Metals, Toxicity and Oxidative Stress

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Abstract: Metal-induced toxicity and carcinogenicity, with an emphasis on the generation and role of reactive oxygen and nitrogen species, is reviewed. Metal-mediated formation of free radicals causes various modifications to DNA bases, enhanced lipid peroxidation, and altered calcium and sulfhydryl homeostasis. Lipid peroxides, formed by the attack of radicals on polyunsaturated fatty acid residues of phospholipids, can further react with redox metals finally producing mutagenic and carcinogenic malondialdehyde, 4-hydroxynonenal and other exocyclic DNA adducts (etheno and/or propano adducts). Whilst iron (Fe), copper (Cu), chromium (Cr), vanadium (V) and cobalt (Co) undergo redox-cycling reactions, for a second group of metals, mercury (Hg), cadmium (Cd) and nickel (Ni), the primary route for their toxicity is depletion of glutathione and bonding to sulfhydryl groups of proteins. Arsenic (As) is thought to bind directly to critical thiols, however, other mechanisms, involving formation of hydrogen peroxide under physiological conditions, have been proposed. The unifying factor in determining toxicity and carcinogenicity for all these metals is the generation of reactive oxygen and nitrogen species. Common mechanisms involving the Fenton reaction, generation of the superoxide radical and the hydroxyl radical appear to be involved for iron, copper, chromium, vanadium and cobalt primarily associated with mitochondria, microsomes and peroxisomes. However, a recent discovery that the upper limit of "free pools" of copper is far less than a single atom per cell casts serious doubt on the *in vivo* role of copper in Fenton-like generation of free radicals. Nitric oxide (NO) seems to be involved in arsenite-induced DNA damage and pyrimidine excision inhibition. Various studies have confirmed that metals activate signalling pathways and the carcinogenic effect of metals has been related to activation of mainly redox-sensitive transcription factors, involving NF-kappaB, AP-1 and p53. Antioxidants (both enzymatic and non-enzymatic) provide protection against deleterious metal-mediated free radical attacks. Vitamin E and melatonin can prevent the majority of metal-mediated (iron, copper, cadmium) damage both *in vitro* systems and in metal-loaded animals. Toxicity studies involving chromium have shown that the protective effect of vitamin E against lipid peroxidation may be associated rather with the level of non-enzymatic antioxidants than the activity of enzymatic antioxidants. However, a very recent epidemiological study has shown that a daily intake of vitamin E of more than 400 IU increases the risk of death and should be avoided. While previous studies have proposed a deleterious pro-oxidant effect of vitamin C (ascorbate) in the presence of iron (or copper), recent results have shown that even in the presence of redox-active iron (or copper) and hydrogen peroxide, ascorbate acts as an antioxidant that prevents lipid peroxidation and does not promote protein oxidation in humans *in vitro*. Experimental results have also shown a link between vanadium and oxidative stress in the etiology of diabetes. The impact of zinc (Zn) on the immune system, the ability of zinc to act as an antioxidant in order to reduce oxidative stress and the neuroprotective and neurodegenerative role of zinc (and copper) in the etiology of Alzheimer's disease is also discussed. This review summarizes recent findings in the metal-induced formation of free radicals and the role of oxidative stress in the carcinogenicity and toxicity of metals.

Keywords: Metals, Fenton chemistry, toxicity, free radicals, antioxidants, redox cycling, oxidative stress, cell signalling, NF-kappaB, AP-1, p53, DNA damage, lipid peroxidation, glutathione depletion, Alzheimer's disease.

INTRODUCTION

Many studies have reported toxic and carcinogenic effects induced when humans and animals are exposed to certain metals. It is also known that several essential transition metals, such as zinc, iron, copper, cobalt and manganese participate in the control of various metabolic and signalling pathways. However, their rich coordination chemistry and redox properties are such that they are capable of escaping out of the control mechanisms such as homeostasis, transport, compartmentalization and binding to the designated tissue and cell constituents. Breakdown of these mechanisms can lead to the metal binding to protein sites other than those tailored for that purpose or displacement of other metals from their natural binding sites. A growing amount of results provide evidence that toxic and carcinogenic metals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules.

The best evidence supporting the hypothesis of the oxidative nature of metal-induced genotoxic damage is provided by the wide spectrum of nucleobase products typical for the oxygen attack on DNA in cultured cells and animals exposed to carcinogenic metals. Detailed studies in the past two decades have shown that metals like iron, copper, cadmium, chromium, mercury, nickel, vanadium possess the ability to produce reactive radicals, resulting in DNA damage, lipid peroxidation, depletetion of protein

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sulfhydryls and other effects. Reactive radical species include a wide range of oxygen-, carbon-, sulfur- radicals, originating from the superoxide radical, hydrogen peroxide, and lipid peroxides but also in chelates of amino-acids, peptides, and proteins complexed with the toxic metals. The toxic effects of metals involve hepatoxicity, neurotoxicity and nephrotoxicity.

The purpose of this review is to provide a detailed overview of current state of knowledge of the role of metals in the formation of reactive oxygen and nitrogen species and tissue damage. The paper is organized in the following manner: firstly the basic principles of the activation of redox-sensitive transcription factors NF-κB, AP-1 and p53 are briefly discussed, followed by an overview of the redox metals, involving iron, copper, chromium, cobalt, vanadium and nickel, all active in Fenton reactions. Further attention is paid to a group of metals involving cadmium, arsenic, mercury, all of which are responsible for depletion of glutathione and bonding to sulfhydryl groups of proteins. Finally the role of zinc, a well-known antioxidant, is discussed together with the role of both zinc and copper in the neurological disorders, including Alzheimer's disease. Attention is also paid to metal homeostasis, metal-induced activation of signalling pathways and the protective role of enzymatic and non-enzymatic antioxidants against metal toxicity and carcinogenicity.

METALS AND THE ACTIVATION OF SIGNALLING PATHWAYS

As a prelude to the discussion of metal-induced activation of signalling pathways outlined below, we briefly review the basic principles of the activation of redox-sensitive transcription factors NF-κB, AP-1.

The carcinogenic effect of metals may be induced by targeting a number of cellular regulatory proteins or signalling proteins participating in cell growth, apoptosis, cell cycle regulation, DNA repair, and differentiation. The carcinogenic effects of certain metals have been related to the activation of transcription factors *via* the recycling of electrons through the antioxidant network sending the signals to redox-sensitive transcription factors NF-κB, AP-1 and p53. These factors control the expression of protective genes that repair damaged DNA, power the immune system, arrest the proliferation of damaged cells, and induce apoptosis [1].

The most significant effect of metals on signalling pathways has been observed in the mitogen-activated protein (MAP) kinase/AP-1 and NF-κB pathways [2]. The nuclear transcription factor NF-κB, is involved in inflammatory responses and AP-1 is important for cell growth and differentiation. P53 is a gene whose disruption is associated with more than half of all human cancers. The p53 protein guards a cell-cycle checkpoint, and inactivation of p53 allows uncontrolled cell division [3].

The activation of AP-1 and NF-κB family of transcription factors is involved in both cell proliferation and apoptosis. The concentration of radicals generated inside cells appears to influence the selective activation of these transcription factors and may therefore help explain the observation that either cell death or cell proliferation may be related to the exposure to carcinogenic metals.

AP-1 and NF-κB Activation by Metals

AP-1 is a collection of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (FosB, Fra-1, Fra-2), Maf, and ATF subfamilies, all of which can bind the tumour-promoting agent (TPA) or cAMP response elements [4]. c-Jun, a potent transcriptional regulator, often forms stable heterodimers with Jun proteins, which aid the binding of Jun to DNA [5]. AP-1 activity is induced in response to certain metals, H_2O_2 as well as several cytokines and other physical and chemical stresses. In addition, *in vitro* transcriptional activity of AP-1 is regulated by the redox state of a specific cysteine64 located at the interface between the two c-Jun subunits, highlighting the importance of redox status on gene transcription; however, recent *in vivo* experiments demonstrated that cysteine64/65 is not required for redox regulation of AP-1 DNA binding *in vivo* [6].

