INTRODUCTION
Oxygen radicals and human disease

It is well established that aerobic organisms produce oxygen species during the course of normal metabolism. These include superoxide anion, hydrogen peroxide and hydroxyl radical (Fridovich, 1989; Sies, 1991; Weiss, 1989). Oxidative DNA damage by these species has been implicated in a number of human diseases, including cancer. The other diseases where such damage may play an important role are rheumatoid arthritis (Gridley, et al., 1993), Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis (ALS) (Sanches-Ramos and Ames, 1994; Mecocci et al., 1994; Robberecht et al., 1994). In this last disease it has been suggested that a lack of superoxide dismutase (SOD) activity or a mutant form of this enzyme may be an important factor (Spencer et al., 1994). In addition, oxygen radical toxicity is considered to be the cause of human male infertility (Aitken and Fisher, 1994).
Amyotrophic lateral sclerosis (ALS) is a degenerative disorder of motor neurons in the cortex, brainstem and spinal cord. It’s cause is unknown and it is uniformly fatal, typically within five years. It has been previously shown that in some but not all cases of familial ALS, the disease is linked to a genetic defect on chromosome 21 q. Rosen et al., (1993) have reported a tight genetic linkage between familial ALS and a gene that encodes a cytosolic, copper/zinc binding superoxide dismutase (SOD1), a homodimeric metalloenzyme that catalyses the dismutation of the toxic superoxide anion to \( O_2 \) and \( H_2O_2 \). These authors have identified 11 different SOD1 missense mutation in 13 different familial ALS families. Recent experiments with transgenic animals suggest that ALS syndrome is not due to lack of SOD activity, but to some toxic property of the mutant copper/zinc SOD enzyme (Gurney et al., 1994).

Oxygen radical production and mitochondrial damage have been proposed to have a role in Parkinson’s disease (DiMonte et al., 1992). Experimental evidence suggest
that mitochondrial damage may cause an increased generation of oxygen radicals. Oxidative stress is also reported to be associated with decreased level of Glutathione, an oxygen radical scavenger, in Parkinson's disease patients. Excess accumulation of tissue iron leading to generation of oxygen radicals has also been implicated in Parkinson's disease (Riederer et al., 1992). It is considered that regardless of whether oxidative stress or mitochondrial damage represents the initial insult, these toxic mechanisms may both contribute to neuronal degeneration via changes in glutathione levels (DiMonte et al., 1992).

Since many years the disease Rheumatoid arthritis has been linked to the formation of oxygen radicals. In this disease the production of synovial fluid is increased and the joint cartilage is eroded. However, the viscosity of the synovial fluid decreases thus reducing its lubricating capacity. The decrease in viscosity is due to the breakdown of the polymer
hyaluronic acid. Exposure of synovial fluid to $O_2^-$ produced by chemical systems or by activated phagocytes in vitro produces a similar breakdown, which can be attributed to the $O_2^-$ dependent formation of $OH^+$ by an iron catalysed Haber Weiss reaction in the synovial fluid (Halliwell et al., 1984). A role of proteases and oxygen radicals in polymorphonuclear neutrophil induced cartilage degradation has been implicated and it was shown that a novel synthetic elastase inhibitor had a protected effect (Iwamura et al., 1993).

Mcleod in 1943, first demonstrated that human spermatozoa could generate and release the reactive oxygen species, hydrogen peroxide. Contemporary analyses of these cells have shown that they are capable of generating oxygen radicals with the superoxide anion being produced as a primary product that subsequently dismutates to $H_2O_2$ under the influence of intracellular superoxide dismutase (Aitken and Clarkson, 1987). The risk of manufacturing oxygen radicals by human spermatozoa is considerable because these cells are
particularly vulnerable to lipid peroxidation. There is good evidence to indicate that oxygen radicals are involved in the initiation of peroxidative damage to the sperm plasma membrane, seen in many cases of male infertility. However, recent data suggest that superoxide anion and hydrogen peroxide also participate in the induction of key biological events such as hyperactivated motility and the acrosome reaction of the spermatozoa. Thus, the spermatozoa use oxygen radicals for a physiological purpose but have the difficult task of ensuring the balance generation of these potentially harmful, but biologically important, modulators of cellular function (Aitken and Fisher, 1994).

