Introduction
Acridine

The discovery of a basic material in the anthracene fraction of coal tar was announced by Graebe and Caro in 1870. On account of its acrid smell and irritating action on the skin and mucous membrane this new substance was called "Acridine" (acris = sharp, or pungent). Then a series of salts were prepared. A most characteristic property of acridine, and many of its derivatives, is the fluorescence which they exhibit in dilute solution (Acheson, 1973).

Ehrlich later discovered the therapeutic property of acridine, acriflavine and proflavine were introduced as antibacterial agents during first world war, when there was an increased demand for active antibacterial agents, which would prove less toxic than known substances to the deep tissues which become exposed in wounds. Aminoacridines penetrate tissues readily, not so readily as many sulphonamides, but better than the triphenylmethane dyes such as crystal violet. It was found that when a small mammal was injected with an aminoacridine, and the tissues quickly frozen and sectioned one hour later, the acridine is seen to have been taken up by many kinds of cells and concentrated in the nuclei. However the cells of the central nervous system did not absorb the dyes. There was little or no further clinical experimentation with acridines until the First World War (1914-1918), when acriflavine and proflavine were introduced as local antibacterial agents, in which there was an increased demand for active antibacterial agents, which would prove less toxic than known substances to the deep tissues which become exposed in wounds. (Alberts, 1966)

Photochemistry of Acridines

Fluorescence is generally observed in those organic molecules which have rigid framework and not many loosely coupled substituents though which vibronic energy can flow out. In general, some substituents in these molecules enhance the fluorescence and they are known to be electron donors such OH, NH₂, etc, which enhance the transition probability or intensity of colour, e.g. acridine and acridine orange as given in figure 1.

For designating energy levels of these compounds, the electronic states are
Figure 1. Diagrams of three acridine with different substitution have difference in fluorescent emission.
Acridine (non fluorescent)

Acridine orange (fluorescent)

Proflavine (fluorescent)
expressed in terms of the initial and final orbitals involved in a transition. An electron from any of the occupied orbital can be promoted to higher unoccupied level on absorption of appropriate radiation. On an electron promotion, the energy state of the molecule changes, and may have singlet or triplet character. (Rohatgi-Mukherjee, 1986).

**Action of acridines**

**Interaction with Nucleic Acids:** The nature of the interaction of acridine derivatives, especially aminoacridines, with nucleic acids has attracted increasing attention since their earliest use as cellular stains. The widespread biological effects of acridine derivatives gradually came to be connected principally with their ability to interact with nucleic acids, but interest in this interaction was heightened when the mechanism of the binding processes involved was understood. The binding ability to nucleic acid depends on the basicity of the acridine which will cause a marked increase in intrinsic viscosity and a decrease in sedimentation rate of DNA, and this indicates an increase in counter length of the DNA double helix (Acheson, 1973).

**Action on Enzymes:** Interest in the biological activity of the acridines has centered on their antibacterial, antimalarial and mutagenic properties. Apart from a few isolated instances, little interest has been taken in their action on enzymes until the last 20 years or so. The interest in its action on enzymes was generated due to our increased understanding of cellular metabolism, as reaction of acridines with enzyme system usually resulted in the inhibition of the enzyme. The inhibition of RNA synthesis in rat liver mitochondria by low concentration of acriflavine revealed that mitochondrial DNA is involved in the process (Acheson, 1973).

**The Antibacterial Action:** An interest in the effect of acridines as wound disinfectants was developed since its antibacterial effect was stronger, in contrast to many other substances and was shown to be retained in the presence of body fluids and pus. Both proflavine and 3,6-diamino-10-methylacridinium chloride became widely used for this purpose. Some pathogen was found to be resistant to acridines (Acheson, 1973).
Proflavine

3,6-Diaminoacridine, having a flavine nucleus, as shown in figure 1, is a known antibacterial agent since Browning's work in 1913, but it is only in later years that its superiority over acriflavine was recognized. It is soluble in 300 parts of water or 35 parts of glycerol, a saturated solution in water is deep orange in color and gives a green fluorescence when freely diluted. Both proflavine and acriflavine were used as wound disinfectants during world wars. The application of not more than 0.5 g of powdered proflavine at a time was recommended, a treatment that gave good results in a number of intractable mixed infections (Albert, 1966).

Observation of Singh-Ranger and co-worker (2001) have presented a case where contact dermatitis in response to proflavine developed after pinnaplasty in which usually the proflavine allergy is uncommon. Proflavine, for umbilical cord care in the umbilical cord separation time was still in use (Hsu et al., 1999).

The acridine dyes proflavine and acriflavine were applied as fluorochromes, by several investigators in the past because of their high quantum efficiency, which made them especially useful for automated cell analysis. It can be used routinely in clinical laboratories because this single stain could help in differentiation of leukocytes as well as counting of reticulocytes without altering the cells morphology (Sagawa and Tatsumi, 1997). The binding of proflavine to human adult hemoglobin in ferrous state will cause the X-band appeared in EPR spectrum to displays the characteristics of T-state of the ligated tetramer. In parallel, oxygen affinity for the deoxygenated derivative of ferrous human adult hemoglobin decreases in the presence of proflavine (Ascenzi et al., 1999). Recently the investigation made on acridines, especially proflavine, is its action on DNA and proteins (Revich and Ripley, 1990; Conti, 1998).

Several investigators studied the binding of proflavine and other acridines such as acriflavine on native denaturated unhydrolyzed DNA in solution. Proflavine were found to bind best to alternating purine-pyrimidine sequences regardless of their nature, the drug is thought to exert its biological action mainly by binding to DNA (Bailly et al., 1992). In neutral aqueous solutions, proflavine exhibits a strong absorption maximum at 444 nm, and upon addition of DNA, a pronounced red shift in the proflavine absorption maximum to 460 nm occurs, that is indicative of the intercalation
of proflavine molecules (Marini and Caplow, 1971; Georghiou, 1997). Studies using CD spectroscopy showed that proflavine relax the superhelical organization of DNA, leading to the formation of a B-like structure without further structural changes as compared to other minor groove-binding drugs such as Hoechst-33258 which is reported to cause other structural changes (Krishna et al., 1993). The introduction of this simple intercalator affects both the conformational features and dynamic properties of the oligonucleotide double helix, as both major and minor grooves became wider with the addition of the intercalating drug (Herzyk et al., 1992). Water uptake accompany the complex formation indicate importance of water as thermodynamic participant (Qu and Chaires, 2001).

Association of proflavine with DNA have been reported to give specific effect, like frameshift mutation during in vitro DNA replication of single-stranded DNA template by the Klenow fragment of Escherichia coli DNA polymerase I. A novel inhibition of polymerization was found opposite to all pyrimidines in the template when proflavine template complexes were exposed for ten seconds in white light (Revich and Ripley, 1990), such frameshift is based on the hypothesis that the polymerase passes by a template base without copying it leading to deletion (Berman et al., 1992). Thus, supporting the proposed mutagenic mechanism for proflavine-induced mutations in which frameshift is produced as a consequence of exonuclease or DNA polymerase activity at the 3' ends of nicks in the DNA is observed in experiment with thymidylate synthase gene of bacteriophage T4 (Brown et al., 1993). Moreover, proflavine caused inhibition of transcription process, and did not cause displacement of the enzyme RNA polymerase from the promoter (Mazumder et al., 1993, Kim et al., 1998). Considerable interest has been focused recently on the role that some host transcriptional factors may play in the initial activation of human immunodeficiency virus (HIV-1) gene expression by interaction with the long terminal repeat of the integrated provirus (Haseltine, 1988), and proflavine was found to do both classical and threading intercalation with the TAR RNA of HIV-1 (Bailly et al., 1996). Indirectly proflavine may cause gene expression of HIV-1 as proposed by Sylvie et al. (1995), intercalated proflavine is known to oxidize the DNA guanine upon irradiation with visible light.
Role of Proflavine in the Generation of Reactive Oxygen Species

Photochemical techniques are emerging as promising approaches for the sterilization of blood products, biological fluids, treatment of cancer and other diseases (Ben-Hur and Horowitz, 1996; Goodrich and Platz, 1997; Fisher et al., 1995). These methods are based on the photodynamic effect in which biological target macromolecules are damaged by light-absorbing, photoexcited endogenous or exogenous chromophores. Single-photon absorption processes utilizing UV or visible light usually excite the photosensitizer molecules. However, the application of these techniques is often limited by a number of factors. For example, UV light, depending on its wavelength, can be intrinsically cytotoxic and/or mutagenic (Young, 1990). Furthermore, strong optical absorption and Rayleigh scattering effects can restrict the penetration depth of UV and visible light photons in biological tissues or fluids (Cheong et al., 1990). The enhanced penetration depth of near-IR light into tissues and the lower extent of Rayleigh scattering offers self-absorption by photosensitizer molecules may limit the absorption of IR light into tissues (Wilson, 1989).

The use of multiphoton excitation of photosensitizer molecules has been recently explored with the goal of overcoming some of the limitations associated with single-photon excitation mechanisms (Leupold and Kochevar, 1997). The simultaneous absorption of two or more near-IR photons can provide an overall excitation energy equivalent to that of a single UV or visible photon.

