CHAPTER 1
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INTRODUCTION

*Allium* is the largest and most important representative genus of the Alliaceae family and comprises 450 species, widely distributed in the northern hemisphere. Besides the well known garlic (*Allium sativum* L.) and onion (*Allium cepa* L.), several other species are widely grown for culinary use, such as leek (*Allium porrum* L.), scallion (*Allium fistulosum* L.), shallot (*Allium ascalonicum*), wild garlic (*Allium ursinum* L.), elephant garlic (*Allium ampeloprasum* L. var. *ampeloprasum*), chive (*Allium schoenoprasum* L.), Chinese chive (*Allium tuberosum* L.), Figure 1.1. Scientific research on these plants started in the second half of the 19th century with the work of Louis Pasteur that in 1858 first noted antibacterial properties of garlic [Pasteur, 1858]. Later on, in 1932 Albert Schweitzer treated amoebic dysentery in Africa with garlic. It was also used for several epidemic diseases (e.g. typhus, cholera, diphtheria, and tuberculosis) [Block, 1985].

Onion (*Allium cepa* L.) is one of the most important vegetables worldwide with an estimated annual production of almost 64 million tons in year 2008. The main production areas are China, India, United States, Russia, Turkey, and Iran. Consumption per capita differs greatly between countries and areas, but a major trend seems to be that onion consumption is increasing worldwide. Onion is of great economic importance, and is the second most important vegetable crop in the world [FAO, 2006]. India being a second major onion producing country in the world has a productivity of 10.16 MT/ha [FAO, 2008]. According to the Food and Agriculture Organization (FAO) onions are grown in at least 175 countries. Of those countries, two of the leading producers are China, which harvested 2.2 million acres of onions in 2005, and India, which harvested 1.3 million acres [Onions, Vegetables, NASS, USDA, April 2008].

Besides making a significant nutritional contribution to the human diet, onions also have medicinal and functional properties [Lanzotti, 2006]. Onion has been recognized as an important source of valuable phytoneutrients as flavonoids [Bilyk et al., 1984; Lanzotti 2006], especially flavonols with a wide range of quercetin, isorhamnetin and kaempferol conjugates [Bilyk et al., 1984], fructo-oligosaccharides (FOS) [Benkeblia et al., 2005], saponins [Corea et al., 2005], thiosulfinates and other sulphur compounds. Flavonoids continue to attract attention as potentially useful agents with implications for inflammation, cardiovascular diseases, and cancer [Middleton et al., 2000; Okamoto, 2005].
1.1 DESCRIPTION OF ONION (Allium cepa L.)

The common onion is biennial, but in commercial production it is mainly grown as an annual plant. The bulb consists of the swollen base of the stem and several fleshy leaves or scales. Onions may differ greatly in bulb shape, color of the outer scales (yellow, red, white), pungency (from mild and sweet to very pungent), bulb storage life, and dry-matter content [Brewster, 1994]. Onion bulbs are of various shapes and sizes, usually globular, the layers being juicy. Onions are grown in most climate zones around the world, from tropical to cool temperate climates. Transition from leaf
growth to bulb formation depends both on temperature and on the day length adaptation of the cultivar. Cultivars are commonly divided into groups based on growing latitude. Flavonoids are present in all terrestrial plants and are found in all plant organs including flower, fruit, leaf, stem, and root. The distribution with respect to structural diversity and quantities may vary considerably within a plant.

1.2 SCIENTIFIC CLASSIFICATION OF ONION

Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Asparagales
Family: Alliaceae
Genus: Allium
Species: A. cepa
Edible Parts: Flowers, Leaves, Root, Seed.

1.3 HISTORY OF ONION (Allium cepa L.)

Since ancient times, garlic and onion have been used as common foods and for the treatment of many diseases. They are among the oldest of all cultivated plants with their origin in central Asia. The first citation of these plants is found in the Codex Ebers (1550 BC), an Egyptian medical papyrus reporting several therapeutic formulas based on garlic and onions as useful remedy for a variety of diseases such as heart problems, headache, bites, worms and tumours [Block, 1985]. Cloves of garlic have been found in the tomb of Tutankhamen and in the sacred underground temple of the bulls of Saqqara. Egyptians thought garlic and onions aided endurance and assumed large quantities of them. Raw plants were routinely given to asthmatics and to those people suffering bronchial-pulmonary complains. Later on, these food plants were known by Greeks and Romans, who used them as important healing agents just as they are still used by most of the people of the Mediterranean area [Griffiths et al., 2002].
Onions are widely used in all parts of the world as a flavoring vegetable in various types of food. According to traditional medical literature they are source of many vitamins and are useful in fever, dropsy, catarrh and chronic bronchitis [Block, 1985]. Roasted or otherwise they are applied as a poultice to indolent boils, bruises, wounds, to relieve hot sensations and applied to the navel for dysentery and fever [Brewster, 1994]. Today Alliums are used for their flavor, aroma and taste, being prepared domestically or forming raw material for a variety of food manufacturing processes (dehydration, freezing, canning and pickling). Also, dehydrated onion production is widely used, especially in the manufacture of other processed foods [Brewster, 1994]. On the other hand, therapeutic and medicinal values of garlic and onions is the subject of many researches. Different clinical studies have shown their benefit in the reduction of cardiovascular disease risk by inducing lowering of serum cholesterol and blood pressure. They have liver protective, immune enhancement and anti-infection, anti-stress and anti-fatigue anti-cancer and cancer preventive effects, brain and neurotrophic and other pharmacological effects [Lau, 1989; Griffiths et al., 2002; Galeone et al., 2006; Shon et al., 2004; Singh et al., 2009]. Many previous studies showed that Alliums have antioxidant effect what could be of the great importance for its use in prevention and treatment of different diseases [Lau, 1989; Geng and Lau, 1997] and contribute to its therapeutic physionomy [Kyo et al., 1998].

