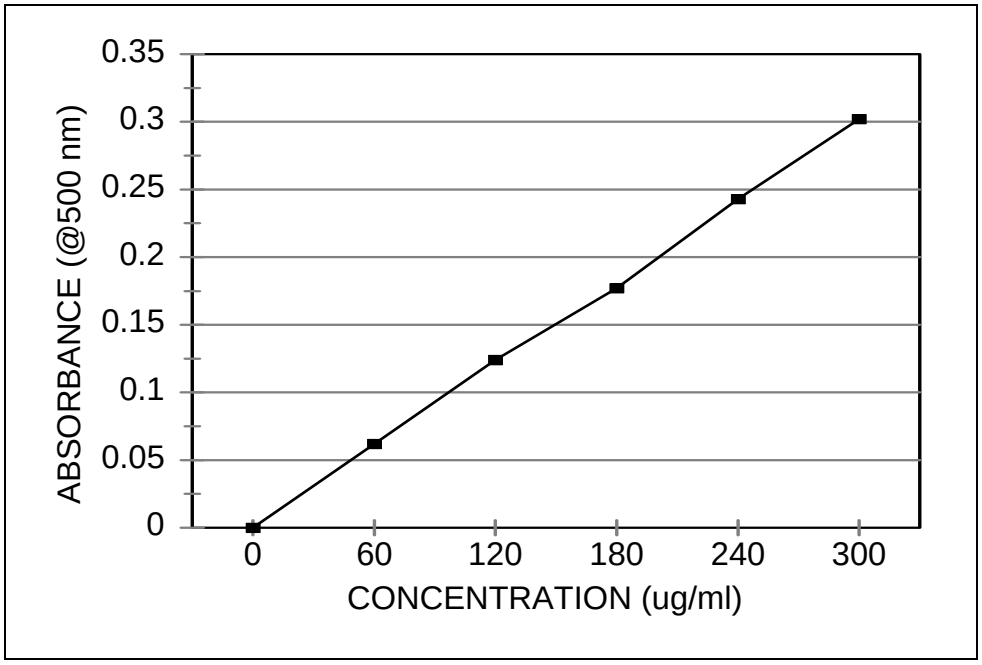
Histological examinations of *in vivo* liver, brain and kidney tissues indicate that copper causes extensive mitochondrial damage. Large, irregular shaped, dense copper-filled lysosomes are very frequent in tissues exposed to copper. In tissues from animals, which were administered with copper/melatonin, less mitochondrial damage is observed, but the large, irregular shaped, dense lysosomes are still evident. Melatonin might also have a role to play in preventing copper-induced mitochondrial damage. Electron transport chain studies indicate that melatonin alone, inhibits the electron transport chain. Copper/melatonin treatment does also inhibit electron transport, but not to the extent of melatonin alone. This signifies that copper co-administered with melatonin, may have a beneficial effect on mitochondrial electron transport, by decreasing the effective rate of ATP production, and preventing the mitochondria from "burnout". These findings

correlate the lipid peroxidation and histological results. Further investigations, need to be carried out to determine where in the electron transport chain, melatonin actually acts.
The various copper-specific stains are not definitive diagnostic tools in the diagnosis of copper toxicity. Rhodanine and silver sulphide appear to be equally sensitive for copper at 2-weeks of copper treatment, but are not sensitive at 6-weeks of copper treatment. It appears that the staining sensitivity does not always correlate to the concentration of copper in the tissues.

The second objective of the study was to investigate the chemical interaction between melatonin and Cu^{1+}. The *in vitro* chemical investigations reveal that melatonin interacts preferentially with Cu^{2+} rather than Cu^{1+}. Melatonin coordinates with Cu^{2+} at the carbonyl group, and not at the amide group, of melatonin. These results were confirmed with the IR analysis. The electrochemistry analysis indicates that melatonin coordinates with Cu^{1+}, and that the bond formed is a fairly strong one, but it was not possible to confirm whether the bond formed is one of simple ligation or chelation. A previous study has indicated that melatonin interacts with Cu^{2+}, and together with these results, it appears that melatonin is able to interact with both Cu^{1+} and Cu^{2+}. Further studies need to be performed to investigate the coordination of melatonin with copper, utilizing other chemical methods like UV studies. *In vivo* studies need to be performed to investigate if the copper-melatonin complex is formed, and then, to isolate and characterize the complex.