Acridine dyes, specifically proflavine, are photodynamic agents that are known to target DNA as well as other biomolecules (Kochevar and Dunn, 1990; Kochevar and Buckley, 1990). Although the exact mechanism of photodamage to DNA initiated by proflavine are not well characterized. Whether its electronically excited state reacts directly with DNA via electron transfer and/or hydrogen atom abstraction mechanisms is not clearly understood (Kochevar and Dunn, 1990). Using 2'-deoxynucleotide-proflavine model systems, Georghiou (1977) found that only guanosine-5'-monophosphate give rise to a substantial quenching of the fluorescence. In contrast, all of the other nucleotides slightly enhanced the fluorescence of proflavine. Furthermore, an enhancement in the proflavine fluorescence decay kinetics is correlated with the G-C content of the DNA (Georghiou, 1977). These results indicate that guanine residues are responsible for the quenching of the fluorescence of proflavine when this molecule
forms complexes with DNA. Furthermore, because guanine is the most easily oxidizable nucleic acid base (Piette et al., 1978), electron transfer from guanine to electronically excited proflavine molecule is likely to constitute the first step in the complex series of reactions that lead to DNA damage by strand cleavage (Piette et al., 1981).

Earlier studies for the role of proflavine in inducing free radicals have been investigated by Piette and co-workers (Piette et al., 1978; Piette et al., 1979; Piette et al., 1981; Piette et al., 1982). They compared different type of dyes, acridines, xanthene derivatives and sulphur-containing dyes. They analyzed the photosensitized induction of free radicals in calf thymus DNA at low temperature, and in the presence of oxygen, their results showed that the photoactivation of proflavine generate different type of free radicals such as singlet oxygen, hydroxyl radical and peroxide radical as detected by using Electron Paramagnetic Resonance, and the use of specific free radical scavengers. But these characteristic results were not obtained when proflavine or DNA were irradiated alone, nor when oxygen was absent. The induction of free radical from proflavine bound to DNA molecule can cause a single strand scission upon irradiation with visible light at high flounce rate as shown by agarose gel electrophoresis (Piette et al., 1981). This is consistent with the hypothesis that a free electron is ejected during the excitation of bound proflavine by visible light. Both superoxide dismutase and ceruloplasmin decrease the e.p.r. signal observed in the reaction system suggesting that proflavine produces superoxide anions when complexed to DNA. Superoxide anion may be formed either by direct reaction between the electron ejected by excited proflavine and molecular oxygen, or by decomposition of the peroxide radical formed by the combination of a DNA base and molecular oxygen (Piette et al., 1982).

**Features of Proflavine**

Recent investigations have shown that the presence of 6 µM intercalator proflavine can cause both direct single- and double-strand breaks in proflavine-supercoiled plasmid DNA complex (Shafirovich et al., 1999). Wainwright et al. (1997) have showed that illumination at a light dose of 6.3 J/cm² resulted in considerable decrease in the minimum lethal drug concentration required, giving enhanced phototoxicity of proflavine against many strains type on the other hand, the aminoacridine and its derivatives were bactericidal at sub-millimolar levels, but were
photobactericidal at the micromolar level. Other researchers have on the negative aspect of photoilluminated proflavine. When irradiated proflavine oxidized the guanine residues of DNA, this will then cause signaling events which will be transmitted into the cytoplasm where the inactive NF-kappa B factor is present, p50/p60 subunits of NF-kappa B will be translocated to the nucleus leading to HIV-1 gene expression in cells surviving the treatment (Legrand-Poels et al., 1995).

Furthermore, the recent observation of Singh-Ranger and co-worker (2001) which have present a case where contact dermatitis in response to proflavine developed after pinnaplasty, in which usually the proflavine allergy is uncommon. It may be possible because free radical production and disturbances in redox status can modulate the expression of a variety of inflammatory molecules, affecting certain cellular processes leading to inflammatory processes, both exacerbating inflammation and effecting tissue damage. It was recently reported that the activity of antioxidant enzymes in allergic patients, was higher than in non allergic controls giving indication for the involvement of free radical in allergic processes (Mates et al., 2000). Other studies have suggested that drug allergy might come from the binding of drug non-covalently to MHC-peptide complexes and to T cell receptors, thereby able to stimulate T cells, hence inducing hypersensitive reactions (Pechler, 2001).

**Do Proflavine Derivatives and Structurally Similar Drugs Have Similar Features like Proflavine?**

Proflavine conjugate, such as 125iodoacetylproflavine, when added to human lymphoblastoid cells was readily taken up by the cells, localized in the nucleus, and was released rapidly following resuspension of the cells in fresh medium. This treatment caused an induced mutant fraction observed on Southern blot. When these results were compared with those observed with other intercalating drugs, the 125iodoacetylproflavine showed a reduced effectiveness per decay, perhaps related to the non-covalent nature of intercalator binding, resulting in reduced energy deposition in the DNA (Whaley et al., 1990).

Studies have been carried out by synthesizing new polycyclic acridine derivatives and investigating their action on DNA. They were found to display a
stronger DNA-binding properties and high selectivity to [Poly (dA-dT)] polynucleotide than to [Poly (dG-dC)] polynucleotide as compared to proflavine (Gimenez-Arnaud et al., 1998). This indicates that the structure elements such as unsaturation or having different terminal group on the aromatic ring can cause a varied drug behavior. This may help in understanding the mechanism of action of different drugs on our body. Tacrine, a drug used for the treatment of Alzheimer’s disease was found to induce elevated serum aminotransferase levels, an indication of potential hepatotoxicity of tacrine. Similar cytotoxicity was found to develop when proflavine and many more structurally similar compounds, were used. Studies have implicated the formation of reactive metabolites in the mechanism of hepatotoxicity (Monteith et al., 1996).

Malaria caused by four species of plasmodial parasites (Wernsdorfer and McGregor, 1988), is estimated to kill between 1.5 and 2.7 million people every year, with over 300 million currently infected. Quinacrine, an antimalarial drug, is reported to cause phototoxicity in the skin and the eyes. The work done by Motten et al. (1999) have demonstrated a correlation between reported toxicity and generation of singlet oxygen and/or free radicals. As malaria is a disease most prevalent in regions of high light intensity, protective precautions such as clothing, sun-block, sunglasses or eye wraps should be recommended when administering antimalarial drugs (Motten et al., 1999).

Oxygen Toxicity

A Radical Explanation

Paramagnetism and the univalent pathway

Rotating electrical charges generate magnetic fields. This applies to electrical current in a coil of wire or to a single spinning electron. The pairing of electrons with opposite spin states neutralizes this effect. Most substances are not influenced by imposed magnetic fields because the electrons they contain are all in spin-opposed pairs. Such substances are diamagnetic. O_2 is unusual in being paramagnetic. It implies unpaired electronic spins. Indeed, O_2 contains two unpaired electrons having the parallel spin state. This electronic structure constitutes a barrier to the insertion of a pair of electrons. Thus, the electrons of the incoming spin-opposed pair would be trying to join
the parallel-spinning unpaired electrons of O₂ and one of them would have the same spin state as its partner to be. This situation, schematized in reaction 1, is energetically very unfavorable, as stated by the Pauly exclusion principle:

\[ \uparrow\uparrow + \uparrow\downarrow \rightarrow \downarrow\downarrow \uparrow\uparrow \]  

(1)

To help this, electrons are added to O₂ one at a time. This works because electronic spins can be inverted by interaction with nuclear spins. However, this is a slow process relative to the lifetime of collisional complexes and is not likely while the reacting partners are in contact. But when the electrons are added one at a time, during separate collision event, there is time between collisions for the inversion of electronic spin. As a result, the facile route of O₂ reduction is by a series of univalent electron transfers (Fridovich, 1998).

**Intermediates on the univalent pathway**

The reduction of O₂ to 2H₂O requires four electrons. Hence, intermediates will be encountered on this univalent pathway and these are superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH). It is these intermediates that are responsible for the toxicity of O₂ and defenses against that toxicity must include minimizing their production to the maximum extent possible and eliminating those whose production cannot be avoided. Most of the O₂ consumed by respiring cells is reduced by cytochrome c oxidase which, by virtue of two ferrihemes and two Cu(II) prosthetic groups, manages the four-electron reduction of O₂ to 2H₂O without releasing intermediates. But there are enzymes that reduce O₂ to H₂O₂, and there are both enzymic and spontaneous processes within cells that produce O₂⁻. It has been estimated that approximately 0.1% of the O₂ reduced by Escherichia coli is reduced to O₂⁻ (Imlay and Fridovich, 1991). Nevertheless, so great is the rate of O₂ utilization by these cells that, were the O₂⁻ stable, this would have corresponded to the production of approximately 5 μmole/l intracellular O₂⁻ per second. Similarly, in mitochondria, a small fraction of total O₂ reduction gives rise to O₂⁻ (Gardner and Boveris, 1990).
Chemistry of free radicals

A free radical is defined as an atom or molecule that contains one or more unpaired electrons. The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field, and sometimes makes the species highly reactive. Such a definition embraces the atom of hydrogen (one unpaired electron) and the ions of transition metals such as iron, copper and manganese. Free radicals can be anionic, cationic or neutral. In biological and related fields, the major free radical species of interest have been those of oxygen. Radicals can easily be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, a process known as homolytic fission. The energy required to dissociate the covalent bond can be provided by heat, electromagnetic radiation or other means (Halliwell and Gutteridge, 1985).