1.4 CHEMICAL COMPOSITION OF ONION

The chemical composition of the onion is variable and depends on cultivar, ripening stage, environment and agronomic. Flavonoids and organosulfur compounds are the two major classes of secondary metabolites found in onions believed to promote beneficial health effects. Their mode of action and biosynthetic pathways are quite different [Lancaster and Shaw, 1989; Randle and Lancaster, 2002], Figure 1.2.

1.4.1 Organosulphur compounds

Evidence from several investigations suggests that the biological and medicinal functions of onions are mainly due to their high organosulphur compounds content
Three sulfoxides present in the tissues of onions were identified in the laboratory of Virtanen and Matikkala [1959], these being (+)-S-methyl-L-cysteine sulfoxide (methiin; MCSO), (+)-S-propyl-L-cysteine sulfoxide (propiin; PCSO) and (+)-S-trans-1-propenyl-L-cysteine sulfoxide or isoalliin (TPCSO). Isoalliin is the major sulfoxide present within intact onion tissues and is the source of the \textit{A. cepa} lachrymatory factor [Virtanen and Matikkala, 1959].

Figure 1.2

Upon cutting and crushing the onion, the vacuolar enzyme allinase, rapidly lyses the cytosolic cysteine sulfoxides, giving highly reactive organosulphur intermediates.

Figure 1.2. Chemical Composition of \textit{Allium} species

With regards to chemical distribution, MCSO is by far the most ubiquitous, being found in varying amounts in the intact tissues of onion. The primary sulphur-containing constituents in onions are the S-alk(en)yl-L-cysteine sulfoxides (ACSOs), such as alliin, and gamma-glutamylcysteines, which, besides to serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs.
from which, and by different metabolic pathways, volatile, such as allicin, and lipid-soluble sulphur compounds, such as diallyl sulphide (DAS), diallyl disulphide (DADS) and others, are originated [Lancaster and Shaw, 1989]. Most likely the compounds work through sulfur-sulfur or sulfur-oxygen linkages [Augusti, 1996]. These compounds are formed when an onion is cut and the cell walls are disrupted, Figure 1.3.

**Figure 1.3**

![Figure 1.3. Enzyme Allinase](image)

**Figure 1.3. Enzyme Allinase:** Convert the primary sulphur-containing constituents in onions, S-alk(en)yl-L-cysteine sulphoxides (ACSOs), such as alliin, and gamma-glutamylcysteines, into allicin, and lipid-soluble sulphur compounds, such as diallyl sulphide (DAS), diallyl disulphide (DADS) and others.

Allinase enzymes produce sulfenic acids via ACSOs which rearrange to various compounds such as thiosulfinates, cepaenes, and onion lachrymatory factor [Block et al., 1997; Lancaster et al., 1998]. These compounds provide to onion their characteristic odour and flavour, as well as most of their biological properties [Lanzotti, 2006]. Common to all Allium species is the enzyme allinase [EC 4.4.1.4], a 50 kDa glycoprotein that catalyses the hydrolysis of cysteine sulphoxide in the presence of the cofactor pyridoxal 5’- phosphate to produce pyruvate, ammonia and sulfenic acids. In intact tissues alliinase is compartmentalised within plant vacuoles and the representative cysteine sulphoxide located in the cytoplasm [Lancaster and Shaw, 1989]. Upon tissue disruption the vacuole and cytoplasmic contents mix, promoting the enzymatic hydrolysis of the respective cysteine sulphoxide. This catalytic reaction leads to the generation of sulfenic acids that condense to form thiosulfinates. The organosulfur compounds are believed to possess anti-
inflammatory, anti-allergic, anti microbial, and anti-thrombotic activity by inhibition of cyclooxygenase and lipoxygenase enzymes [Block et al., 1997].