The stressors (metals or H_2O_2) invoke a signal cascade that begins with the activation of MAP kinases family of serine/threonine kinases, regulating processes important in carcinogenesis including proliferation, differentiation, and apoptosis. Three major subfamilies have been identified: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and the p38 kinases [7]. The induction of AP-1 by H_2O_2, metals, cytokines, and other stressors is mediated mainly by JNK and p38 MAP kinase cascades [7]. It is known that stressors can activate MAP kinases and thereby AP-1 in several manners. One involves a MAP kinase, apoptosis signal-regulating kinase (ASK1) [8]. The other mechanism involves oxidant-mediated inhibition of MAP kinase phosphatases, which leads to increased MAP kinase activation. Whichever mechanism prevails, activation of MAP kinases leads directly to increased AP-1 activity. The role of cellular oxidants and AP-1 activation in the cancer process is now well documented by a number of experiments [9]. An effect of AP-1 activation is to increase cell proliferation. It has been demonstrated that c-fos and c-Jun are positive regulators of cell proliferation [10]. Expression of c-fos and c-jun can be induced by a variety of compounds, involving reactive radicals and nongenotoxic and tumour promoting compounds (various metals, carbon tetrachloride, phenobarbital, TPA, TCDD, alcohol, ionizing radiation, asbestos). In addition to affecting cell proliferation, AP-1 proteins also function as either positive or negative regulators of apoptosis [4]. Whether AP-1 induces or inhibits apoptosis is dependent upon the balance between the pro- and anti-apoptotic target genes, the stimulus used to activate AP-1 and also on the duration of the stimulus. AP-1 proteins have also been found to participate in oncogenic transformation through interaction with activated oncogenes such as Ha-ras [11].

A number of reports during recent years indicate that some metals are able to affect the activation or activity of NF-κB transcription factors. NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in cell survival, differentiation, inflammation, and growth [12].

NF-κB is a DNA binding protein that interacts with the enhancing domain of target genes in the configuration of a dimer of two members of the NF-κB /Rel/Dorsal (NRD) family of proteins [13]. Although there are five known NRD members, RelA (also called p65), cRel, RelB, p50 (also called NF-κB1) and p52 (also called NF-κB2), the classical dimer is composed of p50 and RelA. Only RelA contains a transactivation domain that activates transcription by an interaction with the basal transcription apparatus. In unstimulated cells, NF-κB is sequestered in the cytoplasm because of an interaction with a member of the inhibitory (IκB) family. Activation of NF-κB occurs in response to a wide variety of extracellular stimuli that promote the dissociation of IκB, which unmasks the nuclear localization sequence and thereby allows entry of NF-κB into the nucleus and binds κB regulatory elements.

NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis [14]. NF-κB activation has been linked to the carcinogenesis process because of its role in differentiation, inflammation, and cell growth. Carcinogens and tumour promoters involving toxic metals, UV radiation, phorbol esters, asbestos, alcohol, and benzo(a)pyrene are among the external stimuli that activate NF-κB. On one side expression of NF-κB has been shown to promote cell proliferation, whereas on the other inhibition of NF-κB activation blocks cell proliferation. Several studies documented that tumour cells from blood neoplasms, and also colon, breast, and pancreas cell lines have all been reported to express activated NF-κB. The mechanism for activation of NF-κB by metals and reactive oxygen species is not yet clear. Reactive oxygen species have been implicated as second messengers involved in the activation of NF-κB *via* tumour necrosis factor (TNF) and interleukin-1 [15]. The "decision" to commit to cell death or cell survival will in part depend on the concentration and duration of oxidant exposure and on the cell type involved. The importance of metals and other reactive oxygen species on NF-κB activation is further supported by studies demonstrating that activation of NF-κB by nearly all stimuli can be blocked by antioxidants, including L-cysteine, N-acetyl cysteine (NAC), thiols, green tea polyphenols, and vitamin E [16]. These findings support the linkage of NF-κB activation to reactive oxygen species leading to the carcinogenesis process.

Activation of transcription factors is clearly stimulated by signal transduction pathways that are activated by metals, H_2O_2 and other cellular oxidants. Through the ability to stimulate cell proliferation and either positive or negative regulation of apoptosis, transcription factors can mediate many of the documented effects of both physiological and pathological exposure to metals or chemicals that induce reactive oxygen species and/or other conditions that favor increased cellular oxidants. Through regulation of gene transcription factors, and disruption of signal transduction pathways, reactive oxygen species are intimately involved in the maintenance of concerted networks of gene expression that may interrelate with neoplastic development.

IRON

Iron is the 26th element of the Periodic table. The electronic structure of iron and its capacity to drive one-electron reactions predetermines iron as a major component in the production and metabolism of free radicals in biological systems. In biological systems iron is commonly found in three oxidation states: Fe(II), Fe(III), and to a much lesser extent Fe(IV). At physiological pH, while Fe(III) precipitates as oxyhydroxide polymers, Fe(II) is soluble. On the other hand, Fe(II) is unstable in aqueous media and tends to react with molecular oxygen to form Fe(III) and superoxide.

Iron is an essential element required for growth and survival of almost every organism. The average-weight human contains approximately 4-5 g of iron. Iron deficiency, is a widely spread condition that affects approximately 500 million people around the world. The consequences of iron deficiency can range from anemia to mental retardation in growing children.

Iron overload is a less frequent condition, and involves defects in iron absorption, transport and secondary iron disorders. Toxicity and chronic iron toxicity is a status that can be associated with: (i) primary haemochromatosis, a genetic disorder related to increased intestinal absorption of iron, (ii) high dietary iron intake and (iii) frequent blood transfusions. Cases of acute iron toxicity are relatively rare and mostly related to hepatoxicity [17]. A high content of iron has been associated with several pathological conditions, including liver and heart disease [18], cancer [19], neurodegenerative disorders [20], diabetes [21], hormonal abnormalities [22], and immune system abnormalities [23].

Iron Homeostasis

About 65% of iron is bound to hemoglobin, 10% is a constituent of myoglobin, cytochromes, and iron-containing enzymes, and 25% is bound to the iron storage proteins, ferritin and haemosiderin. Ferritin is a high-capacity and low-affinity storage protein with the storage capacity of about 4500 atoms of iron per molecule. Another protein important in iron homeostasis is transferrin, a high-affinity, low-capacity protein with transporting capacity of only two atoms of iron per molecule in plasma. It is thought that only trace amounts of the metal remain free as non-chelated or loosely chelated iron (see below). To prevent the potential health disturbances produced by both iron deficiency and iron overload, mammals have evolved with numerous, integrated mechanisms regulating iron metabolism.

In mammals, the iron balance is primarily regulated at the level of duodenal absorption of dietary iron [24]. Disorders of iron homeostasis, resulting in iron deficiency or overload, are very common worldwide. Normal iron homeostasis depends on a close link between dietary iron absorption and body iron needs. Over the past few years, an important body of information concerning the proteins involved in iron absorption and in the regulation of iron homeostasis has arisen from the study of inherited defects, both in humans and mice, leading to distinct iron disorders [25].

Dietary free iron, on reduction from the ferric [Fe(III)] to the ferrous [Fe(II)] state on the luminal surface of the proximal small intestine, is transported into the enterocytes by the apical transporter DMT1 (also known as DCT1,

Nramp2). Dietary heme iron is taken up by a not yet discovered transporter and released from the heme molecule within the enterocyte. The iron may be stored within the enterocyte (in the iron storage protein, ferritin) or transferred across the basolateral membrane to the plasma by the transport protein Ireg1 [26] (known also as ferroportin1 and MTP1). This latter process requires oxidation of Fe(II) to Fe(III) by hephaesti. Once iron has entered the circulation, there are no significant physiologic mechanisms for iron loss other than menstruation.

Absorbed iron is bound to circulating transferrin (a high-affinity and low-capacity protein, see above) and passes initially through the portal system of the liver, which is the major site of iron storage. Hepatocytes take up transferrin-bound iron *via* the classical transferrin receptor (TfR1) but likely in greater amounts by the recently identified homologous protein, TfR2 [27]. The major site of iron utilization is the bone marrow, where iron is taken up *via* TfRs on erythrocyte precursors for use in heme synthesis.

