REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

During the last two decades, there has been a growing interest in studies that concern with the prevention of uncontrolled oxidative processes leading to various diseases in the living system. Several studies have shown the role of oxidative stress in the causation and progression of different diseases including atherosclerosis, carcinogenesis, neurodegenerative diseases, chronic inflammatory diseases, radiation damage, aging and various other pathological effects (Yu, 1994). This definitely developed a responsibility in scientists, medical practitioners and clinical epidemiologists, to find out the exact role and the control of oxidant - antioxidant system in human diseases (Irshad and Chaudhuri, 2002).

The putative therapeutic impression of many traditional medicines appears to be attributed to the presence of antioxidant principles (Noguchi and Niki, 2000). Clinical trails and epidemiological studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular diseases, cancer and age related disorders (Kaur and Kapoor, 2001).

Among the most important constituents of the edible plant products, low molecular weight antioxidants are the most important species. It is known that the consumption of fruits and vegetables is essential for normal health of human beings. A vegetarian diet can reduce the risk of cancer, atherosclerosis etc. (Khopde *et al.,* 2001). Crude extracts of fruits, vegetables, herbs, cereals and other plant materials rich in phenolics are increasingly of use in the food industry because they retard oxidative degradation of lipids (Javanmardi *et al.*, 2003).

SOURCES OF OXIDANTS

Oxidants or free radicals are the species with very short half life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. These species may be either oxygen derived (ROS-Reactive Oxygen Species) or nitrogen derived (RNS-Reactive Nitrogen Species). The oxygen derived species include $O_2^{\bullet-}$ (superoxide), $HO^{\bullet}$ (hydroxyl), $HOO^{\bullet}$ (hydroperoxyl), $ROO^{\bullet}$ (peroxyl) and $RO^{\bullet}$ (alkoxyl) as free radicals and H_2O_2 (hydrogen peroxide), HOCl (hypochlorous acid), O_3 (ozone) and $^1O_2$ (singlet oxygen) as non-radicals. Similarly nitrogen derived oxidant species are mainly NO (nitric oxide), $ONOO^{\bullet}$ (peroxynitrite), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide) (Evans and Halliwell, 1999).

Hydroxyl radical ($^{\bullet}OH$) is widely accepted as being the most damaging ROS produced by cells (Halliwell and Gutteridge, 1992). The free radicals in general and $^{\bullet}OH$ in particular react with virtually every molecule in the living cells (i.e., lipids, sugars, aminoacids, and nucleotides) with very high rate constant (Halliwell and Gutteridge, 1985).

REACTIVE OXYGEN SPECIES

The Reactive Oxygen Species (ROS) are formed either by the exogenous sources like electromagnetic radiation, cosmic radiation, cigarette smoke, UV light, ozone (O_3) and low wavelength electromagnetic radiations, or by the endogenous sources like mitochondrial electron transport chain, respiratory burst by phagocytes, β-oxidation of fat in peroxisomes, auto-oxidation of aminoacids, catecholamines, haemoglobin and ischemia reperfusion injury (Mc Cord, 2000).

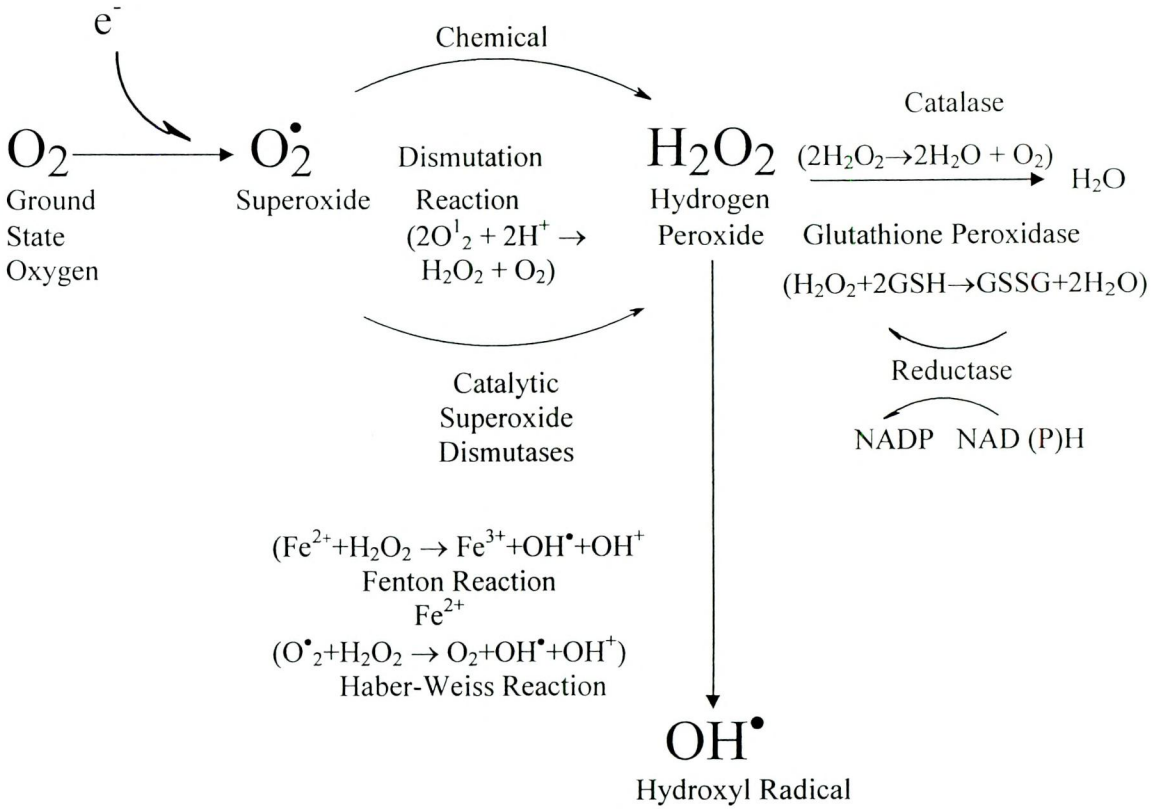


Figure 1 GENERATION OF MAJOR FORMS OF ROS AND THEIR METABOLISM IN CELLS

(Goel and Khanduja, 1998)

SUPEROXIDE ($O_2^{\bullet-}$) ANION is the first reduction product of O_2. The $O_2^{\bullet-}$ can be produced either by univalent reduction of O_2 or by the univalent oxidation of H_2O_2. The most important source of $O_2^{\bullet}$ is oxidative enzymes, among which xanthine oxidase and NADPH/NADH oxidase are the most effective sources (Cross and Jones, 1991). These enzymes possess flavin or transition metals such as Zn, Cu and Fe, which serve as electron donors (Mohazzab and Wlin 1994). Several oxidative enzymes such as aldehyde oxidase and dihydro orotic dehydrogenase have been shown to produce substantial amounts of $O_2^{\bullet-}$ (McCord and Fridovich, 1969). The spontaneous and enzymatic dismutation of $O_2^{\bullet-}$ yields H_2O_2, so a significant by-product of the actual sequence of oxidation–reduction reactions may be the generation of $O_2^{\bullet-}$ and H_2O_2 (Beckman and Ames, 1998).

HYDROGEN PEROXIDE (H_2O_2) may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of $O_2^{\bullet}$. H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases (Oshino *et al.*, 1973).

HYDROXYL RADICAL ($^{\bullet}OH$) is highly reactive with a half life of 10^{-5} seconds and a product from H_2O_2 and $O_2^{\bullet-}$ by Haber-Weiss reaction (Beauchamp and Fridovich, 1970). Some $^{\bullet}OH$ may be produced from hypochlorous acid in phagocytic cells (Irshad and Chaudhuri, 2002). The insufficient stability does not allow it to diffuse through the cells. Therefore, it reacts with an organic matter at the sites or near the sites of its formation. The reactions of $^{\bullet}OH$ are thus site specific (Ray and Hussain, 2002).