Figure 1.4

![Flavour precursors of Onions](image)

Defining the mechanism by which organosulfur compounds (OSCs) derived from *Allium* vegetables inhibit cancer cell growth has been the topic of intense research in the last two decades. Some *Allium* vegetable constituents have also entered clinical trials to assess their safety and anticancer efficacy [Powolny and Singh, 2008]. OSCs have been shown to exert diverse biological effects such as: induction of carcinogen detoxification, inhibition of tumor cell proliferation, antimicrobial effect, free radical scavenging, inhibition of DNA adduct formation, induction of cell cycle arrest, and induction of apoptosis. It has been suggested that these compounds act as chemopreventive agents through a combination of above mechanisms [Moriarty et al., 2007]. It has also been proposed that the chemoprotective effect of onion sulfides is due, at least in part, to the ability of these sulfides to increase tissue activities of phase II detoxification enzymes [Guyonnet, 2001; Teyssier et al., 2001]. These enzymes, which include glutathione S-transferase (GST), epoxide hydrolase (EH), quinone reductase (QR, DT-diaphorase, NAD[P]H:quinone-acceptor oxidoreductase)
and UDP glucuronosyl transferase (UDPGT), inactivate many electrophilic substances, including certain carcinogens, and facilitate their elimination from the body.

Taking into account the relationship between structure and enzyme inducing ability by OSCs it can be drawn the importance of unsaturation in the alkyl chain, showing prop-1-enyl derivatives a higher level of induction than the propyl in many tissues. The importance of the number of sulfur atoms in the molecule was also important; the general rule could be that the higher activity is found with increasing number of sulfur atoms, although the direction of the effect was different in the prop-1-enyl sulfide which was stronger inducer than the disulfide [Munday and Munday, 2004].

There have been varying aroma compounds reported in studies where the occurrence of disulfides from chopped onion, with dipropyl disulfide (DPDS) accounting for the largest amount [Bernhard et al., 1964; Saghir et al., 1964]. Analysis of onion volatiles by Kallio and Salorinne [1990] identified 27 separate aroma compounds, the most prominent of which were DPDS, methyl propyl disulfide (MPDS), 1-propenyl propyl disulfides ((E)- and (Z)- PPDS), methyl 1-propenyl disulfides ((E)- and (Z)-MPeDS), 1-propanethiol (PT), dipropyl trisulfide (DPTS), methyl propyl trisulfide (MPTS) [Kallio and Salorinne, 1990], Figure 1.4. Later, Jarvenpaa et al. [1998] using the novel Solid Phase Micro Extraction (SPME) technique coupled to GC-MS, determined the changes in volatile compound composition in chopped onion. Initially, thiopropanal S-oxide was the major sulfur compound identified in the head space with minor levels of DPDS and PPDS. Within 30 minutes thiopropanal S-oxide had disappeared and was replaced by diprop(eny)l disulfides. These data indicate that the chemical profiles of Allium volatiles change dramatically with time.

1.4.2 Flavonoids

Onions contain high levels of non-nutrient antioxidant compounds (phenolics) which have protective effects against different degenerative pathologies such as cardiovascular and neurological diseases, cancer and other dysfunctions based on oxidative stress [Griffiths et al., 2002]. In recent years, flavonoids have gained much
attention due to their antioxidative, antibacterial, and anticarcinogenic properties, which has led to an increase in research on cancer prevention and reduction of cardiovascular diseases [Scalbert et al., 2005]. Flavonoids are major phenolics in onions and can be classified in different subclasses (flavones, flavanones, flavonols, isoflavones, flavanols, flavones, flavanols, and anthocyanins) according to the degree of unsaturation and degree of oxidation of the 3-carbon skeleton. Flavonols and anthocyanins are the main subclasses of flavonoids present in onions, the latter being found only in red onions. Many of these compounds are glycosylated, and some of these glycosyl derivatives are esterified with aromatic or aliphatic acids whose combinations yield a large variety of compounds [Slimestad et al., 2007]. Within a vegetable family, the quality and quantity of the phenolic pool may change with the cultivar, growth stage, and environmental conditions.