Molecular oxygen has two unpaired electrons in its outer orbital and its reactivity results from this biradical property. Most of the oxygen is taken up by human cells is reduced to water by the action of mitochondrial cytochrome oxidase. This requires the addition of four electrons to each oxygen molecule as seen in equation (2):

\[ O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \]  

(2)

The intermediate steps of oxygen reduction are formation of superoxide anion radical, hydrogen peroxide and hydroxyl radical (OH), corresponding to reduction by one, two and three electrons, respectively. Superoxide anion radical (Table 1) contains three electrons in the orbitals (\( \pi^*, 2p \)) and, when dissolved in organic solvents, is extremely reactive. Two molecules of superoxide are reduced (with two protons) to \( \cdot OH \). At physiological pH, the low concentration of protons reduces the rate of dismutation. Step-wise single electron addition to molecular oxygen generates a unique spectrum of other reactive intermediates (Table 1). Hydrogen peroxide (H\(_2\)O\(_2\)), formed in vivo via enzymes specific for its removal, such as catalase and glutathione peroxidase, is probably more harmful than superoxide. Hydroxyl radical, produced when water is exposed to high energy ionizing radiation, is also highly reactive. \( \cdot OH \) are generated whenever H\(_2\)O\(_2\) comes into contact with copper ions (Cu\(^{2+}\)) or iron ions (Fe\(^{2+}\)). Since both H\(_2\)O\(_2\) and metal complexes are present in human, it is logical to assume that \( \cdot OH \) can be formed in vivo.
Table 1
Types and period of half-life of free radicals.

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Formula</th>
<th>Half-life (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>'OH</td>
<td>$10^9$</td>
</tr>
<tr>
<td>Alcoxy radical</td>
<td>RO'</td>
<td>$10^-6$</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>$O_2^1$</td>
<td>$10^-5$</td>
</tr>
<tr>
<td>Peroxynitrite anion</td>
<td>ONOO'</td>
<td>0.05-1.0</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>ROO'</td>
<td>7</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>'NO</td>
<td>1-10</td>
</tr>
<tr>
<td>Semiquinone radical</td>
<td>--</td>
<td>days</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>$H_2O_2$</td>
<td>enzyme decomposition</td>
</tr>
<tr>
<td>Superoxide anion radical</td>
<td>$O_2^-$</td>
<td>enzyme decomposition</td>
</tr>
</tbody>
</table>
Molecular (triplet) oxygen can be electronically excited to singlet molecular oxygen, either $\Delta$ or $\sigma$-singlet oxygen. Singlet oxygen is also generated from superoxide anion radical and hydroxyl radical. In $\Delta$-singlet oxygen, both electrons are paired with opposite spin and exist in one orbital, leaving the other orbital empty. In the $\sigma$-singlet state, the two electrons occupy different orbitals, as in the ground state, but are of opposite spins. Singlet oxygen is an especially reactive form of oxygen capable of oxidizing many molecules, including membrane lipids. $\Delta$-singlet oxygen is more common because it is thermodynamically more stable, whereas $\sigma$-singlet oxygen rapidly converts to the $\Delta$-state. Singlet oxygens are not truly free radicals, but are included in the term reactive oxygen species (ROS).

Singlet oxygen is most often generated in the laboratory by photosensitization reactions. If certain molecules are illuminated with light of a given wavelength they absorb it and the energy raises the molecule into an excited state. The excitation energy can then be transferred onto an adjacent oxygen molecule, converting it to the singlet state with the photosensitizer molecule returns to the ground state. Popular sensitizers of singlet oxygen formation in the laboratory include the dyes acridines orange, methylene blue, rose bengal and toluidine blue. Many compounds found in vivo are also effective, such as the water soluble vitamin riboflavin and its derivatives FMN. The singlet oxygen produced on illumination of these substances with light of the correct wavelength can react with other molecules present or it can attack the photosensitizer molecule itself. The chemical changes thereby produced are known as photodynamic effects. Hence illuminated solutions of flavins lose their orange color and chlorophylls their green color as they are attacked, this is called photobleaching (Halliwell and Gutteridge, 1985).

Reactive oxygen species can combine with other atoms or larger molecules to form alkyl- or peroxyl-radicals, e.g. in lipids (Table 1). Recent interest has centered on nitric oxide (NO), the active moiety of endothelial derived relaxing factor, and on other nitrogen-centered free radical species, such as peroxynitrate and peroxynitrite anion. These nitrogen radicals possess long lives and high reactivity. Hemolytic cleavage of peroxynitrous acid (ONOOH) will generate the reactive radical ($\mathrm{NO}_2^-$), which may contribute to peroxynitrous acid toxicity.
Experimental methods for studying free radical must overcome major problems associated with their high reactivity, relatively short half-lives, and short migration distances. Free radicals can only be studied in vitro by physico-chemical methods such as electron spin resonance (ESR). Free radicals can be measured indirectly in vivo by “trapping” them with other chemicals and then measuring the product ex vivo using ESR. Thorough assessment of free radical effects must also include measurement of natural defense systems that protect organisms from their damage, such as superoxide dismutase, catalase, glutathione peroxidase, etc. (Bergendi et al., 1999).

**Cellular Sources of Free radicals**

Inside the cell ROS are produced enzymatically and nonenzymatically. As stated earlier, any electron-transferring protein or enzymatic system can result in the formation of ROS as “by-products” of electron transfer reactions. This “unintended” generation of ROS in mitochondria accounts for ~1-2% of total O₂ consumption under reducing conditions (Freeman and Crapo, 1982). Due to high concentrations of mitochondrial SOD, the intramitochondrial concentrations of O₂⁻ are maintained at very low steady-state levels (Tyler, 1975). Thus unlike H₂O₂, which is capable of diffusing across the mitochondrial membrane into the cytoplasm (Chance et al., 1979), O₂⁻ generated in mitochondria is unlikely to escape into the cytoplasm. The potential for mitochondrial ROS to mediate cell signaling has gained significance in recent years, particularly the regulation of apoptosis (Banki et al., 1999). There is evidence to suggest that tumor necrosis factor (TNF)-α and interleukin (IL)-1-induced apoptosis may involve mitochondria-derived ROS (Sidoti-de Fraisse et al. 1998). It has also been suggested that the mitochondria may function as an “O₂ sensor” to mediate hypoxia-induced gene transcription (Chandel et al., 1998).

The endoplasmic reticulum (ER) is another membrane-bound intracellular organell. Unlike mitochondria, is primarily involved in lipid and protein biosynthesis. Smooth ER contains enzymes that catalyze a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The most extensively studied of these are the cytochrome P-450 and b₂ families of enzymes that can oxidize unsaturated fatty acids and xenobiotics and reduce molecular O₂ to O₂⁻ and/or H₂O₂ (Capdevila et al., 1981). Although there may not to be a direct link between ER-derived oxidants and growth factor signaling, there is evidence for redox regulation of ER-related functions.
such as protein folding and secretion (Bader et al., 1999). Bausking et al. (1991) showed that LTK, a nonreceptor tyrosine kinase (non-RTK) expressed mainly in lymphocytes, leukemia cells, and neurons, is activated by forming disulfide-linked multimers in response to thiol-oxidizing agents. It has also been suggested that an \( \text{O}_2^- \) generating microsomal NADH oxidoreductase may function as a potential pulmonary artery \( \text{O}_2 \) sensor in pulmonary artery smooth muscle cells (Mohazzab and Wolin, 1994).

Nuclear membranes contain cytochrome oxidase and electron transport systems that resemble those of the ER but the function of which is unknown (Faulkner and Fridovich 1993). It has been postulated that electron "leaks" from these enzymatic systems may give rise to ROS that can damage cellular DNA in vivo (Halliwell and Gutteridge, 1985).

Peroxisomes are an important source of total cellular \( \text{H}_2\text{O}_2 \) production (Boveris et al., 1972). They contain a number of \( \text{H}_2\text{O}_2 \)-generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-\( \alpha \)-hydroxyl acid oxidase, and fatty acyl-CoA oxidase. Peroxisomal catalase utilizes \( \text{H}_2\text{O}_2 \) produced by these oxidases to oxidize a variety of other substrates in "peroxidative" reactions (Sidoti-do Fraisse et al., 1998). These type of oxidative reaction are particularly important in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules (including ethanol) that enter the circulation. Another major function of the oxidative reactions carried out in peroxisomes is \( \beta \)-oxidation of fatty acids, which in mammalian cells occurs in mitochondria and peroxisomes (Alberts et al., 1994). Specific signaling roles have not been ascribed to peroxisome-derived oxidants, and only a small fraction generated in these intracellular organelles appears to escape peroxisomal catalase (Boveris et al., 1972; Pool, 1975).