Linkage of dietary iron absorption with body stores occurs in the proximal small intestine. Here duodenal crypt cells, the precursor cells for the absorptive enterocytes, sense the iron needs of the body and are "programmed" as they mature into absorptive enterocytes to express appropriate levels of the iron transport proteins [28]. The crypt cells obtain information about body iron needs from two postulated regulators, the "stores regulator," which responds to body iron stores, and the "erythropoietic regulator," which responds to the body's requirement for erythropoiesis [29]. The capacity of the stores regulator to change iron absorption is low relative to the erythropoietic regulator. Regardless of this, it plays an essential role in meeting increased iron needs and in preventing excess.

One of the most significant recent findings in iron homeostasis was reported by Nicolas *et al.* [30]. This group proposed that a central player in the communication of body iron stores to the intestinal absorptive cells may have been identified and have recently isolated a cDNA encoding the peptide hepcidin. Hepcidin acts as a signalling molecule that is required to regulate both intestinal absorption and iron storage in macrophages. Nicolas *et al.* [30] also found the absence of hepcidin expression in mice exhibiting iron overload consequent to targeted disruption of the gene encoding the transcription factor Upstream Stimulatory Factor 2 (USF2).

It has been proposed that increased hepatocellular uptake of transferrin-bound iron by TfR2 leads to increased production and secretion of hepcidin, which interacts with the β_2M-HFE-TfR1 complex and increases iron uptake or retention by reticuloendothelial macrophage duodenal crypt cells. Crypt cells differentiate into daughter enterocytes programmed to have decreased expression of iron transport proteins, leading to decreased dietary iron absorption. It was found that hepcidin is a disulfide-bonded peptide and exhibits antimicrobial activity [30]. The protein is synthesized in the liver in the form of a propeptide that contains 83 amino acids and is converted into mature peptides of 20, 22, and 25 amino acids [30]. Hepcidin was recently reported to be highly synthesized in the livers of experimentally or spontaneously iron-overloaded mice [31].

Iron and Free Radicals

Free radical-mediated tissue damage is generally accepted as a major mechanism underlying the occurrence of certain chronic diseases. The alterations in cell structure and function caused by iron overload seem to be fundamentally related to free radical mediated damage of cell components [32].

Many *in vitro* experiments confirmed the production of the hydroxyl radical ($^{\bullet}OH$) which can be explained in terms of the following reactions [33].

$$Fe(III) + O_2^{\bullet -} \leftrightarrow Fe(II) + O_2 \quad (1)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^- \text{ (Fenton reaction)} \quad (2)$$

The overall reaction of the combined steps is called Haber-Weiss reaction [33]

$$O_2^{\bullet -} + H_2O_2 \leftrightarrow O_2 + {}^{\bullet}OH + OH^- \quad (3)$$

In addition to the above reactions, the following reactions may also take place

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + H^+ + O_2^{\bullet -} \quad (4)$$

$$^{\bullet}OH + Fe(II) \rightarrow Fe(III) + OH^- \quad (5)$$

$$LOOH + Fe(II) \rightarrow Fe(III) + LO^- + {}^{\bullet}OH \quad (6)$$

The Fe(II)-dependent decomposition of hydrogen peroxide (reaction 2) is called the Fenton reaction and yield hydroxyl radical [34, 35] which can react at diffusion-limited rates with various biomolecules, including lipids, proteins, and DNA. This implies that shielding of iron from molecular oxygen and the surrounding media is a critical event in preventing iron-mediated oxidative stress. The tight binding of low molecular chelators *via* coordinating ligands such as O, N, S to iron blocks the iron's ability to catalyze redox reactions. Since the maximal coordination number of iron is six, it is often argued that the hexadentate chelators can provide more consistently inert complexes due to their ability to completely saturate the coordination sphere of the Fe atom. Consequently, a chelator molecule that binds to all six sites of the Fe ion completely deactivates the "free iron". Such chelators are termed "hexidentate", of which desferrioxamine is an example. There are many Fe chelators that inhibit the reactions of Fe, oxygen, and their metabolites [36]. For example, desferrioxamine mesylate (DFO) markedly decreases the redox activity of Fe(III) and is a very effective antioxidant through its ability to bind Fe.

Some chelators saturate only two of the coordination sites on iron. These chelators are called, "bidentate". An example of this type of molecule is ferrichrome. Thus three molecules of ferrichrome coordinate with a single iron to produce complete chemical immobilization of the metal [37]. It has also been found that many bidentate and tridentate Fe chelators inhibit Fe complex-mediated oxidative damage.

In contrast a number of Fe-chelating agents mediate toxicity by stimulating Fe-mediated oxygen radical generation. For example ligands like nitrilotriacetate (NTA) and ATP are known to bind Fe and cause oxidation of lipids and DNA damage [38]. The hexadentate ligand, ethylenediaminetetraacetic acid (EDTA), can also induce Fenton-chemistry mediated radical damage [39]. It was observed that autoxidation of Fe(II) is enhanced by EDTA

and also by NTA, but inhibited by *o*-phenanthroline and deferoxamine. NTA enables Fe(III) to react with H_2O_2 and produce hydroxyl radicals. The redox activity of the Fe–EDTA complex has been explained by the incomplete coordination environment of Fe by this ligand. This allows the temporary binding of water and cellular reductants to the incompletely occupied sixth coordination site. The ability of a Fe chelator to promote redox cycling of Fe and consequent production of reactive oxygen species is a property that may play a role in its antiproliferative activity [40]. For example, the Fe complex of the thiosemicarbazone chelator, Triapine, has recently been shown to cause oxidation of ascorbate, the hydroxylation of benzoate, and the rapid degradation of plasmid DNA [41]. These studies indicate that at least part of the anti-proliferative activity of Triapine could be ascribed to redox cycling and the subsequent damage to biomolecules.

Coordination of iron to biomolecules almost always involves the participation of *d* orbitals of the metal. Since molecular oxygen, through the electrons in antibonding π^* orbitals, can also ligate to iron by overlapping the *d* orbitals of the metal [42], iron may simultaneously bind to biomolecules and molecular oxygen, effectively serving as a bridge between the biomolecule and oxygen. The same applies also to iron bound proteins. When oxygen from an enzyme active site is bound to iron, reactions of the bound iron and reduced oxygen intermediates become less energetically favorable. In addition, proteins have complete control of steric factors and can prevent undesirable side reactions between bound reactive intermediates and nonsubstrate molecules. The "flexibility" of iron not only can vary the oxidation state but also can change its electronic spin properties and relative redox potential in response to interaction with different coordinating ligands. This allows the physical properties of iron to be "fine tuned" in response to the particular needs of the enzyme in which it operates.

From the above discussion it is clear that if iron is not appropriately shielded it can readily participate in one-electron transfer reactions that can lead to the production of toxic free radicals including $^{\bullet}OH$. To avoid these problems, organisms have evolved a series of molecules known as siderophores which are secreted into the extracellular medium where they complex with iron, Fe(III), and are then assimilated by the cells *via* a receptor mediated mechanism.

The participation of iron in the reactions occurring in cells is essential in: (i) the production of hydroxyl radical that can subsequently initiate lipid oxidation or oxidise almost any molecule present in biological systems, (ii) the propagation of free radical reactions by decomposing peroxides. The relevance of iron-catalysed reactions *in vivo* is definitely supported by findings that iron is present inside the cell. In contrast, based on the recent findings of O'Halloran and his group [43], the concentration of copper inside the cell is restricted to one ion per cell, thus the occurrence of Fenton chemistry *in vivo* is restricted predominantly to the presence of iron and possibly other trace metals (see below).

Generally, the hydroxyl radical may react by (i) hydrogen abstraction, (ii) electron transfer and (iii) addition reactions. The reaction of the hydroxyl radical with a biomolecule will produce another radical, usually of lower reactivity. As a result of the high reactivity of $^{\bullet}OH$, it often abstracts carbon bound hydrogen atoms more or less non-selectively, e.g. from glucose. Production of $^{\bullet}OH$ close to an enzyme molecule present in excess in the cell, such as lactate dehydrogenase, might have no biological consequences. However, attack by $^{\bullet}OH$ on a membrane lipid can cause a series of radical chain reactions that can severely damage the membranes [44]. The hydroxyl radical can also add to DNA bases leading to generation of a variety of oxidative products. The interaction of $^{\bullet}OH$ with guanine leads to the generation of 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxo-dG) and 2,6-diamino-5-formamido-4-hydroxypyrimidine (FAPy-G) [33]. 8-oxo-dG is a good marker of oxidative damage. Adenine reacts with $^{\bullet}OH$ in a similar manner to guanine, although oxidative adenine lesions are less prevalent in DNA damage.