One of the theories of etiology of cancer which is being widely accepted, holds that the major cause is damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983; Totter, 1980). Several enzymes produce superoxide anion ($\cdot O_2^-$) during the oxidation of their substrates, for example, xanthine oxidase and peroxidase.
Numerous substances such as reduced flavins and ascorbic acid upon autoxidation produce superoxide anion. This radical further accepts an electron from a reducing agent, such as thiols to yield peroxide ($H_2O_2$). There is \textit{in vitro} evidence that $H_2O_2$ may then react with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($OH^\cdot$) (Wolff et al., 1986). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous occurrence of superoxide dismutase. Indeed, certain white blood cells generate superoxide deliberately by means of a specialized membrane bound NADPH oxidase and this participates in the killing of microorganisms and tumour cells (Wolff et al., 1986).

It has been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these substances. Fat and hydrogen peroxide are among the most potent promoters (Welsch and Aylsworth, 1983). Other well known
cancer promoters are lead, calcium, phorbol esters, asbestos and various quinones. Inflammatory reactions lead to the production of oxygen radicals by phagocytes and this is the basis of promotion by asbestos (Hatch et al., 1980). Many carcinogens which do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos et al., 1980). These include nitroso compounds, hydrazines, quinones and polycyclic hydrocarbons. Much of the toxic effect of ionizing radiation damage to DNA is also due to the formation of oxygen radicals (Totter, 1980). The mechanism of action of promoters involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982; Varshavsky, 1981). Promoters also cause modification of prostaglandins which are intimately involved in cell division, differentiation and tumour growth (Fischer et al., 1982). Most data on radical damage to biological macromolecules concern with the effects of radiation on
nucleic acids because of the possible genetic effects. However, in view of the catalytic role of enzymes, damage to proteins is also considered important. It has been suggested that primary oxygen radicals, produced in cells and their secondary lipid radical intermediates, modify and fragment proteins. The products are often more susceptible to enzymatic hydrolysis leading to accelerated proteolysis inside and outside the cells (Wolff et al., 1986).

Anticarcinogens and antioxidants

The protective defence mechanisms against mutagens and carcinogens include the shedding of surface layer of the skin, cornea and the alimentary canal. If oxygen radicals play a major role in DNA damage, defence against these agents is obviously of great importance (Totter, 1980). The major source of endogenous oxygen radicals are hydrogen peroxide and superoxide which are generated as side products of metabolism (Pryor, 1976-1982). In addition, oxygen radicals also arise from
phagocytosis after viral and bacterial infection or an inflammatory reaction (Tauber, 1982). The exogenous oxygen radical load is contributed by a variety of environmental agents (Pryor, 1976-1982). The enzymes that protect cells from oxidative damage are superoxide dismutase, glutathione peroxidase (Pryor, 1976-1982) D.T. diaphorase (Lind et al., 1982) and glutathione transferases (Warholm et al., 1981). In addition to these enzymes, some small molecules in the human diet act as antioxidative agents and presumably have an anticarcinogenic effect. Some of these compounds are discussed below.

Tocopherol (vitamin E) is an important trap of oxygen radicals in membranes (Pryor, 1976-1982) and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin which are toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimethylhydrazine induced
carcinogenesis have also been observed (Beckman et al., 1982). p-carotene is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen, which is mutagenic and is mainly generated by pigment mediated transfer of energy of light to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer et al., 1981). p-carotene and similar polyprenes are also the main defence in plants against singlet oxygen generated as a byproduct of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be anticarcinogens in rats and mice and may also have a similar effect in humans (Mathews-Roth, 1982; Peto et al., 1981). Glutathione is present in food and is one of the major antioxidants and is antimutagenic in cells. Glutathione transferases are a major defence against oxidative and alkylating carcinogens (Warholm et al., 1981). Dietary glutathione is an effective anticarcinogen against
aflatoxins (Novi, 1981). The cellular concentration of glutathione is influenced by dietary sulphur amino acids (Tateishi et al., 1981). Selenium, which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid hydroperoxides and endogenous hydrogen peroxide and therefore helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Several heavy metal toxins, such as Cd\(^{2+}\) (a known carcinogen) and Hg\(^{2+}\) decrease glutathione peroxidase activity by interacting with selenium (Flohe, 1982). Some other dietary antioxidants include ascorbic acid and uric acid. The former has been shown to be anticarcinogenic in rodents treated with UV light and benzo(a)pyrene (Hartman, 1982). Uric acid is present in high concentrations in the blood of humans and is a strong antioxidant (Ames et al., 1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer; however, too high levels may cause gout.
In addition, edible plants contain a variety of substances such as phenols that have been reported to inhibit or enhance carcinogenesis and mutagenesis in experimental animals (Ames, 1983). The inhibitory action of such compounds may be due to the induction of cytochrome p-450 and other metabolic enzymes (Boyd et al., 1982). The optimum levels of dietary antioxidants have not been determined; however, there might be considerable variation among individuals. On the other hand, high doses of such compounds may lead to deleterious side effects. The differences in cancer rates of various populations are generally considered to be due to environmental and life style factors such as smoking, dietary carcinogens and promoters. However, these differences may also be due, in good part, to insufficient amounts of anticarcinogens and other protective factors in the diet (Maugh, 1979).