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase and tryptophan dioxygenase can generate ROS during catalytic cycle (Freeman and Crapo, 1982). The most extensively studied of these is the \( \text{O}_2^- \) generating xanthine oxidase, which can be formed from xanthine dehydrogenase after tissue exposure to hypoxia (McKelvey et al., 1988). Xanthine oxidase is widely used to generate \( \text{O}_2^- \) in vitro to study the effect of ROS on diverse cellular processes, however, no studies have implicated a direct physiological role for endogenous xanthine oxidase in cell signaling.
Auto-oxidation of small molecules such as dopamine, epinephrine, flavins, and hydroquinones can be an important source of intracellular ROS production (Freeman and Crapo, 1982). In most cases, the direct product of such auto-oxidation reactions is $\text{O}_2^-$. Although there is no known role for auto-oxidation of small molecules in growth factor and/or cytokine signaling, such reactions may induce oxidative stress and alter the overall cellular redox state. There is a suggestion that the pro-oxidant effects of dopamine auto-oxidation may be involved in the dopamine-induced apoptosis that is implicated in the pathogenesis of neurodegenerative disease such as Parkinson’s (Offen et al., 1997).

Plasma membrane-associated oxidases have been implicated in most growth factor- and/or cytokine-stimulated oxidant production (Krieger-Brauer and Kather 1995, Sundaresan et al., 1995), although the precise enzymatic sources have yet to be fully characterized. The best characterized of the plasma membrane oxidases in general is the phagocytic NADPH oxidase, which serves specialized function in host defense against invading microorganisms. This multicomponent enzyme catalyzes the one-electron reduction of $\text{O}_2$ to $\text{O}_2^-$, with NADPH as the electron donor through the trans-membrane protein cytochrome $b_558$. The transfer of electrons occur from NADPH on the inner face of the plasma membrane to $\text{O}_2$ on the outside. During phagocytosis, the plasma membrane is internalized and wall of the phagocytic vesicle, which was once outer membrane surface now facing the interior of the vesicle. This targets the delivery of $\text{O}_2^-$ and its reactive metabolites internally for localized microbicidal activity (Babior, 1999).

Enzymes involved in phospholipid metabolism are known to exist for several decades. Membrane phospholipids, in addition to their structural role in providing membrane integrity, are substrates for the action of phospholipases (PLs) PLA$_2$, PLC, and PLD. Although these enzymes are important for the generation of lipid second messengers, they have generally not been associated with ROS production in nonphagocytic cells. A recent report by Touyz and Schiffrin (1999), however, suggests that ANG II-induced $\text{O}_2^-$ production in smooth muscle cells is dependent on the PLD pathway.

PLA$_2$ hydrolyzes phospholipids to generate arachidonic acid. Arachidonic acid then forms the substrate for cyclo-oxygenase- and lipoxygenase (LOX)-dependent synthesis of the four major classes of eicosanoids: prostaglandins, prosta-cyclins, thromboxanes, and leukotrienes. These synthetic pathways involve a series of oxidation
steps that involve a number of free radical intermediates (Freeman and Crapo, 1982). Arachidonic acid metabolism, particularly involving the LOX pathway, which leads to leukotriene synthesis, was reported to generate ROS (Baud and Ardaillou, 1986; Lim et al. 1983, Nakamura et al., 1985; Singh et al., 1981). LOX activity has also been implicated in redox-regulated signaling by ANG II (Wen et al., 1997), epidermal growth factor (EGR) (Mills et al., 1998), and IL-1 (Bonizzi et al., 1999). There is the suggestion that LOX-derived lipid peroxidation products may be involved in the oxidative stress response to asbestos (Faux and Howden, 1997). TNF-α-induced apoptosis appears to be mediated by LOX-dependent but ROS-independent mechanism (O’Donnell et al., 1995). A lipid-metabolizing enzyme in fibroblasts similar to 15-LOX is shown to generate large amounts of extracellular O$_2^{-}$ that appears to be independent of flavoenzyme activity (O’Donnel and Azzi, 1996).

**Photochemical Production of Oxy Radicals**

Many molecules are known photodynamic sensitizers, including various acridines, porphyrins, phenothiazines, quinones, flavins, and metal derivatives such as ruthenium bipyridyl. An activated sensitizer can react directly with other molecules to produce free radicals by removal of an electron or of hydrogen, or the sensitizer can react with singlet oxygen. Another route to activated oxygen species lies in photoreduction of the molecule in the presence of a suitable hydrogen donor, followed by reaction of the reduced molecule (either one- or two-electron) with di-oxygen. The transfer of an electron to yield superoxide radical anions.

Photoreduction of riboflavin or of flavin mononucleotide is extensively used for production of superoxide anions during the reoxidation of the dihydroflavin with donors such as methions, EDTA, or NADH to facilitate the photoreduction at 365nm. Reagents which are commonly used to detect the O$_2^{-}$ include ferricytochrome c (which is reduced), tetranitromethane (reduced), nitroblue tetrazolium (reduced), and luminol (oxidized with light emission) (Greenwald, 1987).
**Possible Implication of Reactive Oxygen Species.**

**Hydroxyl radical**

'OH is an extraordinarily powerful oxidant, which attacks most organic compounds at diffusion-limited rates (Czapski, 1984). First encountered during studies of the effect of ionizing radiation on water, it can also be produced by the reduction of \(H_2O_2\) by metal cations such as Fe(II) or Cu(I).

\[
\begin{align*}
O_2^- + \text{Me}(n) & \leftrightarrow O_2 + \text{Me}(n-1) \quad (3) \\
\text{Me}(n-1) + H_2O_2 & \rightarrow \text{Me}(n) + \cdot OH + \cdot OH \quad (4)
\end{align*}
\]

Reaction (4) above can be broken down into a series of steps as follows:

\[
\begin{align*}
\text{Fe(II)} + \text{HOOH} & \leftrightarrow \text{(Fe-OOH)}^+ + H^+ \quad (4a) \\
\text{(Fe-OOH)}^+ & \leftrightarrow \text{(FeO)}^{2+} + \cdot OH \quad (4b) \\
\text{(FeO)}^{2+} + H^+ & \leftrightarrow \text{(Fe-OH)}^{3+} \quad (4c) \\
\text{(Fe-OH)}^{3+} & \leftrightarrow \text{Fe(III)} + \cdot OH \quad (4d)
\end{align*}
\]

The powerful oxidant produced by any of the above reactions like \((\text{FeO})^{2+}\) or \((\text{FeOH})^{3+}\) or \'OH are highly damaging to cell and its components. This is particularly the case since Fe(III) would not exist in free solution, but rather exist in bound formed with polyanions such nucleic acids or to phospholipid membrane. In that case, the \'OH, or \((\text{Fe-OH})^{3+}\) or \((\text{FeO})^{2+}\) would be produced adjacent to, and would selectively attack, those critically important targets.

Cells are rich in reductants, such as thiols and enediols, and these are able to reduce Fe(III) to Fe(II), thus obiating the need for reaction (3). None the less, \(O_2^-\) does collaborate with \(H_2O_2\) in producing 'OH within cells and it worked so by oxidizing the \([4Fe-4S]\) clusters of dehydratases, such as aconitase, causig the release of Fe(II). In this way, \(O_2^-\) increases the availability of iron for reactions (4a)-(4d). Liochev and Fridovich have proposed this mechanism (Liochev and Fridovich, 1994) and it was subsequently experimentally verified by Keyer and co-worker (Keyer et al., 1995; Keyer and Imlay, 1996).

**Superoxide anion radical**

\(O_2^-\) is more selective in its reactivity. Means that 'OH is potentially more damaging, thus, 'OH will react with something, perhaps something expendable, within a very small radius of its site of generation, whereas \(O_2^-\) can diffuse a considerable
distance before it encounters a suitable, and possibly critical, target. $O_2^-$ is the conjugate base of the hydroperoxyl radical ($HOO^\cdot$) whose pKa is approximately 4.8, and spontaneous dismutations can occur as proposed by Bielski (1978):

$$\cdot OOH + \cdot OOH \leftrightarrow H_2O_2 + O_2 \quad (5)$$

$$\cdot OOH + O_2^- + H^+ \leftrightarrow H_2O_2 + O_2 \quad (6)$$

$$O_2^- + O_2^- + 2H^+ \leftrightarrow H_2O_2 + O_2 \quad (7)$$

$O_2^-$ is thus intrinsically unstable in protic solvents such as water but, because reaction (7) is so slow, the higher the pH the more stable it becomes. At neutral pH, it is stable enough to oxidize compounds such as polyphenols, thiols, ascorbate, catecholamines, leukoflavins, tetrahydropterins and sulfite. More importantly, it can rapidly inactivate aconitase and similar $[4F-4S]$-containing dehydratases.

The limited reactivity of $O_2^-$ with a range of metabolic intermediates (Bielski and Richter, 1977) led some to argue that $O_2^-$ was benign and that SODs were not needed for defense (Fee 1980; Sawyer and Valentine, 1981). To counter this position, the ability of $O_2^-$ to cause the oxidation of sulfite, thiols, catechols, catecholamines, leukoflavins, and tetrahydropterins was documented (DiGuiseppi and Fridovich, 1984). Upon reaction with $O_2^-$ several enzymes were found to be inactivated (Kono and Fridovich, 1982; Benov and Fridovich, 1999).