It has been demonstrated that in the presence of Fe(III) or Fe(III)–EDTA complex, endogenous reductants such as ascorbate, glutathione (GSH), and the reduced form of nicotineamide adenine dinucleotide (NADH), caused DNA damage at every type of nucleotide with a slight dominance by guanine [45]. Specifically, NADH in the presence of Fe(III)–EDTA and H_2O_2 generated $^{\bullet}OH$ leading to formation of 8-oxo-dG. The DNA damage was inhibited by typical $^{\bullet}OH$ scavengers and by catalase, suggesting that these reductants cause DNA damage *via* the Fenton reaction.

Several *in vitro* experiments also confirmed that ferrous iron has the capacity to reduce molecular oxygen to superoxide radical (see also reaction 1).

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet -} \quad (7)$$

This reaction is accomplished in aerobic conditions at cellular and extracellular levels. However, biological reductants involving ascorbate, thiol compounds and others can restore ferrous iron according to reaction

$$Fe(III) + A(\text{biological reductant}) \rightarrow Fe(II) + A^*(\text{oxidised biological reductant}) \quad (8)$$

where A is biological reductant, for example ascorbate anion (Asc^-) and A^* is oxidised biological reductant. Ascorbic acid can act as both an iron chelator and an iron reductant. Therefore, the physiological form of ascorbic acid, ascorbate anion (Asc^-), can bind Fe(III), and subsequently reduce the iron to Fe(II) which can then reduce oxygen to superoxide. Thus, it appears as if ascorbate autoxidises, in reality the reaction is mediated by iron. Accordingly, the autoxidation of numerous biogenic amines and ascorbate is completely inhibited by the presence of strong metal chelators. Therefore, it has been suggested that biomolecules do not "autoxidise" but that the "oxidation" of biomolecules is mediated by trace amounts of transition metals, such as iron. In fact, Buettner and Jurkiewicz [46] demonstrated that the amount of ascorbate oxidised in a solution correlates with the concentration of metals in that solution.

Fe(II) can further react with hydrogen peroxide generated from two superoxide radicals (enzymatically catalyzed by SOD or much slower spontaneously), a dismutation reaction,

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (9)$$

Superoxide is not extremely reactive by itself and cannot attack DNA, however, it has been shown *in vitro* that it

favors the Fenton reaction by reducing free ferric iron (reaction 1) leading to production of hydroxyl radicals which can then damage any biomolecule. A definitive *in vivo* demonstration of iron-mediated superoxide toxicity was provided by analysis of the phenotype of *sodA sodB E. coli* mutants. The results have shown that the excess of superoxide causes DNA damage that cannot be due to direct effects of superoxide itself [47].

The redox state of the cell is largely linked to iron redox couple and is maintained under strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron, however, *in vivo*, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. For example the release of iron from ferritin, the most concentrated source of iron (up to 4500 Fe(III) atoms per molecule), has been well documented [48]. The release of iron by superoxide has also been demonstrated for [4Fe–4S] cluster-containing enzymes of the dehydratase-lyase family. Inactivation of these enzymes by $O_2^{\bullet-}$ was a rapid process that entailed oxidation of the iron-sulfur cluster and that could be reversed by treatment with Fe(II) plus thiols. The native clusters contain two Fe(II) and two Fe(III) atoms, and the oxidation may be written according to reaction

$$[2Fe(II)\ 2Fe(III)\text{-}4S] + O_2^{\bullet-} + 2H^+ \rightarrow [Fe(II)\ 3Fe(III)\text{-}4S] + H_2O_2 \quad (10)$$

The rate constant for reaction (10) has been estimated in the range of $10^8 - 10^9$ $M^{-1}s^{-1}$ [49]. Because the oxidised protein binds the Fe(III) (by sulfur ligands) more tightly, Fe(II) ions are released from protein according to the reaction [49]

$$[Fe(II)\ 3Fe(III)\text{-}4S] \rightarrow Fe(II) + [3Fe(III)\text{-}4S] \quad (11)$$

The released Fe(II) can participate in the Fenton reaction thus generating hydroxyl radical (reaction 2). The above reactions imply that *in vivo* $O_2^{\bullet-}$ acts rather as an oxidant of [4Fe-4S] cluster-containing enzymes than reductant of Fe(III) ions. Thus, in both the *in vitro* and the *in vivo* conditions, $O_2^{\bullet-}$ facilitates $^{\bullet}OH$ production from H_2O_2 by making Fe(II) available for the Fenton reaction. On the other hand, as mentioned above, *in vitro* conditions confirmed superoxide acting as a reductant of Fe(III) to Fe(II). Moreover, in the cell at neutral pH, Fe(III) has a very low solubility in water ($< 10^{-17}$ M) and precipitates in form of hydroxides.

It has been proposed that "free iron" is following absorption transported into an intermediate, labile iron pool (LIP), which represents a steady state exchangeable and readily chelatable iron compartment [50]. To avoid an excess of harmful "free iron", the LIP is kept at the lowest sufficient level by transcriptional and post-transcriptional control of the expression of principal proteins involved in iron homeostasis. LIP is defined as a low-molecular weight pool of weakly chelated iron that rapidly passes through the cell. Most probably, it consists of both forms of ionic iron (Fe(II) and Fe(III)) chelated with a variety of chelators such as citrate, phosphate, carboxylates, nucleotides and others [34]. In these iron chelates, at least one of the six possible coordination sites is left free to maintain catalytic activity. It has been found that there is a correlation between number of occupied coordination sites and catalytic activity of $^{\bullet}OH$ production [51]. LIP represents only 3-5% of the total cellular iron. Experimental evidence for this iron pool was presented by Konijn *et al*. [52] who recently detected a chelatable intracellular iron pool by introducing a method based upon quenching of the fluorescent chelator calcein by metal ions [53]. Experiments, carried out in human K 562 cell line, have suggested that the concentration of Fe in this pool (LIP) is 0.2–0.5 μM and that it is composed primarily of Fe(II). However, it should be noted that there are still serious methodological problems associated with the estimation of LIP concentrations ranging 0.2–230 μM obtained for the same types of cells and tissues.

Iron-Induced Carcinogenicity

In highly-developed, meat-eating, countries iron excess may be a serious problem because increased body iron stores is associated with an increased risk of cancer. Nelson proposed that intestinal exposure to ingested iron may be a principal determinant of human colorectal cancer [54]. A dose-dependent relationship for serum ferritin level and colon adenoma risk was found. We have proposed an alternative mechanism in which the bile acids (deoxycholic acid), the K vitamins, iron(II) complexes and oxygen interact to induce an oncogenic effect in the colon by the generation of free radicals [19].

The major cause of death in genetic haemochromatosis patients is hepatocellular carcinoma. Epidemiological trials have also shown a link between cirrhosis and genetic haemochromatosis, hepatocellular carcinoma. This implies that cirrhosis may provide a necessary replication stimulus in carcinogenic process [55]. It has been revealed that serum "free iron" in haemochromatosis patients exists largely complexed with citrate.

Occupational exposure to asbestos containing about 30% (weight) of iron is related to increased risk of asbestosis – the second most important cause of lung cancer. It is generally accepted that asbestos-induced carcinogenesis is linked with the free radicals.

Animal studies of iron-induced carcinogenesis are well-documented. Intramuscular injections of an iron-dextran complex, frequently used for the treatment of anemia in humans, caused spindlecell sarcoma or pleomorphic sarcoma in rats at the site of injection [56].

Nitrilotriacetic acid (NTA), synthetic aminotricarboxylic acid forms water-soluble chelate complexes with metal cations and is used in household detergents. The Fe-NTA complex, however, induced renal carcinogenesis. Surprisingly, this complex, regardless of number of saturated binding sites on iron by NTA, works as efficiently as "free iron" *in vitro* at physiological pH catalyzing the breakdown of hydrogen peroxide *via* Fenton reaction [57].