In the past two decades, there has been much emphasis on the induction of cancer by occupational and industrial pollution factors. There is growing
recognition, however, that these may account for only a small fraction of human cancers. It is becoming increasingly clear from epidemiological and laboratory data that diet is an important factor in the etiology of certain human cancers. It has been suggested by Doll and Peto (1981) that in the United States diet accounts for 35% of cancer deaths. According to these authors, there are five possible ways whereby diet may effect the incidence of cancer; (i) ingestion of powerful direct acting carcinogens or their precursors; (ii) affecting the formation of carcinogens in the body; (iii) affecting transport, activation or deactivation of carcinogens; (iv) affecting "promotion" of cells that are already initiated; and (v) overnutrition. Normal individual consumption of potentially mutagenic substances per day from foods and beverages is estimated to be between 1 to 2 gm. In addition, the endogenous conditions favour the formation of still more mutagens in vivo in humans (Oshshima and Bartsch, 1981).
Figure 1: Structure of L-DOPA
L-DOPA
Scope of the Work presented

L-3,4-dihydroxyphenylalanine (L-DOPA) is an important metabolite in various metabolic reactions. Dopamine, one of the catecholamine is formed by the decarboxylation of L-DOPA which in turn is formed by hydroxylation of tyrosine. Dopamine is a neurotransmitter in central nervous system and accounts for 90% of the total catecholamines. It serves as a precursor of hormones, noradrenalin and adrenalin. The neurological disorder Parkinson's disease associated with an under production of dopamine in the human brain (Lehninger et al., 1993). L-DOPA has therefore been found to be an effective drug in the treatment of Parkinson's disease. Another important biochemical reaction for which L-DOPA serves as a metabolite is the synthesis of melanin (Hill, 1992). Protein-bound 3,4-dihydroxyphenylalanine is a major reductant formed during hydroxyl radical damage to proteins (Gieseg et al., 1993).
Figure 2A: Biosynthesis of catecholamines - DOPA, dopamine, norepinephrine and epinephrine - from tyrosine
Tyrosine

\[ \text{O}_2, \text{NADPH}, \text{H}^+ \]
\[ \text{H}_4-\text{biopterin} \]
\[ \text{Tyrosine hydroxylase} \]
\[ \text{H}_2O, \text{NADP}^+ \]
\[ \text{H}_2-\text{biopterin} \]

DOPA

\[ \text{DOPA decarboxylase} \]
\[ \text{PyP} \]
\[ \text{CO}_2 \]

Dopamine

\[ \text{O}_2 \]
\[ \text{Dopamine} - \beta - \text{hydroxylase, Ascorbic acid} \]

Norepinephrine

\[ \text{AdoMet} \]
\[ \text{Ado Hcy} \]

Epinephrine
Figure 2B: Biosynthetic pathways from tyrosine to melanins
Tryosine

\[ \text{O}_2 \rightarrow \text{Tyrosinase, Cu}^{2+} \rightarrow \]

3,4-Dihydroxyphenylalanine (DOPA)

\[ \text{O}_2 \rightarrow \text{Tyrosinase} \rightarrow \]