Of more general importance was the observation that $O_2^-$ and $H_2O_2$ could collaborate in the production of the vastly reactive hydroxyl radical (Beauchamp and Fridovich, 1970). At the time it was not appreciated that metal impurities in the phosphate buffer played a critical role in this process. When that was demonstrated (McCord and Day, 1978; Halliwell, 1978), it became apparent that the process must involve the reduction of ferric chelates by $O_2^-$, followed by the reduction of $H_2O_2$ to $'OH + 'OH$ by the ferrous chelates. Referred to as the metal-catalyzed Haber-Weiss reaction, this process was thought possible in vivo. Others correctly objected that iron, to the extent that it was “free” in cells, would be kept reduced by abundant biological reductants such as glutathione, thus obviating the need for $O_2^-$ (Winterbourn, 1979). Yet the collaborative interaction of $O_2^-$ and $H_2O_2$ in imposing oxidative damage was made apparent by the increased sensitivity of SOD-null mutants of *Escherichia coli* to $H_2O_2$ (Carlioz and Touati, 1986).
Defense Systems

Indigenous System of Defenses

Due to both the background generation of reactive oxygen species from ambient radiation and the endogenous generation of cellular ROS outlined above, cells have evolved an elaborate series of mechanisms to minimize ROS presence and reduce damage if it occurs. Figure 2 outlines the pathways involved in ROS generation and control.

Oxidative stress was considered purely from the toxicological perspective. A relatively small number of free radicals such as the superoxide anion and the hydroxyl radical were recognized as minor by-products of oxidative phosphorylation. Britton Chance and colleagues (1973) have determined that approximately 2% of the oxygen reduced by the mitochondrion forms O$_2^-$ or the dismutation product H$_2$O$_2$. This estimate has been confirmed repeatedly (Hensley et al., 1998). Superoxide and peroxide react with metal ions to promote additional radical generation, with the release of the particularly reactive hydroxyl (Stadtman, 1990). Hydroxyl radicals react at nearly diffusion-limited rates with any component of the cell, including lipids, DNA, and proteins. The net result of this nonspecific free radical attack is a loss of cell integrity, enzyme function, and genomic stability (Stadtman and Berlett, 1991; Halliwell, 1999). Consequently, numerous detoxification mechanisms have evolved to deal with oxyradical stress.

Defenses against O$_2^-$

There are multiple defenses against O$_2^-$. These are the superoxide dismutases (SODs) which catalyze reaction at diffusion-limit rates. Such catalysts are abundant in aerobic cells and they keep the steady-state level of O$_2^-$ in the $10^{-10}$ mol/l range (Imlay and Fridovich, 1991). This would be the case if the spontaneous and the SOD-catalyzed dismutations were the only fates open to O$_2^-$. However, there are targets that would be
Figure 2. Source of reactive oxygen species and mechanism of their removal from cells.
O2

Flavin oxidase
Catalase
GSH peroxidase
GSH
GSSG
NADPH
Cys (glu, gly)

SOD
H2O2

OH

NO

Peroxynitrite

Damage
DNA
Lipid
Protein

Signalling
GSH
Protein Oxidation

Respiration
P450 reductase
Xanthine oxidase
NADPH oxidase
Semiquinones
Catecholamines
Radiation

O2

H2O

Radiation

H2O
attacked by $O_2^-$, if they were not removed by SOD. It has recently been estimated that the SODs in *E. coli* provide approximately 95% protection for all targets susceptible to $O_2^-$ attack in that cell (Liochev and Fridovich, 1997). Returning to the multiplicity of SODs, we note that there are SODs that depend for their activity on active sites containing Cu and Zn, Mn, Fe and even Ni. There are SODs that are cytosolic, localized to specific subcellular organelles and also secreted from the cell. The CuZn SODs are enzymes that have Cu and Zn at their active sites. The copper undergoes valence changes during the catalytic cycle while the Zn is thought to play mainly the structural role. CuZnSODs are found in the cytosols as dimer in eukaryotic cells, in the periplasms of gram-negative bacteria, in the plastids of plants and in the extracellular spaces of mammal’s (Tainer *et al.*, 1982). The Cu(II) and the Zn(II) are ligated to a bridging imidazolate that plays a role in proton conduction. Thus, upon reduction of the Cu(II) by $O_2^-$, the Cu-imidazolate bond is broken and the imidazolate becomes protonated. During reoxidation of the Cu(I) by the next $O_2^-$, the Cu-imidazolate bond is re-established while the protons convert the reduced $O_2^-$ to HO$_2^-$, which leaves the active site and picks up a second proton to become H$_2$O$_2$. This mechanism was proposed on kinetic grounds (Hodgson and Fridovich, 1975) and recently verified though X-ray absorption fine structure (XAFS) and crystallography (Murphy *et al.*, 1997). The MnSODs, which are as active as the CuZnSODs, but are unrelated as judged by sequence, may be dimeric or tetrameric. They contain one Mn(III) per subunit and their structures have been determined by X-ray crystallography (Wagner *et al.*, 1993). The *E. coli* MnSOD is dimeric and it is not ordinarily produced when the cells are growing anaerobically (Fridovich, 1998). However, it is induced under aeration and is further induced by compounds that can increase intracellular $O_2^-$ production. The homotetrameric nickel-containing SOD has recently been described from *Streptomyces griseus*, which also contains a homotetrameric FeSOD.

However, SOD deficiency in the organism results in increased sensibility to hyperoxia as can be noticed in children with monosomy 21. These children may have cardiac murmur, bone abnormalities and other morphological disorders. The increased production of superoxide and hydrogen peroxide leads to, under certain conditions, DNA damage, while these reactive forms of oxygen do not interact directly with DNA
but probably by means of another highly toxic metabolite, hydroxyl radical, which can be produced from them by Fenton type reaction (Bergendi et al., 1999)

**Defenses against H\textsubscript{2}O\textsubscript{2}**

The catalases and peroxidases, dismute 2H\textsubscript{2}O\textsubscript{2} into O\textsubscript{2} + 2H\textsubscript{2}O, using various reductants, are the enzymes that deal with H\textsubscript{2}O\textsubscript{2}. Some of the peroxidases can also reduce alkyl hydroperoxides to the corresponding alcohols. As was the case with the SODs, the catalases and peroxidases constitute a diverse family of enzymes. Most of these are ferriheme enzymes, and their action involves the divalent oxidation of the heme to an Fe(IV) π cation radical by H\textsubscript{2}O\textsubscript{2}, followed by divalent reduction by H\textsubscript{2}O\textsubscript{2}, in the case of catalase, and by two successive univalent reductions by the organic substrate, in the case of the peroxidases (Dolphin et al., 1971).

Mammalian catalase are homotetrameric ferriheme-containing enzymes whose subunit mass is approximately 60 kDa. These enzymes are most efficient when dealing with relatively high concentration of H\textsubscript{2}O\textsubscript{2} because their Km for H\textsubscript{2}O\textsubscript{2} lies in the millimolar range. Hence catalase is packaged into peroxisomes, along with many H\textsubscript{2}O\textsubscript{2}-producing enzymes. Mammalian catalase can also act as a peroxidase towards a few small molecules such as methanol, ethanol, nitrite and formate. Thus, it can use H\textsubscript{2}O\textsubscript{2} to oxidize these substrates, which are small enough to gain access to the heme iron. The structure, as determined by X-ray crystallography, indicates that the heme lies deeply buried in the protein and is thus accessible only to small substrates (Reid III et al., 1981). Catalase contains tightly bound NADPH, which may function to prevent the accumulation of an inactive Fe(IV) form of the enzyme (Kirkman et al., 1987).

**Exogenous System of Defenses**

Studies of antioxidants are important because they can be used to protect polymers and foodstuffs against oxidative damage, e.g. during sterilization of food by ionizing radiation, and for possible use in the treatment of patients who ingested compounds that increase lipid peroxidation in vivo, and recently it has shown possibility of therapeutic benefit in the treatment of cancer (Brennan, 2000).

Ascorbic acid is an important antioxidant of human plasma. It is able to rapidly react with many reactive oxygen species, especially with peroxyl radical, together with the fact that ascorbic acid forms with radicals a low reactive semidehydroascorbate
radical also. Ascorbic acid is a significant antioxidant in the absence of transition metal ions, while in their presence its prooxidative properties are preferred. Recommended high doses of vitamin C (sometime even more than 10 g/day) need not be harmful for a healthy human, whereas they need not be useful in some diseases (Halliwell, 1991).

α-Tocopherol (vitamin E) itself is widely used in many foodstuffs, it is concentrated in the adrenal glands and in blood lipoproteins. It is the major, if not the only, lipid soluble antioxidant in human blood plasma. Vitamin E both quenches and reacts with singlet oxygen and could therefore protect the membrane against this species. It also reacts with the superoxide radical, although this is probably less important since the reaction is slow and $O_2^-$ does not initiate lipid peroxidation. In most membranes vitamin E can react with lipid peroxy radical to form vitamin E radical that are insufficiently reactive to abstract H from the membrane lipid. It thus interrupts the chain reaction of lipid peroxidation by acting as a chain terminator. The vitamin E radical produced are fairly stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure, so increasing stability. Furthermore, the butylated hydroxyanisole (BHA) is very often added to foodstuffs. It acts as an antioxidant by hydrogen donation, which is common to all the phenolic antioxidants. For example, addition of BHA to fat, e.g. butter, increases its storage life from a few months to a few years (Halliwell and Gutteridge, 1985).