Targets of Iron-Mediated Oxidative Damage

Iron-induced oxidative stress has the following implications: (i) failure in redox regulation leading to DNA damage, lipid peroxidation and oxidative protein damage and (ii) free radical-induced activation of signal transduction pathways. First we focus on redox regulation failure of iron affecting lipids, proteins and DNA.

DNA

It has been concluded that the genotoxicity of many chemical compounds is likely enhanced by their ability to decompartmentalize iron [58]. There is abundant evidence that, in addition to synergising the oxidation of polyunsaturated fatty acids, "loose" intracellular iron will also promote DNA damage. In fact, iron has been implicated as an agent in numerous cancers, probably most markedly in the etiology of colorectal cancer [54]. One of the possible mechanisms by which iron could be involved in the initiation or promotion of cancer is through the oxidation of DNA. The known products of reactions between DNA, iron, and oxidants are not yet fully elucidated but include single- and double-strand breaks, oxidatively modified bases, depurination/depyrimidation, DNA-protein cross-links [59] or chemical modification of the sugar moiety [41, 59].

It has been clearly established that oxidant induced damage to naked DNA and intracellular DNA is greatly enhanced by iron [60, 61]. In the absence of transition metals such as iron and copper, DNA is quite unreactive with oxidants such as H_2O_2. However, in the presence of added iron, DNA scission occurs, preferentially in inter-nucleosomal linker regions, producing "ladders" resembling those typical of apoptosis [62, 63]. Many experiments suggest that the most aggressive species responsible for oxidising or modifying DNA is the hydroxyl radical. Superoxide radicals had no or very little effect on the oxidation of DNA in the absence of adventitious metals (see also above). This suggests that the role of superoxide in DNA oxidation is simply as a constituent of the Haber-Weiss reactions to produce the hydroxyl radical.

It has also been documented that the addition of any chemical that will act as an alternate reactant for the hydroxyl radical, such as organic based buffers or "hydroxyl radical traps," inhibits the oxidation of DNA and conversely, the presence of chemicals that increase the iron-mediated production of the hydroxyl radical will promote the oxidation of DNA. Several studies postulated that the iron-mediated oxidation of DNA is a site-specific process [64]. It was proposed that iron (or iron chelates) binds to the DNA, either to phosphate groups on the backbone or to the DNA bases where the iron can serve as a center for cycling formation of the hydroxyl radical resulting in modification of the DNA.

The bleomycin-iron complex was the first well-studied example of site-specific, metal-mediated damage to DNA [65]. The bleomycin- mediated cleavage of DNA is proposed to occur *via* formation of a ternary complex, DNA-bleomycin-iron. It is not known whether bleomycin binds to DNA first and the Fe(II) binds to the bleomycin-DNA complex or whether a bleomycin-Fe(II) complex forms and the complex binds to DNA. Regardless of the binding mechanisms, oxidation of the complexed Fe(II) results in a site-specific oxidation of DNA, most probably by the hydroxyl radical [66]. Bleomycin-iron complex cleaves DNA to release N-propenal-substituted derivatives of thymine, cytosine, adenine, and guanine. It is believed that the base propenals produced are also responsible for some of the cytotoxic effects of bleomycin.

The damage to DNA caused by iron oxidants is decreased or absolutely prevented by effective iron chelators (i.e., chelators, such as desferrioxamine (DFO), which fill all six coordination positions and make the iron chemically unreactive). The iron chelator, DFO, protects cultured cells against oxidant challenge but pharmacologically effective concentrations of this drug cannot readily be achieved *in vivo.* DFO localizes almost exclusively within the lysosomes following endocytic uptake, suggesting that truly lysosomotropic chelators might be even more effective. It was proposed that an amine derivative of α-lipoamide (LAP), would concentrate *via* proton trapping within lysosomes, and that the vicinal thiols of the reduced form of this agent would interact with intralysosomal iron, preventing oxidant-mediated cell damage [67].

Lipids

It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. Pioneering studies of lipid peroxidation by an iron complex started with the observations of Bucher *et al.* [68]. These authors proposed that the process of lipid peroxidation occurs *via* a free radical mechanism promoted by iron. Since then, the study of oxidation of lipids (or lipid peroxidation) has been a topic of many studies [42, 63].

Whilst the ability of the hydroxyl radical (generated *via* Fenton chemistry) to initiate lipid peroxidation is unquestionable, it is also necessary to take into account the diffusion-limited reactivity of the hydroxyl radical toward sugars, nucleotides, or proteins. The mechanism, proposed more than two decades ago by Bucher *et al.* [68], involves the formation of a Fe(II):Fe(III) complex (or a Fe(II)-O_2-Fe(III) species). The maximal rates of lipid peroxidation are observed when the ratio of Fe(II):Fe(III) is 1:1. Bucher *et al*. [68] also demonstrated that ADP-Fe(II) promoted the peroxidation of phospholipid liposomes but only after a lag phase. Catalase, superoxide dismutase, and hydroxyl radical scavengers did not extend the lag phase or inhibit the subsequent rate of lipid peroxidation.

Several experimental models of iron overload *in vivo*, confirmed increased polyunsaturated fatty acids (PUFA) oxidation of hepatic mitochondria, as well as lysosomal fragility [69]. Bacon and co-workers [70] observed that, following oral intake of carbonyl iron in rats, mitochondrial lipid peroxidation occurred. Experiments also showed that this was accompanied by substantial decrements in mitochondrial metabolism. These observations suggest that mitochondrial PUFA are a preferential target for iron-driven peroxidation.

In addition to mitochondrial functions, the deleterious process of the peroxidation of lipids is also very important in arteriosclerosis and inflammation. The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination [33]. Initiation, the first stage, involves the attack of a reactive oxygen species capable of abstracting a hydrogen atom from a methylene group in the lipid. The presence of a double bond adjacent the methylene group weakens the bond between carbon and hydrogen so the hydrogen can be more easily removed from the fatty acid molecule. Fatty acids with no double bonds or with one double bond can undergo oxidation but not a chain lipid

peroxidation process. For example oleic acid (18:1) with 18 carbon atoms and one double bond cannot undergo lipid peroxidation events [71]. The process of hydrogen abstraction leaves behind a fatty acid having one unpaired electron (see Fig. (**1**)). When oxygen is present in the surrounding tissues, the fatty acid radical can react with it leading to the formation of lipo-peroxyl radicals ($ROO^{\cdot}$) during the propagation stage. These radicals are themselves very reactive and are capable of abstracting another hydrogen from the neighboring fatty acid molecule thus creating lipid hydroperoxides ($ROO^{\cdot} + H^{\cdot} \rightarrow RO{-}OH$). Lipid hydroperoxides ($RO{-}OH$) are a relatively short-lived species. They can either be reduced by glutathione peroxidases to unreactive fatty acid alcohols or they react with redox metals to produce a variety of products which are themselves reactive (e.g. epoxides, aldehydes, etc.). The reaction of lipid