Dopaquinone

Leucodopachrome

5,6-Dihydroxyindole

Indole-5,6-quinone

Polymeric red Melanin

Polymeric black Melanin

Nonenzymatic
Copper has been reported to be neurotoxic as evidenced by the brain pathology produced in patients with copper overload as a result of Wilson’s disease (Hartard et al., 1993). Lipid peroxidation is promoted by copper ions (Hochstein et al., 1980; Esterbauer et al., 1989) which also catalyses formation of highly reactive hydroxyl (OH·) radicals from hydrogen peroxide (Halliwell and Gutteridge, 1990). Copper ions and H₂O₂ produce DNA damage; strand breaks (Tachon, 1990) and chemical changes in purine and pyrimidine bases, especially conversion of guanine into 8-hydroxyguanine (Aruoma et al., 1991). Recent work of Halliwell and coworkers (1994) have shown that L-DOPA, dopamine and 3-O-methyl-DOPA cause extensive base modification in DNA in presence of H₂O₂ and traces of copper ions. These authors propose that copper ion release, in the presence of L-DOPA and its metabolites, may be an important mechanism of neurotoxicity, e.g. in Parkinson’s disease and amyotrophic lateral sclerosis (ALS). In this laboratory studies have been carried out on the
mechanism of interaction with DNA of antioxidants of plant and animal origin. These include flavonoids [Rahman et al., 1989; Rahman et al., 1990; Fazal et al., 1990; Said Ahmad et al., 1992; Said Ahmad et al., 1994], Tannic acid [Bhat and Hadi, 1992; Bhat and Hadi, 1994; Bhat and Hadi, 1994] and uric acid [Shamsi and Hadi, 1995]. In addition there are a number of molecules present in human extracellular fluids that are considered to have an antioxidant function [Halliwell and Guttridge, 1990]. These include uric acid, bilirubin, ascorbate and glucose. From previous studies in this laboratory and those with several other antioxidants [Stoewe and Prutz, 1987] suggest that several of the biological antioxidant are themselves capable of causing oxidative DNA damage. Another good example of such a molecule appears to be melanin as it has been suggested to be both a radical scavenger and at the same time capable of DNA damage. Melanin is synthesized from DOPA by spontaneous polymerization of DOPA chrome, an oxidized product of DOPA [Nicolaus,
Hill 1992 has enumerated the following possible functions of melanin: (i) It acts as camouflage and adornment, (ii) It is a sunscreen against UV damage by sunlight, (iii) It is a radical scavenger, (iv) It is a photo- and radio-sensitizer, (v) It binds to drugs, (iv) It transforms one kind of energy into another.

X and gamma rays cause ionization of water to generate a variety of radical species. These include hydroxyl radicals, aqueous electrons, hydrogen radicals, also small amounts of hydrogen peroxide and hydrogen gas. Hydroxyl radicals are the most damaging to cellular constituents. Superoxide anion radical and hydrogen peroxide produced in these reactions can interact with each other, especially in the presence of metal ions, to form hydroxyl radical. Reactive species produced by UVB and UVA in cells are similar to those produced by ionizing radiations. However, some photosensitized interactions may produce singlet oxygen which can also damage cellular macromolecules [Peak et al, 1985]. Free radicals and active oxygen species interactions produce
DNA strand breaks, base damage and DNA protein crosslinks. They are also lethal, mutagenic and carcinogenic. Synthetic melanins have properties that are similar in many ways to natural melanins. However, they are easier to study because some of them are soluble. Sarna et al. (1986) examined the ability of synthetic melanins to scavenge radicals produced by ionizing radiation. Melanin scavenged hydroxyl radicals most efficiently. The rank order of scavenging was the same as the rank order of the rate of production by ionizing radiation of the various radicals. Other studies demonstrate that melanin can scavenge molecular oxygen as well as active oxygen species when it is exposed to UV light [Sarna et al., 1984; Korytowski et al., 1987].

Melanin can absorb free radicals and active oxygen species, but when it does so, free radicals and active oxygen species are also produced. Melanins are unique among biological molecules in that they continuously
emit a free radical signal [Sealy, 1984]. The free radicals associated with this signal are called "melanin free radicals". When melanins are irradiated with UV, or when melanins absorb superoxide anion radical, the melanin-free radical signal is enhanced and, depending on the conditions, superoxide anion radical, hydrogen peroxide and hydroxyl radical are produced [Korytowski et al., 1987]. Melanin-mediated radical production is potentially lethal to cells [Menon et al., 1985].

In this thesis, I describe experiments to show that L-DOPA in presence of Cu(II) alone is capable of causing strand breakage in DNA in vitro and that this breakage results from the generation of reactive oxygen species. I, further, show that Cu(I) is an essential intermediate in this reaction and that L-DOPA is capable of binding to DNA. Since DOPA is the precursor for melanin synthesis it was of interest to compare DNA breakage activity of L-DOPA and melanin in the presence of Cu(II). The L-DOPA-Cu(II) system also causes lambda bacteriophage inactivation indicating that this system
is biologically active. The effects of chemically modifying tyrosine to dihydroxyphenylalanine (DOPA) residue, within a protein, were investigated and the DNA nicking effects of T₃ RNA polymerase-DOPA, EcoRI methylase-DOPA and BSA-DOPA were studied using plasmid DNA.