**Function of Reactive Oxygen Species**

*Development of Limb and Respiratory Muscle Fatigue*

Many reports have indicated that reactive oxygen species represent one of the factors capable of modulating the rate of development of limb and respiratory muscle fatigue. Free radical production by contracting muscle has been demonstrated by direct, e.g. EPR and ESR studies, (Davies et al., 1982; Borzone et al., 1994). Indirectly lipid peroxidation (Brady et al., 1979), protein carbonyl formation (Witt et al., 1992, glutathione oxidation (Anzueto et al., 1992) processes are the index of free radical damage.
**Process After the Phagocytosis by Macrophages**

Most studies on biochemistry of phagocytosis have carried out using neutrophils and macrophages, especially pulmonary macrophages since they are the only macrophages that can readily be obtained from humans. At the onset of phagocytosis, however, the cell shows a marked increase in oxygen uptake that is not prevented by cyanide, and so is unrelated to mitochondrial electron transport. This respiratory burst can be ten or twenty times the resting respiratory rate in neutrophils, but is less marked in macrophages. Some strains of bacteria are quickly killed by hydrogen peroxide, which will be formed from $O_2^{-}$ by the dismutation reaction. Also, the ‘OH, formed from $O_2^{-}$ and hydrogen peroxide by an iron-catalysed Haber-Weiss reaction may sometimes participate in killing (Halliwell and Gutteridge, 1985). Nitric oxide and nitrates are produced in vitro by macrophages incubated in the presence of LPS and IFN-α. They are originated from the semi essential amino acid arginine by the activity of nitric oxide synthase (Stuehr and Marletta, 1987).

**Free Radical and Inflammatory Diseases**

Reactive oxygen species can also damage host tissue especially in the vicinity of the inflammatory site. Reactive oxygen species have been reported to be involved in tissue injury associated with a number of inflammatory diseases including rheumatoid arthritis, atherosclerosis, adult respiratory distress syndrome, ischemia-reperfusion injury of the heart, brain, pancrease, and others (Stvrtinova et al., 1995). The essential role played by the NADPH oxidase in protecting the body from infection is unequivocally demonstrated by a rare genetic disorder known as chronic granulomatous disease. Chronic granulomatous disease is characterized by genetic mutations in one of the required NADPH oxidase component proteins which result in an inactive oxidase and a shortage of hydrogen peroxide for the myeloperoxidase system (Carsky et al., 1993). Patients with Chronic granulomatous disease experience severe recurrent bacterial and fungal infections that are somewhat resistant to conventional treatments (Carsky et al., 1993).

**Free Radicals in Cardiovascular Disease**

Most cardiovascular disease events are the consequence of a thrombotic occlusion in diseased arteries containing established atherosclerotic plaques.
Pathological observations have shown that most lipid material is derived originally from circulating low-density lipoprotein (LDL) particles which are taken up by macrophages and other cells of the vascular wall. However, oxidative modification of LDL particles, which occur mainly through the effect of reactive oxygen species, seems to be a critical event in facilitating their rapid uptake into the cells of the vascular wall via an alternative scavenger receptor (Steinberg et al., 1989). Oxidized LDL has other deleterious effect, it is a chemoattractant and enhances the recruitment of monocyte/macrophages into the intima, it is cytotoxic toward endothelial cells and it inactivates nitric oxide released by endothelium. These effects all contribute to the endothelial dysfunction which has been found in association with the important coronary risk factors. The endothelium normally plays a pivotal role in healthy vascular function by controlling vascular tone, cellular proliferation and haemostasis largely via the production of nitric oxide. The presence of endothelial dysfunction is thought to facilitate the subsequent development of vascular disease (Maxwell, 2000).

Reactive Oxygen Species and Neuronal Death

Neuron cell death contributes to some of the most debilitating diseases of the brain, including stroke, brain trauma, Alzheimer, Parkinson, and Huntington diseases. Even in diseases that are largely considered non-neurological, such as AIDS, there can be neuro-degeneration that due to its irreversibility, may be a lifelong consequence once the pathogenesis is halted (Zhang et al., 1998).

Another active area of research and clinical investigation has been the role of reactive oxygen species in the death of neurons. Energy transport and utilization by living organisms is carried out through oxidative phosphorylation, the transfer of electrons in a series of chemical reactions within the mitochondrion. Of course, injuries that break blood vessels can expose neurons to the free-radical-forming catalyst iron. The Fenton reaction, through which iron catalyzes the production of hydroxyl radical, often is initiated experimentally by exposing neurons to ferric iron. Another clinically relevant class of compounds that can lead to increase in free-radical production within neurons is the amyloid peptides. Free-radical elevation is a shared effect of the prion protein implicated in bovine spongiform encephalopathy (mad cow disease), the Alzheimer -amyloid peptide, and amylin. The peptide -amyloid is the main constituent of plaques that are the hallmarks of Alzheimer disease. It has been shown to directly
generate free radicals in aqueous solution even in the absence of cellular components (Hensley et al., 1994). The stable by-products of reactive oxygen species (oxidized lipids and modified proteins) can be detected in Alzheimer disease as well as other neurodegenerative diseases (Hutchins and Barger, 1998).

**Reactive Oxygen and Signal Transduction**

Reactive oxygen species formed in the mitochondria and in the cytosol are important determinants of the redox state of protein cysteinyl residues, and therefore, they constitute a common regulatory mechanism of protein conformation and function. Reactive oxygen species-dependent redox cycling of cysteinyl thiols is also critical for the establishment of the protein-protein and protein-DNA interaction that determine many aspects of a signal transduction pathway (Ziegler, 1985).

Glutathione is the key regulator of the redox state of protein cysteinyl thiols. These thiols will react with GSSG if their pKa value is enough to generate a reduction potential greater than that of glutathione thiolate anion. Reactivity will tend to increase if electron-withdrawing substituents, such as basic amino acids, are in close proximity to a cysteinyl residue, because they will tend to decrease its pKa (Thomas, 1995). The intracellular level of GSSG increases from metabolism of H2O2 by glutathione peroxidase and decreases from the export of GSSG out of the cell and also through glutathione reductase-and NADPH-mediated reconversion of GSSG to GSH (Cotgreave et al., 1988). By far, GSH is the major form of cellular glutathione, typical GSH/GSSG ratios in normal mouse liver tissue range from 50 to 200 (Jaeschke, 1990). Because of the low concentrations of GSSG relative to GSH, small increase in the oxidation of GSH to GSSG resulting from increase in reactive oxygen species and H2O2 metabolism will tend to produce large increase in GSSG and in steady state ratios of GSSG/GSH. GSSG increase will promote oxidation of protein cysteinyl thiols, shifting the equilibrium of thiol-disulfide exchange significantly in the direction of mixed disulfide formation and, ultimately, changing protein conformation (Thomas et al., 1995).

Reactive oxygen species not only regulate the activity of pre-existing proteins, they also are responsible for inducing the expression of many genes (Keyes and Tyrrell, 1989), and for the perturbation of the signal transduction circuits responsible for maintenance of concerted patterns of gene expression (Schulze-Osthoff et al., 1997). In particular, reactive oxygen species are critical in the regulation of transcription factors
in the AP-1 (Devary et al., 1991). NF-kB (Toledano and Leonard, 1991), and AP-2 (Grether-Beck, 1996) the three transcription factors families that have crucial functions in proliferation, differentiation, and morphogenesis. Reactive oxygen species signaling pathway for AP-1 and NF-kB are activated in enucleated cells and in the absence of protein synthesis, indicating that DNA damage or nuclear factors are not required for their activation (Devary et al., 1993).

**Protein Modification Studies**

There is a great deal of radiobiological literature on free radical interactions with nucleic acids and these interactions have recently been coupled to the demonstration of expected oxidation products in human urine. Hydroxyl radicals are known to fragment polysaccharides (e.g. hyaluronic acid) and free radicals participate in lipid peroxidation (Slater, 1984). In contrast, little is known of the interactions of free radicals with proteins. Proteins may thus be critical targets because they are present inside and outside the cell in high concentrations and, since many are catalytic in nature, modifications by free radicals may have an amplified effect on their activity i.e. susceptibility to proteolysis, inactivation and degradation etc.

**Protein Degradation as an Index of Oxidative Stress**

Reactive oxygen species are known to participate in numerous physiological and pathological processes. Situations that augment oxidant exposure, or compromise antioxidant capacity, are commonly referred to as oxidative stress. Oxidative stress can result from exogenous sources such as redox-active xenobiotics (Davies and Doroshow, 1986; Doroshow and Davies, 1986; Marcillat et al., 1989) or from increased in endogenous oxidative metabolism i.e., mitochondrial electron transport (Davies et al., 1982). Regardless of its source, oxidative stress has been found to effect the behavior of several different cell types.