PEROXYL RADICAL ($ROO^{\bullet}$) is produced by the oxidation of organic molecules. The $^{\bullet}OH$ radical abstracts the hydrogen atom from organic molecules and form the organic radical. The latter product has a single unpaired electron and thus can react with oxygen in triplet ground state. The addition of triplet oxygen to the organic radical can lead to the formation of a peroxy-radical which can readily abstract hydrogen from another organic molecule leading to the formation of a second organic radical. This chain reaction is far more damaging than any other reaction catalysed by reactive oxygen species. This hydrogen abstraction reaction of hydroxyl radical is best demonstrated by lipid peroxidation of linolenic acid in cell membranes(Frankel, 1984).

SINGLET OXYGEN ($^1O_2$) can be generated by photo-excitation and chemiexcitation (Sies, 1986). Molecular ground state of oxygen is kinetically inert. The two unpaired electrons in the outer most orbits have the same quantum number imposing a spin restriction on the reactivity of oxygen. This can be removed by moving one of the unpaired electrons in a way that alleviates the spin restriction. This phenomenon requires an input of energy and generates the singlet states of oxygen. The singlet states of oxygen do not have unpaired electrons and hence do not qualify as a radical (Devasagayam and Kamat, 2002).

HYPOCHLOROUS ACID (HOCl) is an effective chlorinating and oxidizing agent (Weiss *et al.*, 1983). It is produced by the neutrophil dependent enzyme, myeloperoxidase, at sites of inflammation (Hazzen *et al.*, 1996). In the presence of chloride ions, hydrogen peroxide is converted to hypochlorous acid (HOCl), a potent oxidant and antimicrobial agent (Devasagayam and Kamat, 2000).

REACTIVE NITROGEN SPECIES (RNS)

Reactive Nitrogen Species (RNS) are also important. NO rapidly undergoes addition, substitution, redox and chain terminating reactions. These reactions serve as the molecular basis for its biological effects in the human body. The target molecules of NO are intracellular thiol and metal containing proteins and low molecular weight thiols like glutathione and cysteine (Stamler, 1994). NO acts as a "double sword" in health and disease. Both deficiency and excess of NO are believed to be involved in different pathophysiological states (Liaudet and Sariano, 2000). Although the direct toxicity of NO is modest, it gets greatly increased when it reacts with superoxide to form peroxynitrite, a very strong oxidant ($ONOO^{\cdot -}$) (Devasagayam and Sainis, 2002).

NITRIC OXIDE (NO) is an inorganic free radical gas, containing odd number of electrons and can form a covalent link with other molecules by sharing a pair of electrons. It is synthesized by a family of isoenzymes called nitric oxide synthase located in various tissues, and plays an active role in free radical and tumour biology (Felley-Bosco, 1998).An inducible nitric oxide synthase (iNOS) is capable of continuously producing large amounts of NO. In activated immune cells it acts as a "killer molecule" (Anggard, 1994).

PEROXY NITRITE ($ONOO^{\bullet}$) is produced during the reaction of NO with O_2. This is an another powerful oxidant that interacts with a wide range of targets leading to tyrosine nitration, thiol oxidation, lipid peroxidation, DNA strand break, guanosine nitration /oxidation and ultimately cell death. The reaction of $ONOO^{\bullet}$ with excess NO generates NO_2, which can combine with more NO to form N_2O_3 and cause nitrosative stress (Koppenol *et al.*, 1992).

TARGETS OF OXIDANTS

Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Lipids are highly prone to free radical damage resulting in lipid peroxidation that can lead to adverse alterations. Free radical damage to protein can result in loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis (Devasagayam *et al.*, 2004).

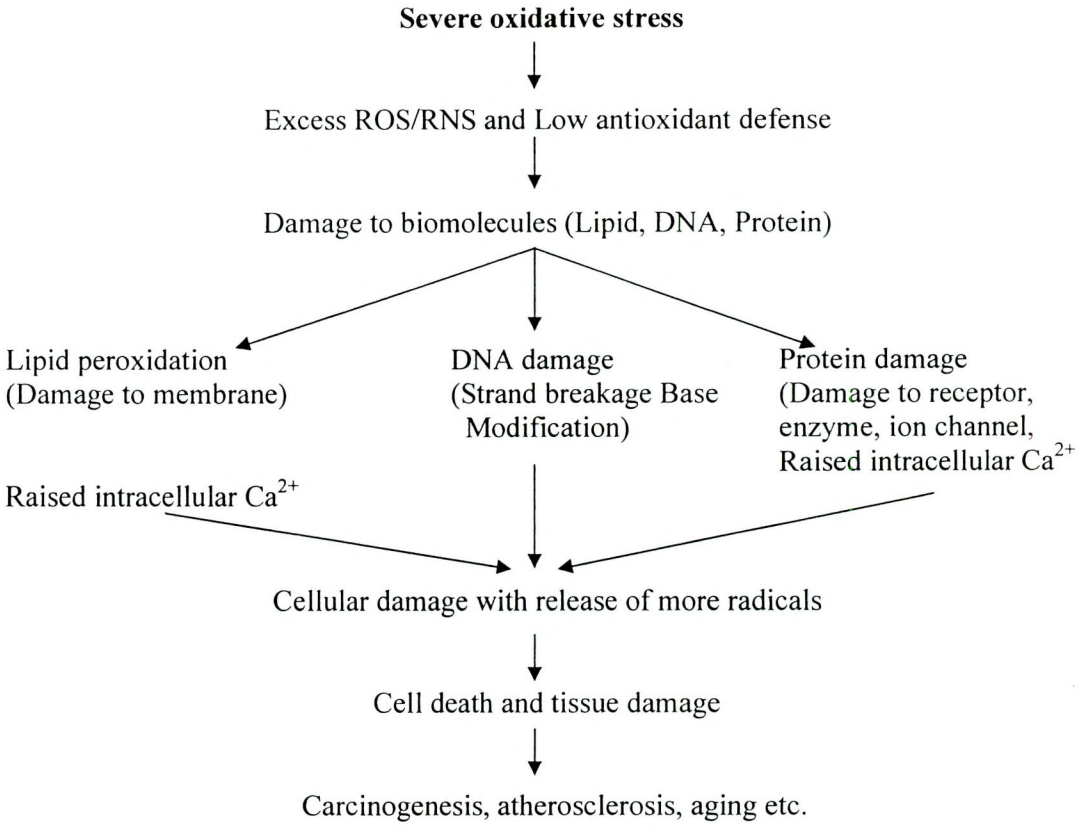


Figure 2 OXIDATIVE STRESS AND ITS EFFECTS ON BIOMOLECULES

(Irshad and Chaudari, 2002)

The ultimate result of these actions are destruction of endothelial cells of blood vessels, macrophage invasion, suppressed immune response, destruction of lung tissue, hastening of aging process and also impotency and infertility (Tripathi, 1998).

OXIDATIVE LIPID DAMAGE Cellular biomolecules like lipids are the most susceptible to oxidative damage. Reaction of ROS with lipids leads to the highly damaging reactions, the lipid peroxidation (LPO) (Devasagayam and Kamat, 2002). The level of LPO is a measure of membrane damage and causes change in the structure and functions of cellular membrane (Sudha *et al.*, 2004). A primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties and can significantly disrupt membrane bound proteins (Tappel, 1975). Malondialdehyde is the major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acids (Vaca *et al.,* 1988).

In a living system, lipid peroxidation is induced by free radicals and reactive oxygen species, which ultimately damage the cells. Metals also induce LPO which may be of two types, enzymatic and non-enzymatic. In the latter case, the reducing agent responsible for converting Fe^{+3} to Fe^{+2} and for further reaction are of chemical nature such as ascorbate, cysteine etc. However, in the enzymatic mechanism, the reducing agent is an enzyme catalyzed reaction such as NADPH-cytochrome P450 reductase. CCl_4 induced lipid peroxidation also an example of enzymatic mechanism because here CCl_4 is metabolically activated to $CCl_3^{\bullet}$ radical (Tripathi, 1998).