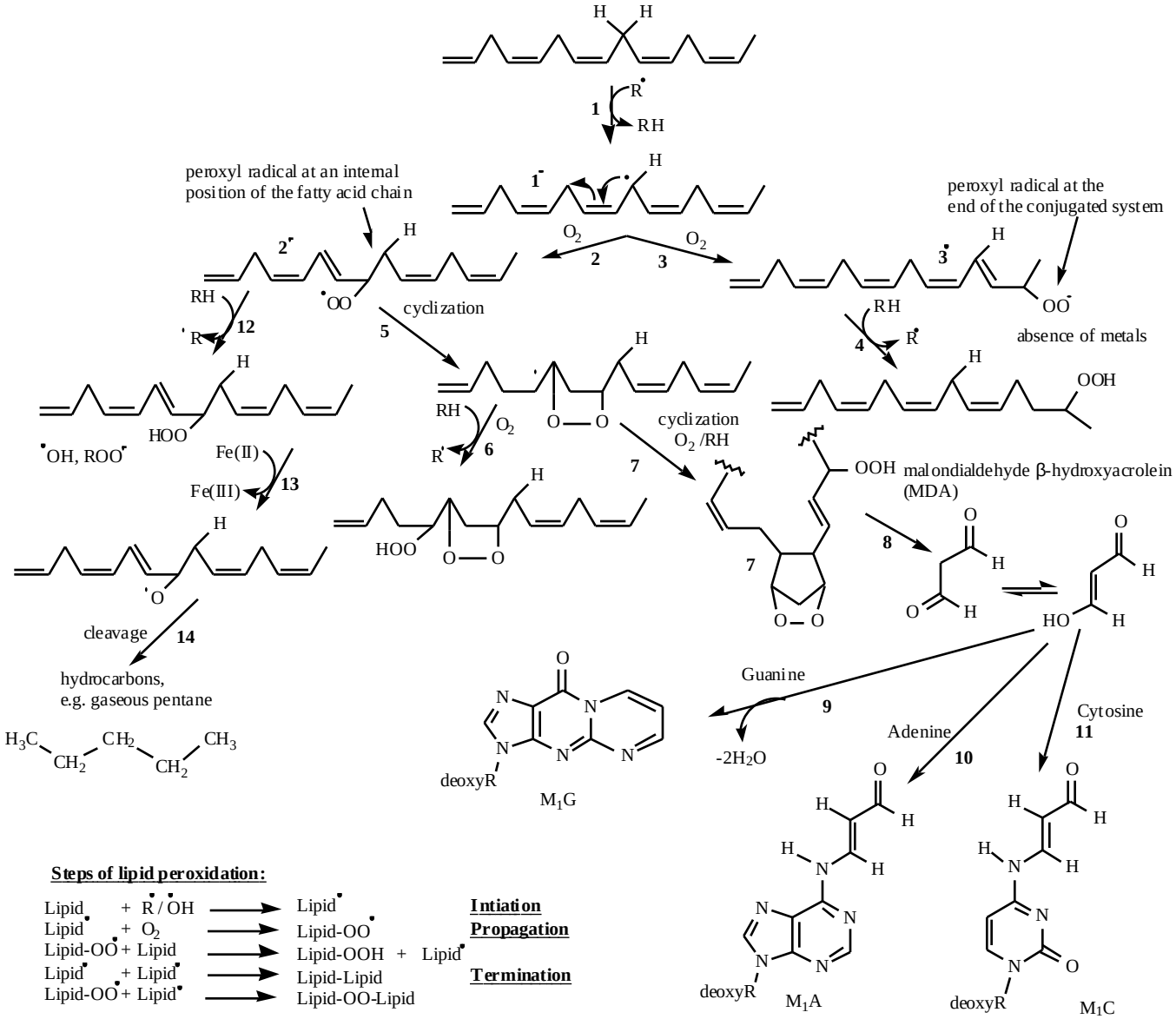


Fig. (1). The methylene groups of polyunsaturated fatty acids are highly susceptible to oxidation and their hydrogen atoms, after the interaction with radical $R^{\bullet}$, are removed to form carbon-centered radicals **1**$^{\bullet}$ (**reaction 1**). Carbon centered radicals react with molecular dioxygen to form peroxyl radicals (**reactions 2 and 3**). If the peroxyl radical is located at the end of a conjugated system (**3**$^{\bullet}$) it is reduced to a hydroperoxide which is relatively stable in the absence of metals (**reaction 4**). A peroxyl radical located at an internal position of the fatty acid chain (**2**$^{\bullet}$) can either react by cyclization to produce a cyclic peroxide adjacent to a carbon-centered radical (**reaction 5**). This can then either be reduced to form a hydroperoxide (**reaction 6**) or, by **reaction 7**, can undergo a second cyclization to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Compound **7** is an intermediate product in the production of malondialdehyde (**reaction 8**). Malondialdehyde can react with DNA bases dG, dA, and dC to form adducts M_1G, M_1A and M_1C. (**reactions 9, 10, 11**). Peroxyl radical located in the internal position of the fatty acid (**2**$^{\bullet}$) can, in addition to the cyclization reactions (**reaction 5**), also abstract hydrogen from the neighboring fatty acid molecule, thus creating lipid hydroperoxides (**reaction 12**). They can further react with redox metals (e.g. iron) to produce reactive alkoxyl radicals ($RO^{\bullet}$) (**reaction 13**) which after cleavage (**reaction 14**) and depending on the chain length, may form e.g. gaseous pentane a good marker of lipid peroxidation.

hydroperoxides with for example Fe(II) and Fe(III) ions lead to the formation of very reactive alkoxyl radicals (RO−OH + Fe(II) → $RO^{\cdot}$ + Fe(III)) and lipo-peroxyl radicals ($ROO^{\cdot}$), respectively. Once formed, lipo-peroxyl radicals ($ROO^{\cdot}$) can be rearranged *via* a cyclization reaction to endoperoxides (precursors of malondialdehyde) with the final product of peroxidation process being malondialdehyde (MDA) (Fig. (**1**)). The major aldehyde product of lipid peroxidation besides malondialdehyde is 4-hydroxynonenal (HNE) [72]. MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynoneal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. In addition, HNE has powerful effects on signal transduction pathways which in turn have a major effect on the phenotypic characteristics of cells. Peroxidation of lipids is an autocatalytic process and is ended by the termination process, for example by the recombination of radicals ($R^{\cdot}$ + $R^{\cdot}$ → nonradical product) or depletion of the substrate.

MDA can react with DNA to form adducts to dG, dA and dC (Fig. (**1**), reactions 9-11) [72]. M_1G and presumably M_1A and M_1C also can be made by the reaction of the corresponding bases with the base propenal, providing an alternate route for their generation by direct oxidation of DNA. M_1G residues were detected in tissue at levels ranging from below the limit of detection to as high as 1.2 adducts per 10^6 nucleosides (which corresponds approximately 6000 adducts per cell). M_1G has also been detected in human breast tissue by ^{32}P-post-labeling as well as in rodent tissues. Site-specific experiments confirmed that M_1G is mutagenic in *E.coli*, inducing transversions to T and transitions to A. The mutation frequencies are comparable with those reported for 8-oxo-dG in similar systems. M_1G is repaired by both bacterial and mammalian nucleotide excision repair pathways and is also repaired in *E.coli* by mismatch repair. Studies employing NMR spectroscopy indicate that M_1G undergoes rapid and quantitative ring-opening to form N^2-oxopropenyl-G when it is present in duplex DNA; however, not when it is present in single-stranded DNA. While the reactive functionality of M_1G is present in the major groove, the reactive functionality of N^2-oxo-propenyl-dG is present in the minor groove of DNA. The interconversion of M_1G and N^2-oxo-propenal-dG within the DNA may lead to the formation of DNA–DNA interstrand crosslinks or DNA–protein crosslinks [72].

Etheno Adducts

There are also other exocyclic DNA adducts that arise from lipid peroxidation. For example etheno-dA, etheno-dC and etheno-dG have been detected by both 32P-post-labeling and GC-MS [73]. While the precise pathway of their formation in DNA is unknown, the adducts can readily be generated *in vitro* by exposure of DNA to a peroxidising lipid. The biological activity of etheno adducts involves transitions to A (induced by N2,3-etheno-dG) and transversions to T in *E.coli* (induced by 1,N2-etheno-dG). It has been demonstrated that etheno-dA and etheno-dC are strongly genotoxic but weakly mutagenic when introduced on single-stranded vectors in *E.coli*. Etheno-dA induces predominantly transitions to G whereas etheno-dC induces transversions to A and transitions to T. Studies dealing with the repair of etheno adducts have shown that etheno-dA is removed by the action of 3-methyladenine glycosylase and its mammalian homologue AAG. In addition to efficient removal by glycosylases, other repair pathways should also be considered.

Propano Adducts

It has been demonstrated that hydroxypropanodeoxyguanosines (HO-PdGs) are present in human and rodent liver DNA [72, 73]. These adducts are most probably derived from the reaction of DNA with acrolein and crotonaldehyde generated by a lipid peroxidation process. Acrolein and crotonaldehyde are mutagenic in bacteria and mammalian cells. However, the mutagenic potency of HO-PdGs has not been evaluated by site-specific approaches, due to the instability of these adducts, which renders their incorporation into oligonucleotides unviable. Therefore a novel, post-oligomerization strategy for the synthesis of oligonucleotides containing the acrolein-derived HO-PdG was reported recently which should make it possible to construct the requisite adducted vectors. Experiments with unsubstituted adduct PdG revealed that this induces base pair substitution mutations in *E.coli* with high efficiency. To date little is known about the repair of HO-PdGs. There may be a possibility that PdG or HO-PdGs are substrates for base excision repair enzymes, however, this needs to be evaluated in more detail.