At least 25 different flavonols have been characterised in onions. Onions ranked highest in quercetin content in a study of 28 vegetables and 9 fruits [Hertog et al., 1992]. The amount of quercetin in onions varies depending on bulb colour and type, being distributed mostly in the outer skins and rings [Lombard et al., 2005; Patil and Pike, 1995]. Quercetin concentration in onions ranges from trace amount in white to 2.5–3 mmol/kg in red varieties in which it occurs as various O-glycosides with d-glucose as the main sugar residue. Quercetin in onions exists in four predominant forms: quercetin aglycone, quercetin-3,4-O-diglucoside, querceti-3-O-glucoside and querceti-4-O-monoglucoside, although several forms of the diglucoside and monoglucoside conjugates have been reported in smaller amounts [Fossen et al., 1998]. Quercetin-4-O-glucoside and quercetin-3,4-O-diglucoside are in most cases reported as the main onion flavonols in various literatures [Bonaccorsi et al., 2005; Bonaccorsi et al., 2008; Slimestad et al., 2007; Caridi et al., 2007]. Figure 1.5. These glucosides of quercetin represent about the 90% of the overall contents in different Allium species [Bonacorsi et al., 2008]. Their glycosyl moieties are almost exclusively glucose, which is mainly attached to the 4', 3, and/or 7 positions of the aglycones. Analogous derivatives of kaempferol and isorhamnetin have been identified as minor onion pigments [Slimestad et al., 2007]. Distribution of quercetin and its glycosides within the onion bulb changes during onion processing, different cooking methods and exposure to fluorescent light [Ewald et al., 1999; Nemeth and Piskula, 2007].
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Quercetin is one of the abundant flavonol-type flavonoids commonly found in vegetables and fruits [Moon et al., 2000]. In general, quercetin has been investigated for their widespread health benefits which have generally been ascribed to its combination of antioxidant and anti-inflammatory activities [Davis et al., 2009]. Quercetin shows a variety of pharmacological effects such as growth inhibition of tumour and microbial cells, reduction of cancer risk, scavenging of free radicals, and protection against cardiovascular disease, which are attributed to specific sulfur-containing compounds and flavonoids [Ly et al., 2005; Caridi et al., 2007; Moskaug et al., 2004; Prakash et al., 2007; Wenzel et al., 2004]. Quercetin beneficial health effects include protection against various diseases such as osteoporosis, certain forms of cancer, pulmonary, and CVD but also against aging. Especially the ability of quercetin to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects [Boots et al., 2008].

Figure 1.5

![Flavonol Structures](image)

Figure 1.5. Main flavonols present in Onions: Quercetin-4′-O-β-glucoside (QMG) and quercetin-3, 4-O-diglucoside (QDG)

The flavonol quercetin has antiproliferative effects in many cancer cell lines. Antioxidant or pro-oxidant activities and kinase inhibition have been proposed as molecular mechanisms for these effects. In addition, an estrogenic activity has been
observed. Findings by Galluzo et al. [2009] suggest that quercetin results in HeLa cell death through a transfected estrogen receptor-dependent mechanism involving caspase and kinase activation. These findings indicate new potential chemopreventive actions of flavonoids on cancer growth.

1.4.3 Fructans and Fructooligosaccharides

Bulb dry matter content is an important quality parameter of onion, also important in the onion dehydration industry because it directly relates to the energy needed for drying. About 65 to 80% of bulb dry matter consists of non-structural carbohydrates [Kahane et al., 2001; Benkeblia et al., 2005]. The onion predominant non-structural carbohydrates are glucose, fructose, sucrose and low-molecular-weight fructans, while starch and raffinose are absent. Fructans, also known as fructooligosaccharides (FOS), are polyfructosyl sucrloses of varying molecular size that constitute the main carbohydrate reserve of onion. Fructans accumulate during bulbing and are then catabolized during regrowth and sprout development of the bulbs [Benkeblia et al., 2005]. There is a clear predominance of kestose in every onion tissue and no occurrence of highly polymerized fructans. The tissues richest in fructans are the fleshy layers, so that the outer two fleshy layers turn out to be the best onion by-product as a possible fructan source [Jaime et al., 2001; Jaime et al., 2002]. The fructan degree of polymerization level in onion is mostly in between 3 and 15. Short chain fructans, with a degree of polymerization less than 5, are potentially used as natural low-calorie sweeteners. Onion bulbs with fructans may be used for lipid replacement with consequential health benefits [Kahane et al., 2001].

Onion showed a better soluble/insoluble dietary fibre (SDF:IDF) ratio than other vegetables that will be connected with different metabolic and physiological effects. SDF increases the viscosity of the stomach contents, thereby allowing down-mixing and absorption of nutrients, whereas IDF reduces intestinal transit time and increases the bulk of the food mass [Jaime et al., 2002]. Fructans could act as osmoregulators due to their solubility in water inside the vacuole. Fructans act stimulating the growth of specific microorganisms in the colon (Bifidobacteria and Lactobacilli) with a general positive health effect, including on colonic inflammation [Lara-Villoslada,
Administration of FOS significantly lowered fasting glycemia and total cholesterol, increasing the intestinal absorption and bone density of calcium and magnesium.

### 1.4.4 Other Onion Bioactive Compounds

Many studies reported that several interesting novel compounds have been isolated from onion. Among them, saponins and peptides have been isolated and studied for their potentially beneficial health effects. 5-hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one, and four others compounds, were isolated and confirmed to be quinone reductase and glutathione S-transferase inducers *in vitro* [Xiao and Parkin, 2007], therefore they could act as chemopreventive agents. This warrants further research to isolate and identify more agents for their potential for phase II enzyme induction *in vitro and in vivo*. Several research reports have demonstrated antifungal, antitumor, cytotoxicity, blood coagulability, antispasmodic and cholesterol-lowering effects of saponins isolated from onion and garlic [Lanzotti, 2006].