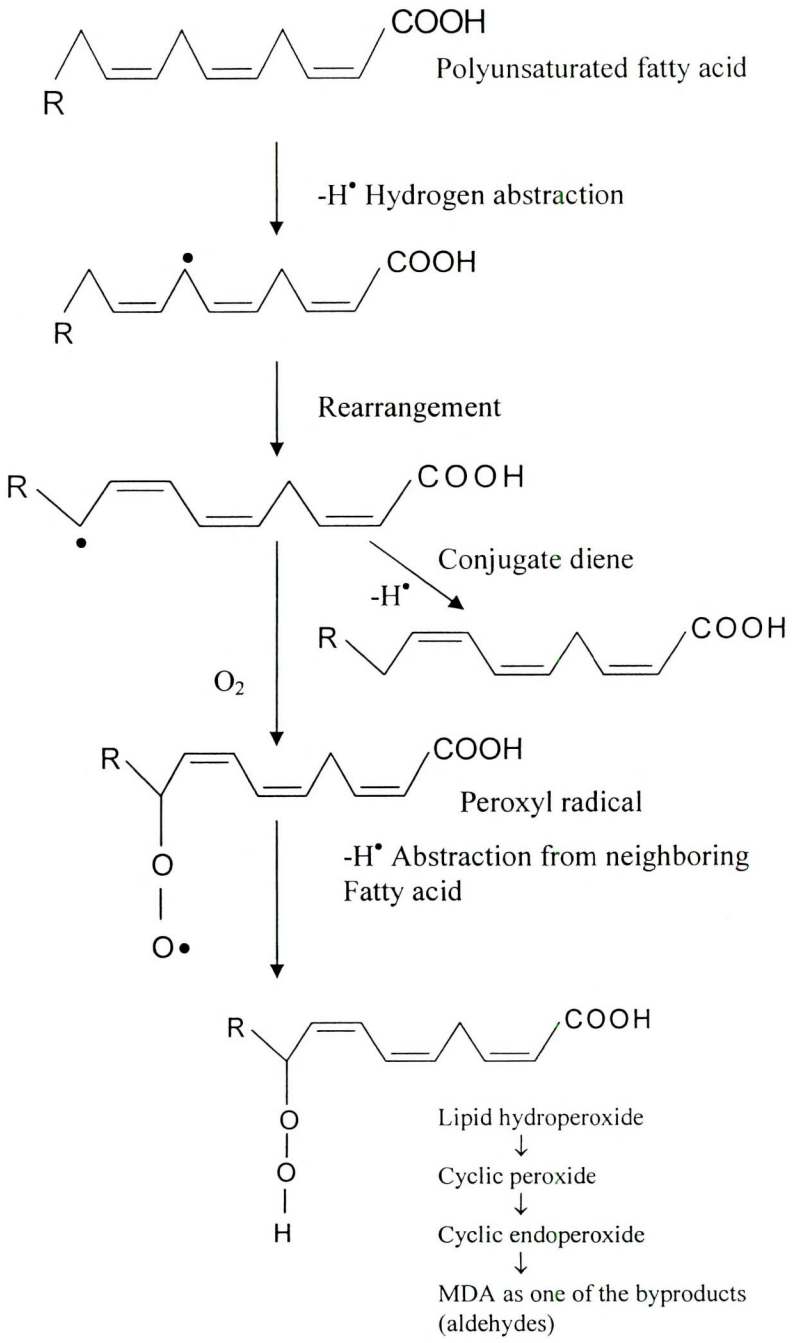


Figure 3 **OXIDATIVE LIPID DAMAGE**

(Formation of conjugated dienes, hydroperoxides and malondialdehydes following hydrogen abstraction from a polyunsaturated fatty acid)
(Ray and Hussian, 2002)

OXIDATIVE DNA DAMAGE This includes adducts of base and sugar groups, single- and double-strand breaks in the backbone and cross links to other molecules. The spectrum of adducts in mammalian chromatin oxidized *in vitro* and

in vivo includes more than 20 known products including damage to all four bases and thymine-tyrosine cross-links (Dizdaroglu, 1992). Hydroxyl radical is known to react with all components of the DNA molecule: the purine and pyrimidine bases, as well as the deoxyribose backbone (Lombardi *et al.*, 1998). An example illustrating the mechanisms of the formation of 8-hydroxyguanine (7, 8-dihydro-8-oxoguanine, 8-OH-G) is given in the figure below.

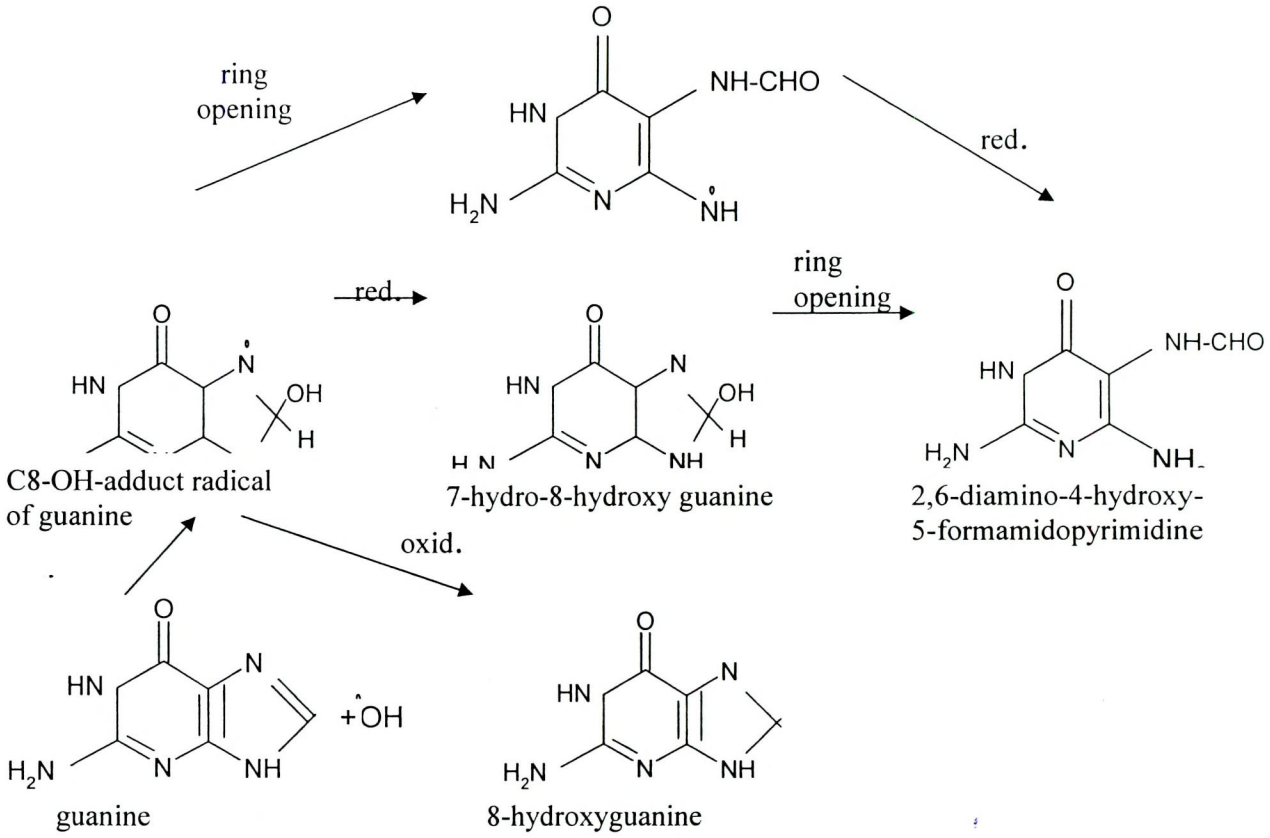
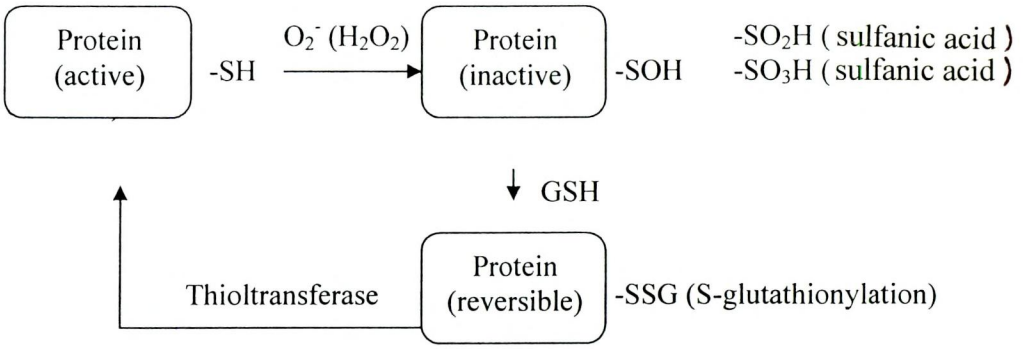