Proteins

Proteins are resistant to damage by H_2O_2 and simple oxidants unless transition metals are present. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine cross links, the introduction of carbonyl groups, and the formation of protein-centered alkyl, $R^{\cdot}$, alkoxyl, $RO^{\cdot}$, and alkylperoxyl, $ROO^{\cdot}$, radicals [63]. Protein damage is likely to be a repairable and is a non-lethal event for a cell, however, evidence has been reported that two mitochondrial proteins–aconitase and adenine nucleotide–translocase may be important targets of long-term oxidative damage [74].

Studies on the metal-induced protein denaturation led to the discovery that degradation occurs when the protein has been oxidised [75]. Iron-mediated oxidation of a protein may be a site-specific process as proline, histidine, arginine, lysine, and cysteine residues in proteins are highly sensitive to oxidation by iron [75]. It is believed that the iron(II) binds to high– and lower–affinity metal–binding sites on the protein, most probably involving the above–mentioned amino acids. The Fe(II) –protein complex reacts with H_2O_2 *via* the Fenton reaction to yield an active oxygen species, e.g., $^{\bullet}OH$, ferryl ion, etc., at the site. While it has been proposed by many authors that the hydroxyl radical represents the major species responsible for the oxidation of protein, clear experimental evidence is still missing. Experimental studies revealed oxidised side chains of amino acids involving carbonyl derivatives, loss of catalytic activity, and increased susceptibility of the protein to proteolytic degradation [75].

A recent study by Welch *et al.* [76] demonstrated the site-specific modification of ferritin by iron which involved the oxidation of cysteine, tyrosine, and also some other residues. Whilst the hydroxyl radical scavenger HEPES protected the protein against oxidation, catalase did not, confirming the site-specific oxidation of ferritin.

The oxidation of myoglobin by H_2O_2 yields ferrylmyoglobin, which contains two oxidising equivalents: the ferryl complex and an amino acid radical. Using EPR spectroscopy Giulivi and Cadenas [77] showed that the spectra of the amino acid radicals consisted of a composite of three signals attributable to a peroxyl radical, a tyrosyl radical, and radicals in an aromatic amino acid-containing peptide. The aromatic amino acid radical was observed to be relatively long lived and in close proximity to the heme iron. Hence, it has been proposed that this is the first site of the protein radical. Reduction of the ferryl complex by Tyr described by the reaction.

$$Fe(IV)=O + Tyr\text{-}OH + H^+ \rightarrow Fe(III) + H_2O + Tyr\text{-}O^{\bullet} \quad (12)$$

and alternatively by other amino acids leads to the subsequent formation of other amino acid radicals within an electron-transferprocess throughout the protein. This view suggests that the protein radical(s) is highly delocalized within the globin moiety in a dynamic process which encompasses electron tunnelling through the backbone chain, or H-bonds, leading to the formation of secondary radicals.

Iron-Induced Oxidative Stress and Antioxidants

An antioxidant is any substance capable of preventing oxidation. Deleterious free radical-mediated oxidations occur in aerobic organism as a result of normal oxygen metabolism. Iron, especially ferrous iron, is able to trigger oxidations by reducing, as well as by decomposing, previously formed hydrogen peroxide. Generally, an antioxidant can protect against iron toxicity by (i) chelating ferrous iron and preventing the reaction with molecular oxygen or peroxides, (ii) chelating iron and maintaining it in a redox state that makes iron unable to reduce molecular oxygen and (iii) trapping formed radicals.

A large number of substances present within the cell, including also antioxidants, can chelate iron *in vivo* thus limiting its participation in free radical reactions. For example desferrioxamine mesylate (DFO) is a powerful iron-chelating substance capable of almost completely suppressing iron-mediated oxidations in biological systems. DFO is often used as a therapeutic tool in the treatment of iron overload [78]. In contrast, EDTA is a synthetic compound, normally present in food, that can chelate iron, but is unable to prevent iron mediated oxygen reduction; therefore its efficiency as an antioxidant is low (discussed above).

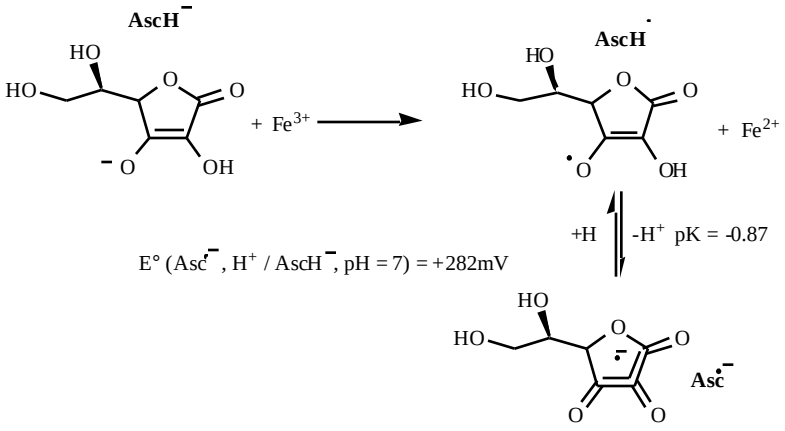
There are a number of substances that have been defined as antioxidants, because of their capacity to protect biomolecules from free radical-mediated damage both *in vivo* and *in vitro*. One of the most effective antioxidant synthesised by mammals are thiol compounds, especially glutathione, which provides significant antioxidant protection. This protection is related to the ability of glutathione to trap radicals, reduce peroxides, and to maintain the redox state of the cells [79].

The interaction between iron overload and the dietary antioxidant vitamin E has been well characterised. Vitamin E is most important lipid-soluble antioxidant [80]. Several studies have shown that vitamin E can prevent the majority of iron-mediated damage both, in *in vitro* systems, and in iron-loaded animals [81].

Regular intravenous iron injections in haemodialysis patients is known to enhance the extent of lipid oxidation. Administration of vitamin E (1200 U/day) in these patients attenuated the extent of lipid oxidation thus providing protection from free radical-mediated damage. In this connection a very recent epidemiological study on the intake of vitamin E supplements showed that taking daily vitamin E doses of 400 IU or move can increase the risk of death, and consequently should be avoided, which came as a great surprise [82].

The biological consequences of the interaction of vitamin C (ascorbate, ascorbic acid) and iron are much less clear. Ascorbic acid has two ionizable hydroxyl groups and is therefore a di-acid ($AscH_2$). At physiological pH, 99.9% of vitamin C is present as $AscH^-$, and only very small proportions as $AscH_2$ (0.05 %) and Asc^{2-} (0.004%). The antioxidant chemistry of vitamin C is thus the chemistry of ascorbate monoanion, $AscH^-$. Ascorbate under physiological conditions ($AscH^-$) can reduce "free iron" (ferric) to ferrous iron according to the reaction (Scheme (**1**)) [33].

This reaction also forms the semidehydroascorbate radical ($Asc^{\bullet -}$), a poorly-reactive radical that can either be converted back to ascorbate by NADH-dependent enzymes or undergo disproportionation to form dehydroascorbate (DHA) [83]. Since Fe(III) has very low solubility, the ability of ascorbate



Scheme 1.

to reduce Fe(III) to Fe(II) has significance in iron sorption in gut [84].

Reduced iron, Fe(II), can further promote the initiation (hydroxyl radical formation *via* Fenton chemistry) and propagation (*via* lipid alkoxyl radical formation) of free radical reactions [83]. Although the Fenton chemistry is known to occur *in vitro*, its significance under physiological conditions is not clear, assuming the negligible availability of "free catalytic iron". As already discussed above, the amounts of free iron are thought to be low due to its sequestration by the various metal-binding proteins (e.g. ferritin, transferrin). However, organisms overloaded by iron (haemochromatosis, b-thalassemia, hemodialysis) contain higher amounts of "free available iron" which in the presence of vitamin C can have deleterious effects. Thus in these cases, vitamin C tissue levels should be maintained in the low range, and vitamin C supplementation should be avoided.