Four furostanol saponins, two of which were new compounds, named ceparoside A and ceparoside B were isolated from the seeds of *Allium cepa* L. [Yuan et al., 2008]. Other new saponins were found years earlier by Corea et al. [2005], they were reported to possess antispasmodic activity in the guinea pig isolated ileum; such an effect might contribute to explaining the traditional use of onion in the treatment of disturbances of the gastrointestinal tract. It was also reported that an onion gamma-glutamyl peptide from onion [Welti et al., 2005] inhibits the development and activity of osteoclasts *in vitro* [Langos et al., 2007].

### 1.5 FREE RADICALS AND REACTIVE OXYGEN SPECIES

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from
oxygen represent the most important class of such species generated in living systems [Valko et al., 2007]. In living cells, the forming of free radicals takes place mainly through such processes as the homolysis of chemical bonds, photolysis, and radiolysis, and as a result of redox reactions. In the cells, free radicals are made up continuously, as by-products of oxygen metabolism during the oxidative phosphorylation taking place in mitochondria.

Reactive oxygen species (ROS) is a collective term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals. Intracellular generation of ROS mainly comprises superoxide (O$_2^-$) radicals and nitric oxide (NO$^+$) radicals, which are further converted to powerful oxidizing radicals like hydroxyl radical (•OH), alkoxy radicals (RO•), peroxyl radicals (ROO•), singlet oxygen (¹O$_2$) by complex transformation reactions. Some of the radical species are converted to molecular oxidants like hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO•), hypochlorous acid (HOCl). Sometimes these molecular species are not free radicals but can easily lead to free-radical reactions in living organisms and act as source of ROS.

1.5.1 Physiological functions of ROS

Typically, low concentration of ROS is essential for normal physiological functions like gene expression, cellular growth and defense against infection. Sometimes they also act as the stimulating agents for bio-chemical processes within the cell, functions in a number of cellular signaling systems and induction in mitotic response [Droge, 2002]. ROS exert their effects through the reversible oxidation of active sites in transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) leading to gene expression and cell growth [Schreck and Baeuerle, 1991]. ROS can also cause indirect induction of transcription factors by activating signal transduction pathways [Schreck and Baeuerle, 1991]. ROS also appear to serve as secondary messengers in many developmental stages. Apart from these; ROS also participate in the biosynthesis of molecules such as thyroxin, prostaglandin that accelerate developmental processes.
In thyroid cells, regulation of H$_2$O$_2$ concentration is critical for thyroxine synthesis, as it is needed to catalyze the binding of iodine atoms to thyroglobulin [Schreck and Baeuerle, 1991]. ROS are also used by the immune system as they have been shown to trigger proliferation of T cells through NF-κB activation. Macrophages and neutrophils generate ROS in order to kill the bacteria that they engulf by phagocytosis. Furthermore, tumor necrosis factor (TNF-α) mediates the cytotoxicity of tumor and virus infected cells through ROS generation and induction of apoptosis [Schreck and Baeuerle, 1991; Sevanian and Ursini, 2000].

1.5.2 Sources of ROS

ROS can be produced from both endogenous and exogenous substances, Figure 1.6:

a) **Endogenous Sources**

Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [Inoue et al., 2003]. Mitochondria have long been known to generate significant quantities of hydrogen peroxide. Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide [Conner and Grisham, 1996].

Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome P450 enzymes, the possibility for the production of ROS, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle. In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H$_2$O$_2$ concentration produced *in vivo* at hyperoxia sites [Gupta et al., 1997]. Peroxisomes are known to produce H$_2$O$_2$, but not O$_2$•⁻, under physiologic conditions.

b) **Exogenous Sources**

ROS can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce ROS.
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in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localised in the matrix) [Klaunig et al., 1997; Klaunig and Kamendulis, 2004].

Figure 1.6

Figure 1.6. The sources and cellular responses to reactive oxygen species (ROS)
1.5.3 ROS induced oxidative stress

Oxidative stress is defined as an exaggerated production of oxygenated free radicals, accompanied by a dislocation of anti-oxidation agents, i.e. imbalance between generation of ROS and the activity of the antioxidant defenses. All forms of life maintain a steady state concentration of ROS determined by the balance between their rates of production and their rates of removal by various antioxidants. Thus each cell is characterized by a particular concentration of reducing species like reduced glutathione (GSH), Nicotinamide adenine dinucleotide (NADH), Flavin adenine dinucleotide (FADH), etc. stored in many cellular constituents which determines the redox state of a cell [Kohen and Nyska, 2002].