Figure 4 OXIDATIVE DNA DAMAGE
[Mao *et al* (1998); Boiteux and Radicella (2000)]

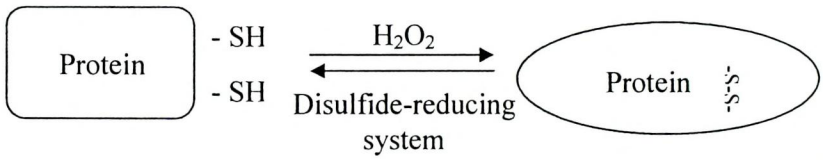
OXIDATIVE PROTEIN DAMAGE ROS can alter protein structure and function by modifying critical amino acid residues, inducing protein dimerization and interacting with Fe-S moieties or other metal complexes. Oxidative modifications of critical aminoacids within the functional domain of proteins may occur in several ways. By far, the best described of such modification involves cysteine residues. Such alterations may alter the activity of an enzyme or protein if the critical cysteine is located within its catalytic domain or the ability of a transcription factor

to bind DNA if it is located within its DNA binding motif (Abate *et al.*, 1990). Hence, the transduction of chemical signal into biologically relevant information is mediated by conformational changes in the proteins (Kaushik and Khanduja, 2004).

A. MODIFICATION OF PROTEIN BY OXIDATION OF CYSTEINE RESIDUES



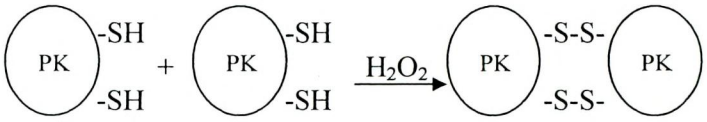
B. FORMATION OF INTRA-MOLECULAR DISULFIDE LINKAGES



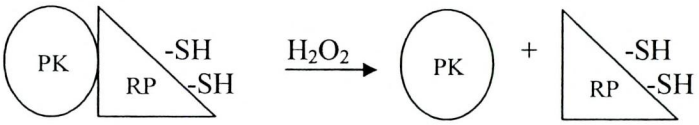
Alteration in activity by conformational changes in protein structure

C. PROTEIN DIMERIZATION BY INTER-MOLECULAR DISULFIDE LINKAGES

1.Direct



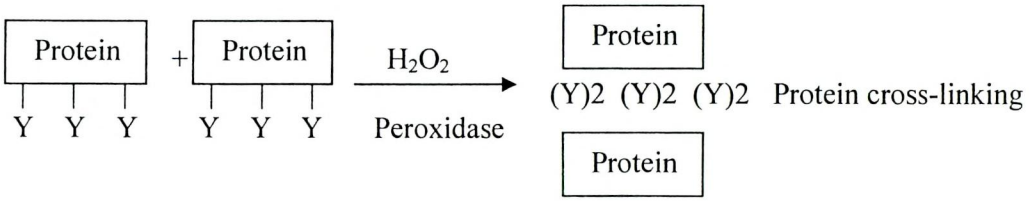
2.Indirect



Bound (inactive)

Unbound (active)

D. DITYROSINE FORMATION BY H_2O_2/PEROXIDASE-DEPENDENT REACTIONS



E. METAL-CATALYZED OXIDATION OF PROTEINS BY 'FENTON-LIKE' CHEMISTRY

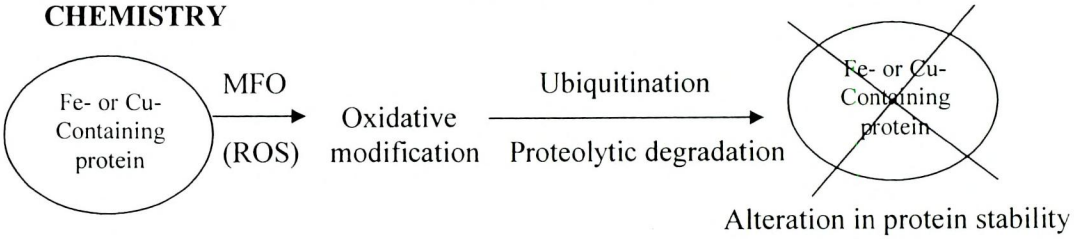


Figure 5 OXIDATIVE PROTEIN DAMAGE (Gerschman *et al.*, 1954)

OTHER AGENTS CAUSING OXIDATIVE STRESS

ETHANOL TOXICITY: Ethanol is absorbed rapidly in the gastrointestinal tract and 90% of it is metabolized in the liver. Its primary metabolite is acetaldehyde, which is produced by the action of alcohol dehydrogenase in hepatocytes. Further, acetaldehyde is oxidized to acetate by aldehyde dehydrogenase (Lieber, 1993).

There are other metabolic routes available for ethanol and acetaldehyde. After chronic alcohol consumption, the hepatic microsomal P450 enzymes become more pronounced (Krikun *et al.*, 1984). This leads to the production of free radicals such as the superoxide anion, hydrogen peroxide and the 1-hydroxyethyl radical (Rao *et al.*, 1996). Similarly oxidation of acetaldehyde to acetate may be accompanied by the generation of free radicals, by some chemicals (Nakano *et al.*, 1995) and enzymatic systems (Albano *et al.*, 1994).

These systems are known producers of reactive oxygen species, such as the superoxide anion and hydrogen peroxide, which in the presence of transition metal ions generate the hydroxyl radical which attacks acetaldehyde with acetyl and methyl radical formation (Nakao *et al.*, 2000).

As potent electrophilics, reactive oxygen species react readily with nucleophilic groups in proteins, phospholipids and nucleic acids to produce adducts, some of which have been detected in alcoholic patients (Fang and Vaca, 1997; Aleynik *et al.*, 1998). This may lead to tissue damage, especially in the liver.

CARBON TETRACHLORIDE (CCl_4) TOXICITY: The chlorinated methanes, particularly carbon tetrachloride and chloroform, are classic models of liver injury and have developed into important experimental hepato-oxidants over the past 50 years. Hepatocellular steatosis and necrosis are features of acute lesion. Mitochondria and the endoplasmic reticulum are the target sites (Plaa, 2000).

Both Slater (1996) and Recknagel (1967) proposed independently, that the putative carbon tetrachloride derived free radicals could attack membranes, leading to peroxidation and resulting in necrosis or steatosis. The trichloromethyl free radical ($^{\bullet}CCl_3$) was eventually identified by spin trapping in rat liver microsomes incubated with carbon tetrachloride and in livers from animals treated with the haloalkane. The free radicals react very rapidly with oxygen to yield a highly reactive trichloromethyl peroxyl radical ($^{\bullet}CCl_3O_2$), which is an initiator of lipid peroxidation (Cheeseman *et al.*, 1985).

The biotransformation of carbon tetrachloride occurs in the endoplasmic reticulum and is mediated by cytochrome P450; the principle isoform implicated as the catalyst is CYP2E1, but evidence for CYP2E1/2 exists as well (Kim *et al.*, 1996a).