Oxidative stress, as already mentioned, is the most ubiquitous stress to which mammalian cells are subjected (Sies, 1991). The mechanism of triggering of the stress response is under active discussion, both in general (Staus et al., 1987; Morimoto et al.,
and as a consequence of oxidative stress (Morimoto et al., 1990; Tartaglia et al., 1991).

**The Involvement of Reactive Oxygen Species**

All aerobic cells possess a number of repair and detoxification mechanisms trying to prevent the cells from oxidant-induced damage. In a normally functioning physiological system, there exits a balance between the production of oxidising species and defense against them. Cellular macromolecules, including proteins, are damaged permanently by free radicals. Oxidative damage to nucleic acids is subject to repair by highly efficient excision/insertion mechanisms, the repair of damage to proteins appears limited to the reduction of oxidized derivatives of the sulfur-containing amino acid residues. Repair of other kinds of protein oxidation has not been demonstrated. Instead, the damaged proteins are targeted for degradation to amino acid constituents by the action of various endogenous protease, including, cathepsin c, calpain, trypsin and especially the 20s proteosome (Grune et al., 1996; Rivett, 1986), whose activity is also under metabolic control by diverse regulatory factors, including the concentrations of enzyme substrates, ubiquitination, and various inhibitors like crosslinked proteins (Friguet et al., 1994), glycation/glycoxidation of protein conjugates (Kristal and Yu, 1992), etc.

In a complex structure like a protein, in addition to modification of amino acid side chain, oxidation reactions were able to destroy, at least partially, the secondary and tertiary structure of the macromolecule. This process is accompanied by an exposure of hydrophobic domains to the protein surface (Pacifici et al., 1993). Hydrophobic surface domains of proteins tend to stick together and form non-covalent aggregates. This process is often accelerated by electrostatic interactions. In addition to the cross-linking reactions which are able to form an insoluble net of covalently cross-linked protein molecules. These crosslinking reactions may be due to the action of the oxidant itself, like the formation of the 2,2-bi-phenyl crosslink from two tyrosyl radicals, or due to the action of cross-linking sugars or lipids (Grune et al., 1997). The direct reaction of several oxidants with proteins may results in protein fragments, that means, oxidants are able to cleave the polypeptide backbone chemically. The cleavage occurs at the Cα-atom of the polypeptide. Therefore, the result of an oxidative cleavage of a polypeptide are modified peptides, like in the case of degradation by proteolytic enzymes (Grune et al., 1990).
1997; Stadtman, 1993). Although the formation of peptide fragments was reported in vitro, evidence does exist, that such peptide fragments are formed due to oxidative stress in living cells (Stadtman, 1993).

**Susceptibility of Radical-Damaged Proteins to Enzymatic Proteolysis**

A number of processes known to occur in vivo may, contribute either directly or indirectly, to the formation of damaging free radicals. Examples include the reparatory burst of phagocytes (Clark et al., 1985), mitochondrial electron transport (Dean and Pollak, 1985), mitochondrial degradation during reticulocyte maturation, seems to depend on radicals produced by an endogenous lipoxygenase (Rapoport et al., 1985). Furthermore, Dean and Pollak (1985) found that isolated rat liver mitochondria in state four (limited by availability of ADP) degrade their endogenously synthesized protein faster when radical fluxes are enhanced (by chain blockers and uncouplers) as compared to when they are minimized. Chloroplastic 32 Kda protein is also degraded more rapidly when radical fluxes are enhanced by illumination (Matto et al., 1984). In a few cases, endogenous agents which may increase radical generation are known to enhance proteolysis, for instance, phenylhydrazine increases breakdown of haemoglobin in reticulocytes (Goldberg and Boches, 1982). It has been also shown that free radicals can fragment monoamine oxidase, a protein in outer mitochondrial membrane (Dean et al., 1986). Cellular proteolysis requires ATP and so changes in ATP concentration can influence proteolysis (Khairallah et al., 1984). It is interesting that depletion of cellular reductants (such as NADH and Glutathione (GSH)) is often associated with enhanced proteolysis (Khairallah et al., 1984). One explanation might be the decreased repair of radical damage to proteins under conditions of oxidative stress. Similarly, zinc is known to possess some antioxidant properties and there can be an inverse correlation between zinc status and proteolytic rate (Wolff and Dean, 1986). Model antioxidants, such as vitamin E, have been shown to play a critical role in retarding radical induced proteolysis, particularly that involving lipid intermediates (Dean and Cheesman, 1987).

It was demonstrated that after free radical or oxidant attack on proteins, the hydrophobicity of surface increases. Surface hydrophobicity is the key factor for the recognition and degradation of the substrate by several proteases, especially by the
proteasome (Pacifici et al., 1993). With increasing radical exposure the degradation by
the proteasome is increasing (Pacifici et al., 1993; Grune et al., 1997). Oxidized
proteins tends to aggregate and form covalent cross-links, which are actually poor
substrates for the proteasome, cells prevent the accumulation of oxidized proteins. It
could be demonstrated that the protein turnover is increased after treatment with
hydrogen peroxide or other oxidative stresses (Grune et al., 1995; Grune et al., 1997).

Rivett postulated first the selective degradation of oxidatively damaged
glutamine synthetase by the proteasome (Rivett, 1985). It was shown that this
degradation in vitro is ATP-independent, and that in fact ATP inhibits the degradation
of all oxidized protein to about 10-20% (Grune et al., 1997; Grune et al., 1995).
Nevertheless the accumulation of ubiquitinated proteins and activation of the ubiquitin
system was measured after oxidative stress (Shang et al., 1997). The role of the
proteasomal system in the degradation of oxidized proteins in living and dividing cells
was clearly demonstrated by antisense oligonucleotides against an essential proteasome
subunit (Grune et al., 1995). Recent studies demonstrated the function of the
proteasome for the degradation of oxidized proteins in cell nucleus (Ullrich et al.,
1999). Therefore, it seems to be an important part of the normal function of the
proteasome to degrade oxidized proteins within the cytosolic and the nuclear
compartment.

**Metal-Catalyzed Oxidation of Proteins**

Presence of the transition metals, Fe(II) or Cu(I), and under normal conditions
can catalyse the conversion of hydrogen peroxide and alkylperoxides to the highly
reactive hydroxyl radical (reaction 8) or alkoxyl radical (reaction 9) which are capable
of reacting with almost any organic substance.

\[ \text{H}_2\text{O}_2 + \text{Fe(II)/Cu(I)} \rightarrow \cdot\text{OH} + \cdot\text{OH} + \text{Fe(III)/Cu(I)} \]  
\[ \text{ROOH Fe(II)/Cu(I)} \rightarrow \text{RO}^+ + \cdot\text{OH} + \text{Fe(III)/Cu(I)} \]

Virtually all kinds of amino acids residues of proteins are potential targets for
oxidation by \( \cdot\text{OH} \) generated by ionizing radiation or by high concentrations of \( \text{H}_2\text{O}_2 \) and
Fe(II) (Huggins et al., 1993). However, since the low concentrations of iron or copper
ions and \( \text{H}_2\text{O}_2 \) are present under most physiological conditions, protein damage is likely
to be limited to the modification of amino acid residues at metal binding sites on the
protein, which effectively concentrate the ions. This consideration gave rise to the proposition, that the oxidation of proteins under physiological conditions is a site-specific process in which, the binding of Fe(II) or Cu(I) to metal binding sites on the protein is followed by reaction with peroxides to generate reactive species, such as ‘OH and RO' radical, that will react preferentially with amino acid residues at the metal binding site (Bachur et al., 1979; Levine et al., 1981). Thus, the metal-binding site supports a biologically important “caged reaction”. Many enzymes, especially those requiring a metal ion for activity, this will lead to loss of catalytic function (Fucci et al., 1983).

**Carbonyl Groups as Markers of Oxidative Damage**

Reactive oxygen species-mediated oxidation of proteins leads to the conversion of histidine residues to 2-oxohistidine (Uchida and Kawakishi, 1993; Berlett et al., 1996), tryptophan residues to kynurenine or N-formylkynurenine (Kikugawa et al., 1994; Winchester and Lynn, 1970), tyrosine residues to dihydroxy derivatives (Huggins et al., 1993; Dean et al., 1993), methionine residues to methionine sulfoxide or methionine sulfone derivatives (Berlett et al., 1996), leucine and valine residues to hydroxy derivatives (Garrison, 1987), and cysteine residues to disulfide derivatives (Garrison, 1987; Swallow, 1960). Of particular significance is the fact that oxidation of some amino acid residues (lysine, arginine, and proline residues) leads to the formation of carbonyl derivatives (Amici et al., 1989; Creeth et al., 1983; Uchida et al., 1990). In addition, carbonyl derivatives of proteins are also produced as a consequence of oxidative cleavage of the peptide backbone via the amidation pathway (Garrison, 1987; Swallow, 1960), or cleavage associated with the oxidation of glutamyl residues (Garrison, 1987; Uchida et al., 1990). Carbonyl derivatives can also be formed as a consequence of secondary reactions of some amino acid side chains with lipid oxidation products, such as 4-hydroxy-2-nonenal (Friguet et al., 1994; Schuenstein and Esterbauer, 1978), or with reducing sugars or their oxidation products (Mullarkey et al., 1990; Creeth et al., 1983). Likewise, reaction of one of the two aldehyde groups of the lipid peroxidation product, malondialdehyde, with lysine α-amino groups of proteins will yield a Schiff base possessing a carbonyl function. The interaction of reducing sugars or dicarbonyl compounds derived from the sugars can also lead to the formation of protein carbonyl derivatives. It is noteworthy that carbonyl groups of proteins
generated by one of these mechanisms may react further with the α-amino group of lysine residues in the same or another protein molecule to form intra- or inter-molecular crosslinked protein derivatives. Some of these derivatives are not only resistant to proteolytic degradation by the 20s proteosome, but may also inhibit the ability of the proteosome to degrade the oxidized forms of other proteins (Friguet et al., 1994; Friguet et al., 1994(B)), they may therefore, contribute to the accumulation of oxidized forms of proteins during aging and age-related diseases (Friguet et al., 1994(B)).