Under normal conditions, the redox state of a biological system is maintained towards more negative redox potential values. However, with increase in ROS generation or decrease in antioxidant protection within cells, it is shifted towards less negative values resulting in the oxidizing environment. This shift from reducing status to oxidizing status is referred as oxidative stress [Kohen and Nyska, 2002]. The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components and alters many cellular functions.

Depending upon their nature, ROS reactions with biomolecules such as lipid, protein and DNA, produce different types of secondary radicals like lipid radicals, sugar and base derived radicals, amino acid radicals and thiyl radicals. These radicals in presence of oxygen are converted to peroxyl radicals. Peroxyl radicals are critical in biosystems, as they often induce chain reactions.

During elevated oxidative stress, there is loss of mitochondrial functions, which results in to ATP depletion and necrotic cell death, while moderate oxidation can trigger apoptosis. Severe oxidative stress can cause cell damage and death. It has been implicated in numerous human diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions [Halliwell and Gutteridge, 1999].
1.5.4 Antioxidants and Natural Defense from ROS induced Damages

Continuous exposure to various types of oxidative stress from numerous sources has led the cell and the entire organism to develop defense mechanisms for protection against reactive metabolites [Cadenas, 1997]. Defence mechanisms against free radical-induced oxidative stress involve:

(i) **Preventative mechanisms**: for example, altering the activity of enzymes, which indirectly produce oxygen metabolites; one such enzyme is xanthine oxidase [Kohen, 1999].

(ii) **Repair mechanisms**: An efficient repair system, one of the most important methods for the organism to cope with oxidative damage, consists of enzymes and small molecules that can efficiently repair an oxidative-damage site on macromolecules. The DNA repair system, for example, can identify a DNA-oxidized adduct [e.g., 8-hydroxy-2-deoxyguanosine, thiamine glycol, and a purinic and a pyramidenic sites], remove it, and incorporate an undamaged base [Dizdaroglu et al., 2002]. Molecules that can donate hydrogen atoms to damaged molecules are also considered repair compounds; one such example is the donation of a hydrogen atom by ascorbate or tocopherol to a fatty acid radical that was previously attacked by a radical and lost its hydrogen [Kohen and Nyska, 2002].

(iii) **Physical defences**: Cellular membranes are also an important mechanism allowing the cell to cope with oxidative stress. Compounds such as tocopherols can provide enhanced stability to cellular membranes, and steric interference can prevent ROS from approaching the target.

(iv) **Antioxidant defences**: Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies:

- Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT).
• Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants.

• Other antioxidants include coenzyme Q₁₀, several bioflavonoids, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B₁, B₂, B₆, B₁₂). They work in synergy with each other and against different types of free radicals.

**Figure 1.7**

Fig. 1.7. Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and enzymatic antioxidants in the management of oxidative stress in erythrocytes and liver.
Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health. Human defenses against ROS-induced damage include the enzymes catalase and glutathione peroxidase (both of which remove $\text{H}_2\text{O}_2$, as well as SOD, which catalyzes the dismutation of $\text{O}_2^{•−}$ to form $\text{H}_2\text{O}_2$ and $\text{O}_2^{•−}$). Glutathione peroxidase is generally thought to be more important than catalase as a $\text{H}_2\text{O}_2$ removing system in humans. Catalase is located in peroxisomes, whereas glutathione peroxidase is localized in the mitochondria and cytosol, a similar distribution to that of SOD. In mammalian (including human) tissues, SOD is mainly an intracellular enzyme. Only small amounts are present in extracellular fluids, such as plasma, cerebrospinal fluid or synovial fluid. In addition, some low-molecular-mass substances, such as uric acid, ascorbate (vitamin C), glutathione, tocopherol (vitamin E), ubiquinol, ergothioneine, hypotaurine, and lipoic acid, may act as antioxidants in the human body. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation and inhibit Fenton and Haber-Weiss-type reactions; vitamins A and E scavenge free radicals [Halliwell and Gutteridge, 1990; Maritim et al., 2003; Valko et al., 2007].

1.6 ERYTHROCYTES OR RED BLOOD CELLS

Red blood cells (RBCs), also known as erythrocytes in mammals are non-nucleated biconcave shaped cells, highly flexible and lacking intracellular organelles. They are flattened and depressed in the center. Erythrocyte content consists mainly of hemoglobin, Figure 1.7. The precursors (Pronormoblast) of erythrocytes mature in the bone marrow, in a process called erythropoiesis, closely attached to a macrophage, these precursor cells manufacture hemoglobin until it accounts for some 90% of the dry weight of the cell, and as it matures the nucleus is squeezed out of the cell and is ingested by the macrophage. In addition the no-longer-needed proteins are expelled from the cell in vesicles called exosomes [Guyton and Hall, 2006].