The carbon tetrachloride derived free radicals can bind irreversibly to hepatic proteins and lipids and can initiate a process of autocatalytic lipid peroxidation by attacking the methylene bridges of unsaturated fatty acid side chains of microsomal lipids (Plaa, 2000). Lipid peroxidation is not the only process associated with the formation of free radicals after carbon tetrachloride intoxication. The reactive products also bind covalently to hepatic macromolecules. Binding to lipids, proteins and nucleic acids have been demonstrated (Castro, 1984). Binding to cytochrome P450, which leads to its destruction, occurs rapidly *in vivo* and in some instances can be shown to be independent of lipid peroxidation (Fuji, 1997).

Kutob and Plaa (1962) demonstrated that administration of a non-lethal dose of ethanol to mice prior to their subsequent exposure to small dose of chloroform resulted in potentiation of the haloalkane-induced liver injury. Later these findings

were extended to carbon tetrachloride involved experiments, where elevations in the plasma aminotransferase activity to quantify liver injury were employed (Klaassen and Plaa, 1966). Regarding mechanisms involved in potentiations observed with ethanol, isopropanol, 1, 3-butanediol, various aliphatic ketones, and chlordecone, increased production of haloalkane-derived reactive metabolites (via cytochrome P450) are certainly of major importance (Wang *et al.*, 1997). The induction of CYP2EI by ethanol and acetone is well established (Imaoka and Funae, 1991).

OXIDATIVE STRESS: THE IMBALANCE BETWEEN PRO- AND ANTI-OXIDANT FORCES IN FAVOUR OF THE FORMER

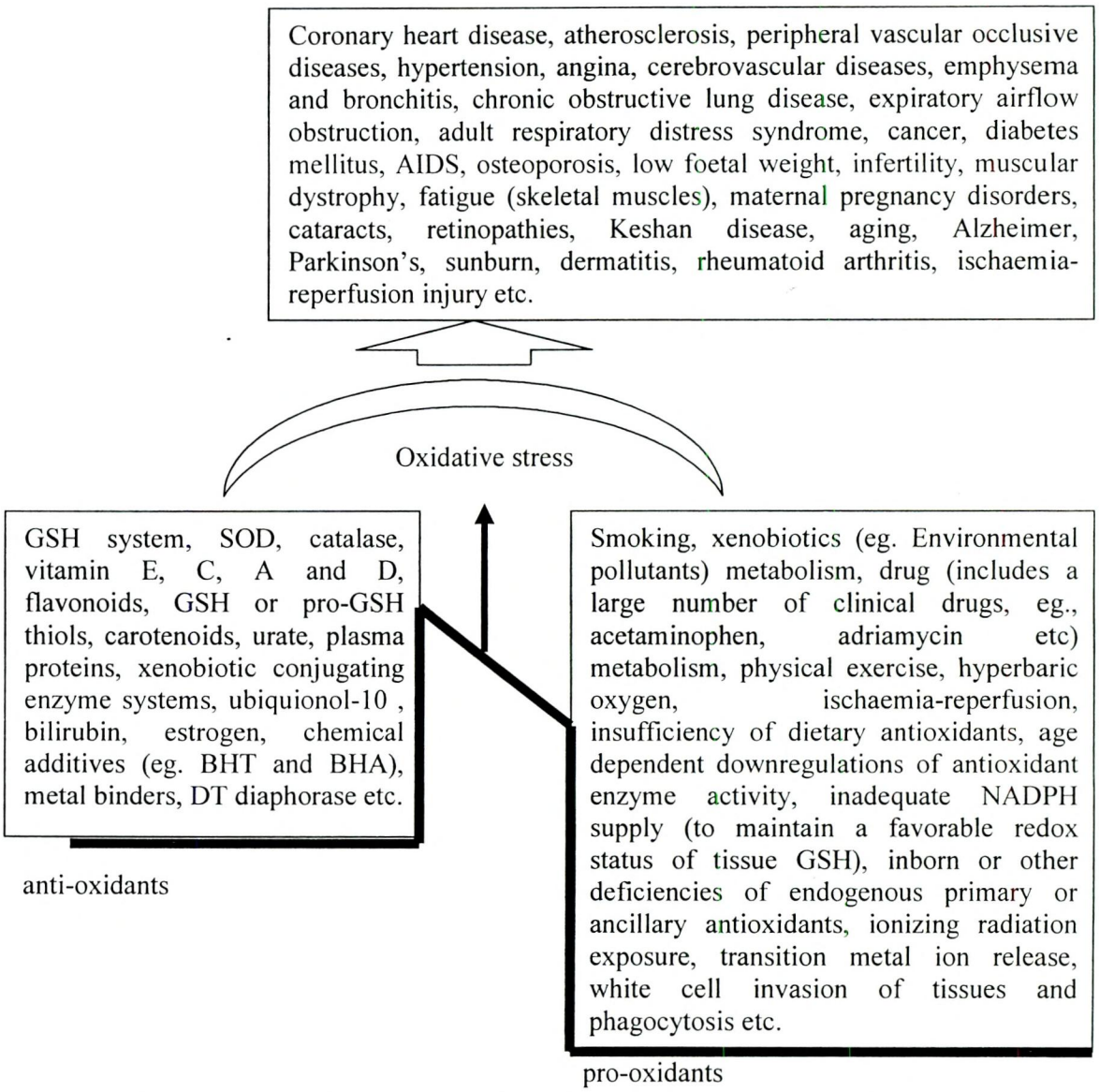


Figure 6 PRO- AND ANTI-OXIDANT IMBALANCE AND DISEASES (Sen, 1995)

OXIDATIVE STRESS AND DISEASES

In a normal cell, there is an appropriate prooxidant: antioxidant balance. However this balance can be shifted towards the pro-oxidant when the production of oxygen species is increased or when the levels of antioxidants are diminished. This state is called "oxidative stress" and can result in serious cell damage if the stress is massive or prolonged. Oxidative stress is implicated in the etiopathogenesis of a variety of human diseases (Frei, 1994; Beck and Levander, 1998).

DIABETES MELLITUS: Oxidative stress is considered to be a unifying link between diabetes and its complications. An increase in oxidative stress may contribute to the development of oxidative protein damage in diabetic patients (Dursun *et al.*, 2005). Over production and / or insufficient removal of free radicals in hyperglycemia result in vascular dysfunction, damage to cellular proteins, membrane lipids and nucleic acids (Johansen *et al.*, 2005).

Different studies have given a lot of evidence of increased oxidative stress with depleted antioxidant enzymes and vitamins, in both type 1 and type 2 diabetes mellitus. Oxidative stress is now acknowledged as a pathogenic mechanism in diabetic complications like diabetic retinopathy, nephropathy and microangiopathy (Ceriello, 2000)

CARDIOVASCULAR DISEASES: Free radicals contribute to the endothelial dysfunction and there is a reduced antioxidant capacity associated with the extent and severity of CVD (Vassalle *et al.*, 2004). In cardiovascular diseases, there is oxidative modification of low density lipoproteins (LDL) in the plasma, which promotes the arterial wall alterations (Rengstrom *et al.*, 1992).

CVD remains the number one killer of human population world wide, Peroxynitrite generated from the biradical reaction of NO and superoxide radical is crucially involved in the pathogenesis of various forms of cardiovascular disorders, including atherosclerosis, myocardial ischemia–reperfusion injury and cardiomyopathy (Ferdinandy and Schulz, 2003).

LIVER DISEASES: Free radicals are found to be involved in the pathogenesis of liver injury (Poli, 1993). Several studies showed the role of oxidative stress in alcohol induced cirrhosis, which is considered as the terminal irreversible stage of liver diseases. It has also been suggested that ROS and LPO may play a role in the pathogenesis of hepatic fibrosis with loss of normal liver architecture (Lieber, 2000; Farzaneh and Moore, 2001).

In viral hepatitis, caused by both RNA and DNA viruses, there is an associated enhanced production of ROS/RNS via long term oxidant stress. Oxidative stress was also observed in peripheral blood mononuclear cells from chronic hepatitis C patients (Boya *et al.*, 1999; Zhang *et al.*, 1999).

RESPIRATORY DISEASES: Oxidative stress is assumed to play an important role in the pathogenesis of a number of lung diseases, like chronic obstructive pulmonary diseases (COPD), bronchial asthma and acute respiratory distress syndrome, not only through direct injurious effects but also by involvement in the molecular mechanisms that control lung inflammation (MacNee, 2000).