The fact that carbonyl groups are major products of reactive oxygen species-mediated oxidation reactions have led to the development of several highly sensitive methods for the determination of protein carbonyl groups (Levine et al., 1994), and the presence of carbonyl groups has become a widely accepted measure of oxidative damage under conditions of oxidative stress, aging and disease (Berlett and Stadtman, 1997). It is clear that protein carbonyl is a marker for oxidative modification of proteins. As outlined above carbonyl groups arise from a variety of oxidative processes, so that the carbonyl measurement provides a generalized or integrated assessment of oxidative damage. The absolute value of the carbonyl measurement will therefore usually be much higher than the measurement of any single product. A typical value for the carbonyl content of organs from healthy young animals is 2 nmole/mg protein (~0.10 mol carbonyl/mol protein), so that on average one of every ten protein molecules carries a carbonyl group. In contrast, a specific oxidation product may be present at very low levels. For example, dopa can be formed from the oxidation of tyrosine, and it also reacts with carbonyl reagents. In low density lipoprotein, there is approximately 1 dopa per 1,600 tyrosine residues (Dean et al., 1997). Thus, it is not surprising that measurement of dopa in tissue protein would give values which are orders of magnitude less than the protein-associated carbonyl in the same sample (Dean et al., 1997). Also, as outlined in this section, the oxidative modification giving rise to the carbonyl group is emerging as more than a marker of oxidative stress. The oxidative modification may be the key to dysfunction induced by oxidative stress.

**Antioxidant Activity of Methionine Residues of Proteins**

Methionine residues of proteins are particularly sensitive to oxidation by virtually all kinds of reactive oxygen species. Ozone (Mudd et al., 1969), hydrogen peroxide (Kido and Kassell, 1975), alkyl peroxides (Chao et al., 1997), peroxynitrite...
Interaction between intermediates in Lipid Peroxidation and Protein Degradation

Food technology studies have shown that peroxidizing lipid damages proteins. Most emphasis has been placed on protein cross-linking but there is also evidence of fragmentation (Hunt et al., 1988; Wolff and Dean, 1987). These reactions with proteins may involve both, the radicals (Bedwell et al., 1989) and the aldehyde generated (Hunt et al., 1988) during lipid peroxidation.

Perhaps the most widely studied biological lipid/protein system is that of low density lipoprotein (LDL). During peroxidation of the lipid component, the apoprotein of LDL becomes fragmented (Bedwell et al., 1989; Parsharsarathy et al., 1985) and there is crosslinking and residue modifications. Many studies have shown that cell-mediated alterations, in which LDL is incubated with endothelial cells, smooth muscle cells or mononuclear phagocytes from a number of species, lead to the generation of modified form(s) of LDL which are more rapidly endocytosed by macrophages (Heinecke et al., 1984; Hinsbergh et al., 1986).

There is much evidence of inactivation of membrane enzymes during lipid peroxidation (Dean et al., 1986), because the mitochondrion is an important source of cellular superoxide anion and hydrogen peroxide. Wolff and Dean (1986) have studied interactions between mitochondrial proteins and free radicals derived either from...
electron transport or from lipid peroxidation. Mitochondrial membrane monoamine oxidase was used as a model in these studies. Fragmentation was produced by \( \cdot \text{OH}/O_2 \) and \( \cdot \text{OOH} \). In addition, much lipid peroxidation has been observed during radical attack (Dean, 1986).

**Proflavine Action on Proteins**

Serine proteases have attracted a great deal of interest as drug targets due to their widespread involvement in biological processes. Proflavine has been an extremely effective probe for elucidating the mechanism of action of these enzymes. The binding of proflavine to the serine proteinases, especially trypsin, thrombin and chymotrypsin, was accompanied by a substantial shift in the visible absorption spectrum. This spectral shift has been utilized by many investigators as a means of detecting complex formation and dissociation between proteases, proflavine, and substrates or inhibitors (Bernhard et al., 1966; Fink, 1974; Koehler and Magnusson, 1974). Proflavine competitively inhibits the activity of several proteolytic enzymes. In thrombin, proflavine binds to a single specific site with micromolar affinity competing both with the charged inhibitor \( p \)-amino benzamidine and with the neutral indole and related compounds. The binding of benzamidine to the specificity pocket of thrombin and the displacement of proflavine by indole lead to the idea that the acridine moiety binds adjacent to the catalytic center in a polar region of the active site. The crystallographic studies of the proflavine binding to the active site of human \( \alpha \)-thrombin have suggested that proflavine molecule binds in the S1 pocket of the enzyme with one of the proflavine amino groups hydrogen bonded to the carboxylate of Asp-189 and with the protonated ring nitrogen which is hydrogen bonded to the carbonyl of Gly-219. Such studies indicate that the proflavine displacement assay can be used for monitoring the binding of other low molecular weight inhibitors to the S1 pocket of \( \alpha \)-thrombin (Coni et al., 1998). Moreover, thermodynamically, this binding is characterized by a change in the standard heat capacity change (\( \Delta C_p \)) which proposed that a burial of a large surface area of non-polar residues brought about the observed heat capacity change (Cristofaro and Landolfi, 1994). A recent crystal structure of proflavine and thrombin have shown that the sodium atom identified in an extended solvent channel beneath the S1 pocket may play a role in binding of these ligands (Nienaber et al., 2000). Interestingly, investigations for the action of proflavine with thiol proteases, papain and ficin, have given rise to apparent
enhancement in the catalytic activity of these two enzymes toward certain synthetic ester substrates. It was acting as a noncompetitive activator of papain by inducing a decrease in Km for the enzyme hydrolysis of its substrate (Hall and Anderson, 1974; Skalski et al., 1973).

Differential action of proflavine on serine and thiol proteases have attracted Brantner and his co-worker to develop a biotechnical method based on proflavine for the separation of proteolytic enzymes. Their procedure was applicable for purifying serine proteinases from natural materials or for removing serine proteinase contaminants from enzyme preparations, and all this was performed by preparing an affinity chromatography utilizing Sepharose aminocaproyl proflavine resin, so the enzymes which are inhibited by proflavine (serine proteinases) are tightly bound to the proflavine column and the enzymes which are activated by proflavine (The thiol proteases ficin and papain) are slightly retarded by the column, and finally those enzymes that are unaffected by proflavine are not bound. The column that they have prepared is stable, and has been used repeatedly for nine months giving the same results (Brantner et al., 1976).

**Diseases Related to Protein Oxidation**

Elevated levels of protein carbonyls are also associated with a number of age-related diseases, often correlating well with the progression of the disease. An increase in the carbonyl content of protein is associated with Alzheimer’s disease (Hensley et al., 1994), Parkinson’s disease (Alam et al., 1997; Floor and Wetzel, 1998), diabetes (Baynes and Thorpe, 1999), rheumatoid arthritus (Chapman et al., 1989), muscular dystrophy (Murphy and Kehrer, et al., 1989), cataractogenesis (Garland et al., 1988), induction of renal tumors (Uchida et al., 1995), bronchopulmonary dysplasia (Gladstone et al., 1994), amyloidosis (Kong et al., 1996), chronic ethanol ingestion (Grattaglianno et al., 1996), acute carbon tetrachloride toxicity (Sundari and Ramakrishna, 1997), amyotrophic lateral sclerosis (Bowling et al., 1993), and the progerias (Oliver et al., 1987). In some of these diseases, more than one kind of oxidative modification has been demonstrated. For example, Alzheimer’s disease has been shown to be associated with an increase in protein carbonyl content (Smith et al., 1991), in advanced glycation end products (Vitek et al., 1994), in protein HNH adducts (Montine et al., 1998), in nitrated tyrosine derivatives (Good et al., 1996), and in redox active iron (Smith et al., 1997).
Cataractogenesis is associated with an increase in protein carbonyl group (Garland et al., 1988) and methionine sulfoxide (Lund et al., 1996), Parkinson's disease is associated with an increase in both protein carbonyl groups (Floor and Wetzel, 1998; Kong et al., 1996) and HNH adducts (Yoritake et al., 1996). It is noteworthy that in the available studies of Parkinson's disease, all patients had been treated with dopa, which is known to provoke protein oxidation (Kong et al., 1996; Lavoie and Hastings, 1999). Thus, in Parkinson's disease the observed protein oxidation may be a consequence of the underlying pathophysiology or it may be a result of drug treatment.