1.6.1 Functions of Erythrocytes

A major function of erythrocytes is to transport hemoglobin, which in turn carries
oxygen from the lungs to the tissues. In some lower animals, hemoglobin circulates as free protein in the plasma, not enclosed in red blood cells. When it is free in the plasma of the human being, about 3 percent of it leaks through the capillary membrane into the tissue spaces or through the glomerular membrane of the kidney into the glomerular filtrate each time the blood passes through the capillaries. Therefore, hemoglobin must remain inside red blood cells to effectively perform its functions in humans.

**Figure 1.8**

![Red Blood Cells or Erythrocytes](image)

**Figure 1.8. Red Blood Cells or Erythrocytes**

RBCs have other functions besides transport of hemoglobin. For instance, they contain a large quantity of carbonic anhydrase, an enzyme that catalyzes the reversible reaction between carbon dioxide (CO₂) and water to form carbonic acid (H₂CO₃), increasing the rate of this reaction several thousandfold. The rapidity of this reaction makes it possible for the water of the blood to transport enormous quantities of CO₂ in the form of bicarbonate ion (HCO₃⁻) from the tissues to the lungs, where it is reconverted to CO₂ and expelled into the atmosphere as a body waste product. The hemoglobin in the cells is an excellent acid-base buffer (as is true of most proteins), so the red blood cells are responsible for most of the acid-base buffering power of
whole blood. RBCs also play some role in immune response by release of free radicals from damaged cells to destroy invading pathogens and also release S-nitrothiols that facilitate vasodilation when they (RBCs) are deoxygenated [Guyton and Hall, 2006].

### 1.6.2 Properties of Erythrocytes

#### 1.6.2.1 Shape and Size of RBCs

Normal RBCs are biconcave discs having a mean diameter of about 7.8 µm and a thickness of 2.5 µm at the thickest point and 1 µm or less in the center. The average volume of the red blood cell is 90 to 95 µm³. The shapes of RBCs can change remarkably as the cells squeeze through capillaries.

#### 1.6.2.2 Concentration of RBCs in the blood

In healthy men, the average number of RBCs per cubic millimeter is 5,200,000 (±300,000); in women, it is 4,700,000 (±300,000). Persons living at high altitudes have greater numbers of RBCs.

#### 1.6.2.3 Quantity of Hemoglobin in the Cells

RBCs have the ability to concentrate hemoglobin in the cell fluid up to about 34 gm in each 100 milliliters of cells. The concentration does not rise above this value because this is the metabolic limit of the cell’s hemoglobin-forming mechanism. Furthermore, in normal people, the percentage of hemoglobin is almost always near the maximum in each cell. However, when hemoglobin formation is deficient, the percentage of hemoglobin in the cells may fall considerably below this value and the volume of the red cell may also decrease because of diminished hemoglobin to fill the cell.

When the hematocrit (the percentage of blood that is in cells—normally, 40 to 45 percent) and the quantity of hemoglobin in each respective cell are normal, the whole blood of men contains an average of 15 grams of hemoglobin per 100 milliliters of cells; for women, it contains an average of 14 grams per 100 milliliters. Each gram of pure hemoglobin combines with 1.34 ml of oxygen. Therefore, in a normal man a maximum of about 20 milliliters of oxygen can be carried in combination with
hemoglobin in each 100 milliliters of blood, and in a normal woman 19 milliliters of oxygen can be carried.

1.6.3 Life Span of RBCs

When RBCs are delivered from the bone marrow into the circulatory system, they can never divide, and live for about 120 days after which they and engulfed and phagocytosed by cells of the RES predominantly in the spleen, bone marrow and liver. Even though mature red cells do not have a nucleus, mitochondria, or endoplasmic reticulum, they do have cytoplasmic enzymes that are capable of metabolizing glucose and forming small amounts of ATP. These enzymes also (1) maintain pliability of the cell membrane, (2) maintain membrane transport of ions, (3) keep the iron of the cells’ hemoglobin in the ferrous form rather than ferric form, and (4) prevent oxidation of the proteins in the red cells. Even so, the metabolic systems of old red cells become progressively less active and the cells become more and more fragile, presumably because their life processes wear out.

Once the red cell membrane becomes fragile, the cell ruptures during passage through some tight spot of the circulation. Many of the red cells self-destruct in the spleen, where they squeeze through the red pulp of the spleen. There, the spaces between the structural trabeculae of the red pulp, through which most of the cells must pass, are only 3 micrometers wide, in comparison with the 8-micrometer diameter of the red cell. When the spleen is removed, the number of old abnormal red cells circulating in the blood increases considerably [Guyton and Hall, 2006].