NERVOUS SYSTEM DISEASES: Due to high oxygen consumption, low glutathione (antioxidant status) content, high levels of free iron oxidisable substances like PUFA in the central nervous system, neuronal cells appear to be particularly vulnerable to oxidative stress. This condition has a role in the genesis and progression of Alzheimer's diseases (Smith *et al.*, 1996; Zandi *et al.*, 2002), Parkinsons' diseases (Cohen, 2000), brain neoplasm (Rao *et al.*, 2000), Down syndrome (Busciglio and Yankner, 1995) and also some other neurodegenerative disorders (Christen, 2000; Chamorro *et al.*, 2002; Pratico *et al.*, 2002).

INFLAMMATORY DISEASES: In chronic inflammatory conditions phagocyte developed ROS have been implicated in inflammation related injury. The development of mutations in p53 tumour suppressor gene and other key regulatory genes promotes inflammation into chronic disease in rheumatoid arthritis and other inflammatory disorders (Tak *et al.,* 2000; Kumar *et al.*, 2002; Taysi *et al.*, 2002).

CANCER: Oxyradicals of endogenous cellular sources are found to cause significant levels of DNA damage. These oxyradicals attack DNA causing change in genomic sequences leading to mutation, deletion, gene–amplification or rearrangement. Thus the oxidative DNA damage was found important in the etiology of many human cancers (Marnett, 2000; Moyad, 2002; Stram *et al.*, 2002).

DNA is the major target of free radical damage. The types of damages include strand breaks (single- or double-strand breaks), various forms of base damage, yielding products such as 8-hydroxy guanosine, thymine glycol or abasic sites, damage to deoxyribose sugar as well as DNA-protein cross links. These damages can result in mutations that are heritable changes in the DNA that can yield cancer in somatic cells or foetal malformations in the germ cells (Bohr *et al.*, 2002).

A multi-stage process such as cancer development is characterised by cumulative action of multiple events occurring in a single cell and can be described by three stages: initiation, promotion and progression. ROS can act in all these stages of carcinogenesis (Klaunig and Kamendulis, 2004). The three stage model of carcinogenesis is shown in Fig 7.

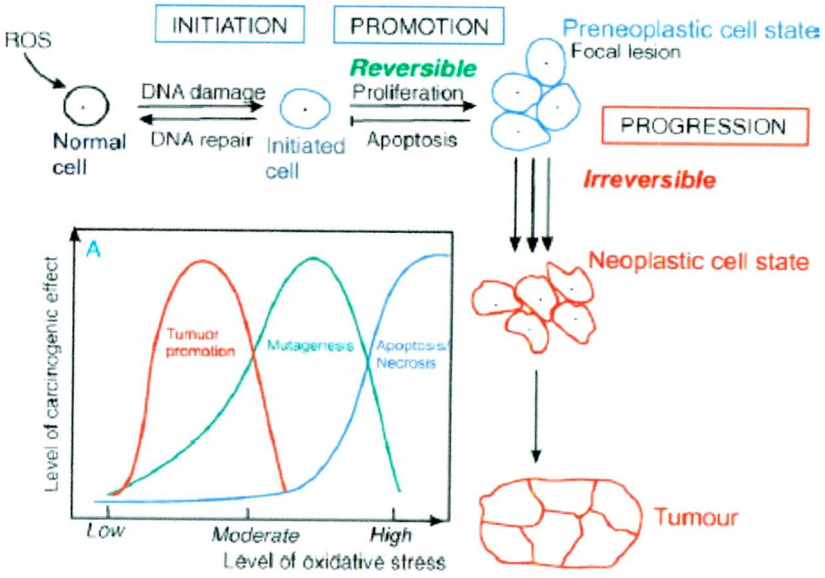


Figure 7 THREE STAGES MODEL OF CARCINOGENESIS

ANTIOXIDANT DEFENSES

Cells are equipped with an impressive repertoire of antioxidant enzymes, as well as small antioxidant molecules mostly derived from dietary fruits and vegetables (Yu, 1994). To counteract the harmful effects of ROS and RNS, the antioxidant defense mechanism operates to detoxify or scavenge these reactive oxygen species. The antioxidant system comprises different types of functional components classified as first line, second line, third line and fourth line defenses.

The first line defense comprises preventive antioxidants that act by quenching of $O_2^{\bullet-}$, decomposition of H_2O_2 and sequestration of metal ions. The antioxidants belonging to this category are enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase and non-enzymic molecules like minerals and some proteins. The antioxidant minerals include Se, Mn, Cu and Zn and function primarily in the metalloenzymes (Irshad and Chaudhuri, 2002).

SUPEROXIDE DISMUTASE (SODs) are a family of metallic enzymes that convert $O_2^{\bullet-}$ to H_2O_2. SOD is the most important enzyme because it is found virtually in all aerobic organisms. There are four families of SOD: Cu-SOD, Cu-Zn-SOD, Mn-SOD and Fe-SOD. The transition metal of the enzyme reacts with $O_2^{\bullet-}$ taking its electron. $O_2^{\bullet-}$ is the only known substrate for SOD (Oberley and Oberley, 1984). The SOD is considered to be a stress protein, which is synthesized in response to oxidative stress (McCord, 1990). SOD has been detected in a large number of tissues and organisms and thought that it is present to protect the cell damage caused by $O_2^{\bullet-}$ (Fridovich, 1972).

CATALASE (CAT) is an enzyme, which is present in most cells and catalyses the decomposition of hydrogen peroxide to water and oxygen. CAT is a heme containing protein. CAT is found to act 10^4 times faster than peroxidase. It is localized mainly in mitochondria and in subcellular respiratory organelles (Pryor, 1986).

GLUTATHIONE (GSH) DEPENDENT ENZYMES are glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GR).

GLUTATHIONE PEROXIDASES (GPXs) are selenoenzymes that catalyse the reduction of hydroperoxides at the expense of GSH (Flohe, 1989: Ursini *et al.*, 1995). In this process, hydrogen peroxide is reduced to water whereas organic hydroperoxides are reduced to alcohols. GSH peroxidase resides in the cytosol and mitochondrial matrix (Mills, 1960).

GLUTATHIONE S-TRANSFERASES (GSTs) are a group of enzymes that catalyse the conjugation of reduced glutathione with a variety of compounds bearing suitable electrophilic centres in them (Jakoby, 1978). The primary step of this reaction is involved in the formation of mercapturic acids, a pathway through which hydrophobic xenobiotics are inactivated and excreted from the body (Devi *et al.*, 2002). The important nucleophilic primary reactant for GST is glutathione. GSTs are a family of detoxification enzymes involved in cellular protection (Soni *et al.*, 1999).

GLUTATHIONE REDUCTASE (GR) is a flavoprotein enzyme, containing one mole of flavin adenine dinucleotide per enzyme subunit. The function of this enzyme is to regenerate GSH which has been converted to GSSG by oxidation and by thiol transfer reactions (Rana *et al.*, 2002).

GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD) is a cytosolic NADP dependant enzyme. This is the first enzyme in the HMP shunt that generates NADPH, which is necessary for the regeneration of reduced glutathione from oxidized GSSG. Maintenance of GSH in the reduced state is an important function of G6PD (Sultana *et al.*, 1995).

SECOND LINE DEFENSE (or) RADICAL SCAVENGING ANTIOXIDANTS

The antioxidants belonging to second line defense include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E (mainly α-tocopherol), carotenoids, flavonoids and ubiquinol.

Vitamin C, vitamin E and β-carotene (provitamin A) are some important scavenging antioxidant vitamins which cannot be synthesized by most of the

mammals including human beings and therefore are required from diet (Irshad and Chaudhuri, 2002). Glutathione is the most abundant non-protein thiol, synthesized in the liver and serves as a scavenger of different free radicals (Beauchamp and Fridovich, 1970).