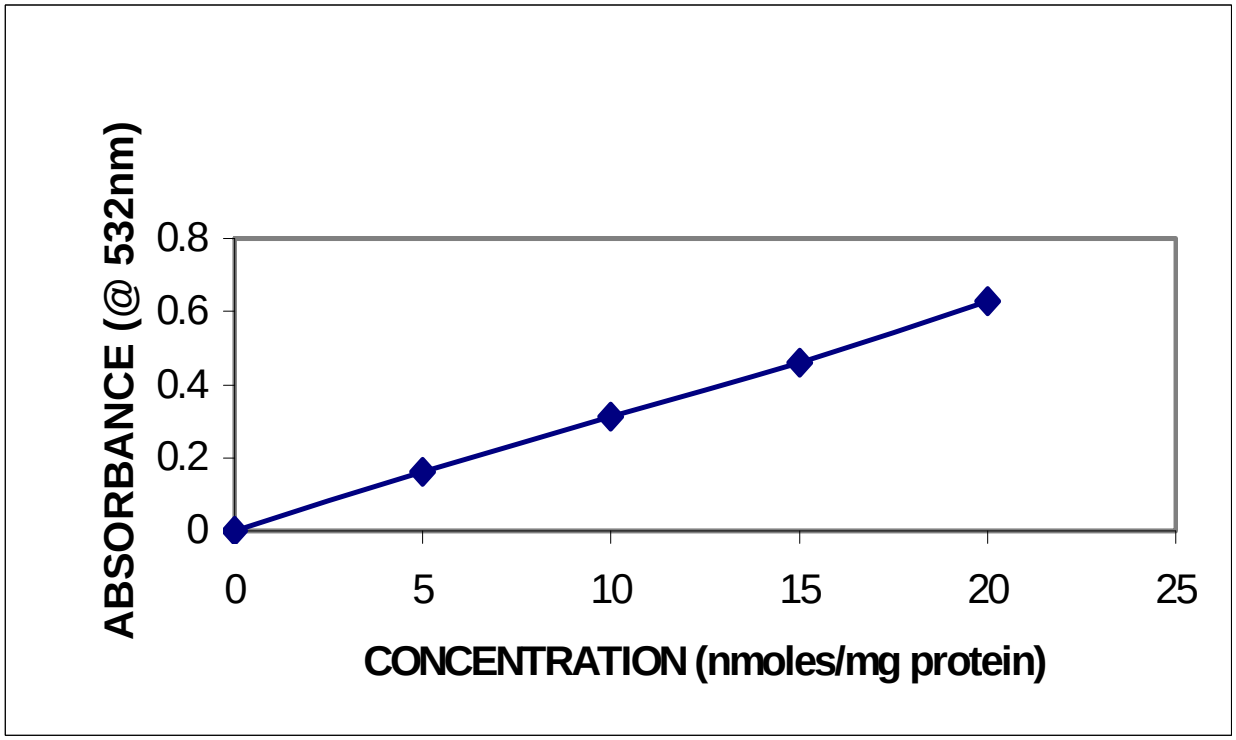
The electrochemistry indicates that melatonin can bind/chelate copper in both oxidation forms, thus theoretically, it appears that melatonin might have a beneficial effect on copper overloading.

APPENDIX 1



Protein standard curve using BSA as a standard (r=0.99948)

APPENDIX 2



MDA standard curve (r=0.9994898)

APPENDIX 3

UZMAN'S RUBEANIC ACID METHOD FOR COPPER

PROTOCOL

Rubeanic acid (dithiooxamide)	0.1gm
Ethanol 70%	100 ml

STAINING PROCEDURE

1. Deparaffinize in xylene, 2 changes
2. Equal parts xylene-absolute ethanol, 2 changes
3. Absolute ethanol, 2 changes
4. Rubeanic acid solution for 20 minutes
5. Add solid sodium acetate (0.2 gm/100 mls) to the staining jar and allow to settle to the bottom-leave slides for 24 hours
6. 70% ethanol, 2 changes for 1-1.5 hours each
7. Absolute ethanol, 24 hours
8. Clear in xylene, 2 changes
9. Mount with DPX

RESULTS

Copper is **fine granular black precipitate**

Reference : Uzman, 1956

MODIFIED RHODANINE

Rhodanine (5-4-dimethylaminobenzylidene rhodanine)

PROTOCOL :

Stock solution : 0.1 gm in 100 ml absolute ethanol (0.1% saturated solution)

Keep in fridge.

Working solution : **10 ml stock + 15 mls distilled water**

STAINING PROTOCOL :

-Dewax in xylene twice

1. Place sections in distilled water
2. Place in Rhodanine solution at 60°C for 1 hour
3. Rinse in distilled water
4. Stain nuclei in Meyer's Haematoxylin for 2 minutes
5. Blue in Scott's tap water for 30 seconds and rinse
6. Dehydrate (ascending concentrations of ethanol from 70%), clear and mount with DPX

Scott's water : sodium bicarbonate 3.5 gm, magnesium sulphate 20 gm and tap water 1000 mls

Add 10 ml formalin to prevent mould from growing

RESULTS :

Copper appears red and nuclei are blue

Reference : Lindquist, 1969

MODIFIED TIMM'S METHOD FOR COPPER

STAINING PROTOCOL :

-Dewax slides in xylene twice

1. Place slides in distilled water
2. Place slides in 0.3% sodium sulphide water solution for 30 minutes
3. Wash in deionised water for 30 minutes
4. Place slides in 15% trichloroacetic acid for 15 minutes
5. Wash in distilled water for 10 minutes
6. Place slides in complexed mixture for 1.5 hours in the dark at 22°C- 25°C
7. Wash in deionized water
8. Counterstain nuclei with Haematoxylin
9. Dehydrate (ascending concentrations of ethanol from 70%), clear and mount with DPX

COMPLEXED MIXTURE:

$ Watery solution of gum arabic 20 %(100 mls)- prepared on hot plate day before

$ Watery solution of silver nitrate 10 % (1 ml)

$ Watery solution containing 5 ml of 5% citric acid and 5 ml of 2% hydroquinone

RESULTS:

Copper appears as black precipitate

Reference: Fujii *et al.*, 1993