1.6.4 The Erythrocyte membrane

Erythrocytes are highly specialized cells containing viscous ‘liquid crystal’ – hemoglobin – surrounded by protein skeleton connected with lipid bilayer. The complex structure of the membrane contains lipid bilayer, attached proteins and peripheral proteins forming membrane skeleton, Figure 1.8. Direct interactions between some proteins of the skeleton and lipid bilayer are additional stabilization [Rybicki et al., 1988; An et al., 2004]. The erythrocyte membrane consists of a lipid bilayer composed of 50% protein, 40% lipid, and 10% carbohydrate. More than 95% of cytoplasmic protein is hemoglobin (Hgb) [Telen and Kaufman, 1999]. Hgb is one of the most widespread and specialized heme containing proteins that exist in nature.
These unique proteins permit the reversible binding to O$_2$ to heme while maintaining iron in the +2 oxidation state. This protein also facilitates exchange of CO$_2$ produced in tissues with the lungs. Heme iron must be maintained in the reduced ferrous form in order to bind O$_2$ reversibly [Telen and Kaufman, 1999; Nohl and Stolze, 1998].

Membrane skeleton proteins make up to 60% of erythrocyte membrane internal surface, performing a wide diversity of functions, such as the role in transporting, adhesion, signaling. They also can exhibit enzymatic activity. Membrane-associated cytoskeletal proteins include spectrin, ankyrin, band 3 (anion exchanger protein), glycophorin C, and protein band 4.1 have important roles in control of cell shape, attachment to other cells and substrates, and in organization of specialized membrane domains [Smith and Marks, 2005].

**Figure 1.9**

- Band 3 is the main membrane transporting protein. It makes up to 25% of the cell membrane surface. This anion exchanger protein also binds membrane skeleton to erythrocyte membrane. It is also the main place where hemichromes and hemoglobin
bind to erythrocyte membrane. N-terminal domain consists of 403 amino acids and is anchored in cytoplasm due to connection with ankyrin and proteins 4.1 and 4.2. Terminal part of this domain, containing 23 amino acids, binds hemoglobin and glycolytic enzymes [Tanner, 2002].

- Spectrin, the most prominent component of erythrocyte membrane skeleton, has two isoforms (alpha and beta) which form a loosely wound helix. Two alpha-beta helixes are linked end to end to form a single tetramer which has binding sites for actin microfilaments forming network on cytoplasmatic surface of the membrane.

- Ankyrin molecule is composed of three functional domains, two of which contain binding places for band 3 protein, spectrin, tubulin and intermediate filament proteins. The third functional domain regulates ankyrin binding to spectrin and band 3 protein [Delaunay, 2002]. Band 5 protein, actin, binds to spectrin and 4.1 protein [Ohanian et al., 1984]. 4.1 protein is a globular protein bound to spectrin close to the place of actin binding. These three proteins stabilize horizontal structure of erythrocyte cytoskeleton.

- Band 7 protein is not homogenous, during two-dimensional electrophoresis it divides into at least 10 fractions, of which stomatin (7.2) is the most important. Its deficiency causes stomacytosis, the disease associated with excessive permeability of cellular membrane [Delaunay, 2002]. Band 8 has not been well known, however, it was found that its increased amount was associated with higher concentration of globin bound to membrane in patients with anaemia [Antonelou et al., 2003]. Glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) (band 6) is one of the three erythrocyte membrane proteins with enzymatic activity, one of the glycolysis enzymes, which catalyses the transformation of 3-phosphoglyceric aldehyde to 1,3-bisphosphoglyceric acid (1,3-BPG). G-3-PD is bound to cytoplasmatic domain of band 3 protein [Rogalski et al., 1989].

- Band 9 protein, globin, is a small-molecule protein generated as a result of hemoglobin degradation. It is bound to erythrocyte cytoskeleton, frequently to spectrin. The amount of the protein increases during echinocyte transformation of erythrocytes, in stress, and in hereditary spherocytosis [Margetis et al., 2007].
All lipids in the mature erythrocyte are found in the membrane bilayer and consist of phospholipid and cholesterol in 1.2:1 molar ratio. Approximately one-half of the fatty acids in the membrane are unsaturated [Telen and Kaufman, 1999]. Interestingly, outer surface lipids exchange freely with the plasma lipid compartment [Bunn, 1991]. In addition, the structure of the lipid bilayer is critical to the cytoskeletal network organization within the red blood cell [Smith and Marks, 2005].

Glucose, the only fuel utilized by mature red cells, is primarily metabolized via anaerobic glycolysis. Following facilitated diffusion, glucose is immediately converted to glucose-6 phosphate. Approximately 80–90% percent is then converted to lactate via the glycolytic pathway. The remaining 10% undergoes oxidation via the pentose phosphate shunt. Glucose metabolism effectively maintains glutathione in the reduced form thereby protecting hgb sulfhydryl groups and red cell membranes from oxidation. A significant portion of the adenosine triphosphate (ATP) generated by glycolysis is spent in operating the sodium potassium pump necessary to preserve the cytoplasmic ionic milieu thus preventing colloidal osmotic lysis. In addition, some metabolic energy is expended on maintenance and repair of the red cell membrane [Bunn, 1991].