VITAMIN C (ASCORBATE) has come to be known as a "wonder worker" (Kronhausen and Kronhausen, 1989). As an antioxidant, primary role of vitamin C is to neutralize free radicals. Since ascorbic acid is water soluble, it can work both inside and outside the cells to combat free radical damage. Vitamin C is an excellent source of electrons; therefore, it can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity (Bendich, 1990). Vitamin C protects DNA of the cells from damage caused by free radicals and mutagens (Schectman *et al.*, 1999).

It is the only endogenous antioxidant in the aqueous phase that can protect lipids from detectable peroxidative damage induced by aqueous peroxyl radicals. Ascorbate appears to trap the peroxyl radicals in the aqueous phase with a rate large enough to intercept virtually all these radicals before they can diffuse into the plasma lipids. The peroxyl radicals that escape the antioxidants in the aqueous phase diffuse into the lipids, where they initiate lipid peroxidation (Tappel, 1962).

VITAMIN E is a fat soluble vitamin composed of several tocopherols and toco trienols: the most biologically active being the alpha tocopherol. In the cells, a large proportion of vitamin E is found in the membranes and can attach to the free radicals to protect the membrane from oxidation (Wang and Quinn, 1999).

The major function of vitamin E is its role as a physiological membrane bound antioxidant, protecting the cell membrane lipids from oxidative damage initiated by Reactive Oxygen Metabolites (Traber, 1997). Vitamin E can directly act with a variety of oxy radicals including the peroxy radicals ($ROO^{\bullet}$), $CCl_3^{\bullet}$, $^{\bullet}OH$ (McCay, 1984), $O^{\bullet}_2^{-}$ (Fukuzwa and Gepicki, 1983) and singlet oxygen (Littarru *et al.*, 1984).

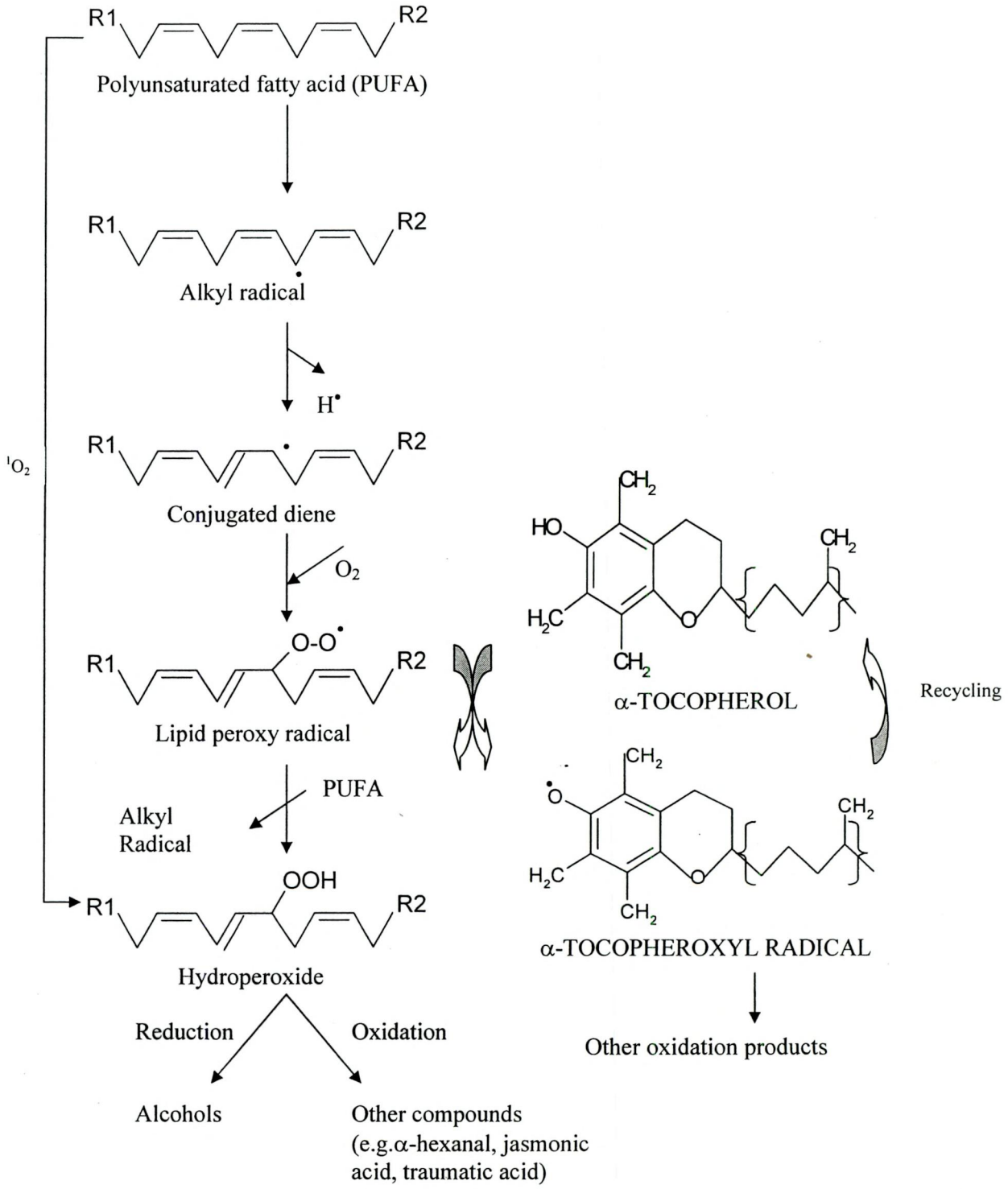


Figure 8 **ROLE OF VITAMIN E AS AN ANTIOXIDANT**

(Munne-Bosch and Alegre, 2002)

VITAMIN A (RETINOL) is a polyisoprenoid compound containing a cyclohexanyl ring. It is a fat soluble vitamin that is formed from β-carotene. The β-carotene scavenges the hydroxyl radicals, superoxide and peroxyl radicals. One example of a bodily process in which β-carotene scavenges free radicals is during lipid peroxidation. Lipid peroxidation occurs when the cell membrane is attacked by oxidizing agents. β-carotene can stop the chain reactions by directly scavenging lipid peroxyl radicals (Stryker *et al.*, 1987).

β-carotene acts as a free radical scavenger by showing up after the radicals are formed to trap the molecules and break their damaging chain reactions. β-carotene can absorb singlet oxygen due to its structure. It is a large molecule that contains eleven double bonds with single bonds in between. β-carotene can absorb the singlet oxygen's energy, spread it throughout the chain of bonds, and release the energy in the form of heat, with its structure remaining unharmed. Because of its unique ability to remain stable after absorbing the ROS, it is able to dismutate or chemically alter upto thousand singlet molecules (www.thedoctorslounge.net).

GLUTATHIONE (GSH) is considered as the most important defense against damage by reactive oxygen species. It is a tripeptide (glytamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing a new target for radical attack. Reaction with radical oxidizes the reduced glutathione and it is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH (Shukla *et al.*, 2004).

GSH acts as a cofactor for the enzyme peroxidase, which catalyses the decomposition of H_2O_2 (Barhoumi *et al.*, 1993) and it interacts with $^{\bullet}OH$, $ROO^{\bullet}$ and HClO and can be regenerated to its reduced form (Gul *et al.*, 2000). GSH maintains ascorbate and α-tocopherol in their reduced form, which also exert an antioxidant effect by quenching the free radicals. It maintains the redox cycle as an antioxidant armoury (Hammond *et al.*, 2001).

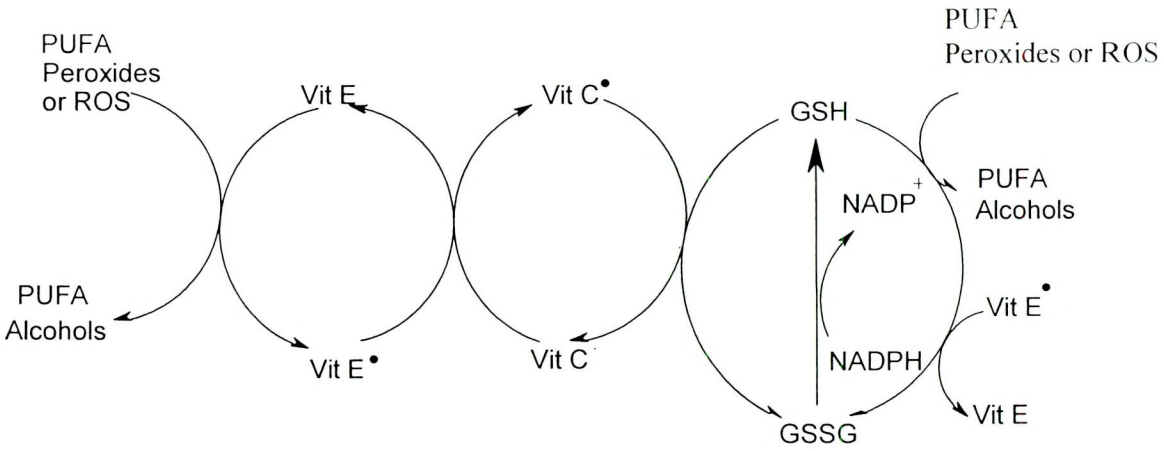


Figure 9 CENTRAL ROLE OF GLUTATHIONE IN ANTIOXIDANT NETWORK

POLYPHENOLS are the most abundant antioxidants in our diet. The main classes of polyphenols are phenolic acids (like caffeic acid) and flavonoids (like catechins, proanthocyanidins and anthocyanins). Polyphenols are reducing agents, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids referred to as antioxidants, protects the body's tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation (Tapiero *et al.*, 2002). The dietary polyphenolics have been recognized largely as beneficial antioxidants that can scavenge harmful active oxygen species including $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ (Sakihama *et al.*, 2002; Shetty and Wahlquist, 2004).

IMPORTANCE OF SILYMARIN

The flavonoid, silymarin and one of its structural components, silibinin, are substances with documented hepatoprotective properties. Their mechanisms of action are still poorly understood. However, the data in the literature indicates that the silymarin and silibinin act in four different ways: (i) as antioxidants, scavengers and regulators of the intracellular content of glutathione; (ii) as cell membrane stabilisers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes; (iii) as promoters of ribosomal RNA synthesis, stimulating liver regeneration; and (iv) as inhibitors of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibres leading to cirrhosis (Fraschini *et al.*, 2002).

MEDICINAL PLANTS

Herbal medicines have been used since the dawn of civilization to maintain health and to treat diseases. The use of herbs for medical benefit has played an important role in nearly every culture on earth (Wargovich *et al.*, 2001). Most of the herbal drugs are a mixture of a number of plant ingredients whose cumulative effect increases their efficacy in treating diseases (Yi *et al.*, 2003).

A whole range of chronic and difficult-to-treat diseases such as cancers, cardiovascular disease, diabetes, rheumatism and AIDS, all require new effective drugs. Most developing countries have relied and will continue to rely on traditional natural medicines due to the high costs of modern allopathic medicines (Grabley and Thiericke, 1999).

Natural products research continues to explore a variety of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry. While microbial products have been the mainstay of industrial natural products discovery, in recent years, phytochemistry has again become a field of active interest (Borris, 1996).

Plant based antioxidant rich foods traditionally formed a major part of the human diet, and are hypothesized to have an important role in maintaining human health (Benzie, 2003). Now-a-days plants with antioxidant properties are attractive sources of new drugs (Amin and Hamza, 2005). Thousands of herbal and traditional compounds are being screened worldwide to validate their use as antioxidants (Jagtenberg and Evans, 2005). This involves the isolation and identification of secondary metabolites from the plants and their use as active principle in medicinal preparations (Seo *et al.*, 2003). During recent years, active principles with diverse chemical structures have been isolated form plants possessing both the hepatoprotective and antioxidant effects (Sunitha *et al.,* 2001).

In tune with this effort, in the present investigation, *Solanum nigrum* (L) was evaluated for its antioxidant and anticancer effects. This plant is considered to be an underutilized species (Edmonds and Chewya, 1997).

GENERAL CHARACTERISTICS OF *Solanum nigrum*

The plant, *Solanum nigrum* (L) belongs to *Solanaceae* family. *Solanum nigrum* (L) and its related species are worldwide weeds of arable lands, garden, rubbish tips soils rich in nitrogen, in moderately light and warm situations which occur from sea to mountain level. In India, it is found throughout the dry parts, upto an elevation of 2100 meters.

Solanaceae, to which the genus *Solanum* L. belongs, is a cosmopolitan family containing many essential vegetables and fruits. Within this family, *Solanum* constitutes the largest and most complex genus; it is composed of more than 1500 species, many of which are also economically important throughout their cosmopolitan distribution. The section *Solanum*, centering around the species commonly known as the black, garden or common nightshade, *Solanum nigrum* L., is one of the largest and most variable species groups of the genus. The boundaries between many of the species are still ill-defined, with many of the 'new' extra proving to be no more than slight morphological variants of those already described. The situation has been further complicated by a number of authors who have persistently treated different members of the section as belonging to one highly variable species, namely *S. nigrum* (Tandon and Rao, 1974; Rao and Tandon 1969; Rao *et al.,* 1977; Venkateswarlu and Rao, 1972).

In addition, many species exhibit considerable genetic variation, both florally and vegetatively. This variation may occur in different populations of the same species or may characterize different intraspecific categories of a species. Sometimes, the character may be genetically controlled in one species, but be phenotypically plastic in another. Thus leaf margins may vary from entire to sinuate-dentate in different populations of *S. americanum* Mill., *S. furcatum* Dun. and *S. nigrum,* while the different subspecies of *S. nigrum* and *S. villosum* Mill. are characterized by different indumentum types. These two species also display a range of berry colours within each of their subspecies with that in *S. nigrum* varying from green through purple to black, and that in *S. villosum* from yellow through orange to red (Henderson, 1974).

MEDICINAL USES

In India, the *S.nigrum* is noted for its antiseptic and antidysenteric properties and is given internally for cardalgia and gripe. An infusion of the plant is used as an enema for infants with abdominal upsets. The plant is also a household treatment for anthrax pustules when it is applied locally. It is further reported to have emollient, diuretic and laxative properties and its decoction is regarded as both antispasmodic and narcotic. Freshly prepared extracts of the plant are apparently effective in the treatment of cirrhosis of the liver and also serve as an antidote to opium poisoning. An alcoholic extract of leaves is active against *Staphylococcus aureus* and *Escherichia coli.* Berries apparently possess tonic, diuretic and cathartic properties and are also useful in heart diseases and as a domestic treatment for fevers, diarrhoea, ulcers and eye troubles (Anon, 1956a; 1956b). The seeds are reportedly used to treat gonorrhoea and dysuria (Jain and Borthakur 1986). In Pakistan Akhtar and Muhammad (1989) showed that a powder from the aerial parts of the plant could be antiulcerogenic.

Some reports also attribute antitumour and anticancer effects to herbal extracts of the Chinese *Solanum nigrum*. Though the accuracy of this information is uncertain, in view of the crudeness of the herbal preparations used and the results observed, such potential value clearly deserves further investigation (Edmonds and Chewya, 1997).

In the presently reported investigation, two forms of *Solanum nigrum* (L) were subjected to various investigations to analyse their antioxidant and anticancer efficacy. The two varieties employed were the *Solanum nigrum* (L), most commonly available one, containing white flowers and black berries with ovate leaves (BBL) and the other variety, related to *Solanum nigrum,* containing white flowers and red berries with wavy or lobed margined leaves (RBL). According to the recent taxonomic survey by Reemakumari (2006), the red berry bearing plants are identified as *Solanum nigrum* L. subsp.*punicerum* (Kirschl), also known as *Solanum villosum* var.*punicerum* (Kirschl). Both the plants and leaves are presented in Plate 1.

The layout of the study, the materials used and the methodology adopted are explained, with appropriate references quoted in the next chapter.

PLATE 1

Solanum nigrum (L) PLATNS

Solanum nigrum

BBL

Solanum nigrum
subsp.*punicerum*
(Solanum villosum
var. *punicerum)*

RBL