Over the past years in addition to the antioxidant role of ascorbate, several studies explored pro-oxidant properties of ascorbate [85]. Concerns have been raised, mainly over potentially deleterious transition metal ion-mediated pro-oxidant effects, about ascorbate. A recent report on the pro-oxidant effect of the iron and vitamin C interaction *in vivo*, revealed that a daily combined supplement of iron (100 mg as fumarate) and vitamin C (500 mg as ascorbate) in the third trimester of pregnancy caused a 20% increase in plasma lipid oxidation [86]. Since only the plasma content of substances reactive to 2-thiobarbituric acid (TBARS) was determined in this study the outcomes should be treated with caution.

The pro-oxidant effect of vitamin C supplementation after muscle injury was reported by Childs *et al*. [87]. These authors tried to ascertain whether vitamin C and antioxidant N-acetyl-cysteine (NAC) could act as pro-oxidants in humans during inflammatory conditions. Acute-phase inflammatory response was induced by an eccentric arm muscle injury. The resulting muscle injury caused increased levels of serum bleomycin-detectable iron. The amount of iron was higher in the subjects consumed vitamin C and NAC immediately following the injury compare to a group who consumed a placebo. The concentrations of lactate dehydrogenase (LDH), creatine kinase (CK), and myoglobin were also significantly elevated, however they returned to the baseline levels within 7 days. The results of this acute human inflammatory model strongly suggests that vitamin C and NAC supplementation immediately after injury, transiently increases tissue damage and oxidative stress.

On the other hand a predominant number of papers including *in vivo* studies reported either antioxidant or nil effect of vitamin C, even in the presence of iron (or copper). A recent study by Proteggente *et al*. [88] explored the effect of iron supplementation on oxidative damage to DNA in healthy individuals with plasma ascorbate levels at the upper end of the normal range. Oxidative damage to DNA bases from white blood cells was measured by gas chromatography/mass spectrometry with selected-ion monitoring (GC/MS-SIM), using isotopically-labelled standards for quantification. Iron supplementation did not affect any of the iron status parameters and there were no detrimental effects over the period under investigation in terms of oxidative damage to DNA. However, the effectsof larger doses or of longer supplementation periods should also be investigated. In line with these findings Lin and Ho [89] evaluated *in vivo* the antioxidative action of melatonin on iron-induced neurodegeneration in the nigrostriatal dopaminergic system. Melatonin dose-dependently suppressed autoxidation and iron-induced lipid peroxidation. Melatonin was as effective as GSH but was less effectivethan Trolox, a water-soluble analogue of vitamin E suggesting that melatonin could, at least partially inhibit iron-induced neurodegeneration.

The role of free radicals in sepsis and the benefit of antioxidant repletion has been suggested by previous studies, thus the effect of intravenous antioxidant therapy on antioxidant status, lipid peroxidation, hemodynamics and nitrite was investigated in patients with septic shock by Galley *et al*. [90]. The authors confirmed markedly depleted vitamin C concentrations in plasma and elevated levels of redox-active iron. Lipid peroxides were elevated in all patients with septic shock but did not increase further in the patients receiving antioxidant supplements. Plasma total nitrite, heart rate and cardiac index increased in the patients receiving antioxidants. Enhanced production of nitric oxide has been implicated in septic shock.

Cardoso *et al.* [91] studied the combined effect of ascorbate/iron and vitamin E on the mitochondrial damage under oxidative stress conditions examined in synaptosomes. A loss of membrane integrity was observed in peroxidised synaptosomes which was prevented by pre-incubation with vitamin E. ATP levels decreased in synaptosomes exposed to ascorbate/iron, as compared to controls. However, vitamin E, idebenone and GSH prevented the reduction of ubiquinol cytochrome c reductase activity observed in synaptosomes treated with ascorbate/iron. These results may indicate that the inhibition of the mitochondrial respiratory chain enzymatic complexes that are affected in different ways by oxidative stress can be recovered by specific antioxidants.

Several other studies confirmed, both in animal models and in humans, no evidence of increased free radical damage due to the combined ingestion of iron and ascorbate [92]. These results are in agreement with the very recent *in vitro* study of Suh *et al.* [93] who studied possible pro-oxidant effects of ascorbate in the presence of iron on a representative biological fluid, human plasma. The results have shown that even in the presence of redox-active iron (or copper) and H_2O_2, ascorbate acts as an antioxidant that prevents lipid peroxidation and does not promote protein oxidation in human plasma *in vitro*. Alul *et al.* [94] found that vitamin C protected LDL from homocysteine-mediated oxidation through covalent lipoprotein modification involving dehydroascorbic acid. Protection of LDL from homocysteine-mediated oxidation by vitamin C may have implications for the prevention of cardiovascular disease. In patients undergoing haemodialysis, lipoperoxidation (LPO) processes were determined in plasma and red blood cells before and after dialysis [95]. To assess the lipoperoxidation process, polyunsaturated fatty acids (PUFA), the end product of LPO, malondialdehyde (MDA), and the hydrophobic antioxidant systems, levels of vitamins A and E were determined. The results showed that the plasma of patients undergoing haemodialysis exhibited increased levels of vitamin A only

(before and after dialysis) in red blood cells. These data suggest that increased vitamin A in red blood cells may provide some degree of protection against oxidative stress in erythrocytes, but not in plasma where LPO was observed.

In another study [96] it was stated that atherosclerotic patients have a lower concentration of vitamin C and higher levels of labile iron pool compared to the control group. The oxidative stress was manifested by a higher level of 8-OH-dG in blood lymphocytes, suggesting that these factors may create an environment that promotes the development of atherosclerosis. Similarly, the role of the metal-catalyzed production of hydroxyl radicals on gastric ulceration caused by restraint-cold stress in rat was studied by Das *et al.* [97]. Stress causes a 50% increase in the thiobarbituric acid reactive species (TBARS), a measure of the lipid peroxidation, a nearly 70% increase in protein oxidation as measured by its carbonyl content and about 40% decrease in the glutathione content of the fundic stomach, suggesting oxidative damage by stress. Increased level of TBARS and the inactivation of gastric peroxidase are also prevented by desferrioxamine, a nontoxic transition metal ion chelator or by antioxidants such as glutathione or vitamin E, suggesting the critical role of metal ion and $^{\bullet}OH$ in the oxidative damage. The results indicate that the stress-induced gastric ulceration is a consequence of the oxidative damage of the gastric mucosa.

COPPER

Copper (cuprum) is the 29th element of the Periodic table. Its electronic configuration is $3d^{10}4s^{1}$. Cuprous ion (Cu(I), Cu^{+}) has completely filled *d*-orbitals with 10 electrons ($3d^{10}$), while the cupric ion (Cu(II), Cu^{2+}) has only 9 electrons in the *d* orbitals, ($3d^{9}$) with one electron being unpaired. Consequently bivalent copper (Cu(II)) is paramagnetic and represents the most stable oxidation state of copper.

Copper is not only a ubiquitous metal in the technological environment, it is also essential for the function of most living organisms [98]. In the same way that it allows the movement of electrons through wires, it also helps catalyze the movement of electrons within biological molecules. Equipped with a high redox potential, copper serves as a cofactor for proteins involved in a variety of biological reactions, such as photosynthesis and respiration, connective tissue formation, iron metabolism, free radical eradication and neurological function [99].

Copper Homeostasis

Copper, in excess of cellular needs, mediates free radical production and direct oxidation of lipids, proteins, and DNA. Therefore the balance between intracellular and extracellular contents of copper is driven by cellular transport systems that regulate uptake, export and intracellular compartmentalization [100]. The balance between copper necessity and toxicity is achieved both at the cellular level and at the tissue and organ levels [101].

Cells regulate the traffic of transition metal ions (such as copper and iron), maintaining the amount necessary for biological function while avoiding excess levels that are toxic. Among the factors required to achieve such metal ion homeostasis are the metallochaperones, proteins that, like chaperones in ordinary life, guide and protect transition metal ions within the cell, delivering them safely to the appropriate protein receptors. “Metallo-Chaperones” can also prevent dangerous reactions that can cause damage to the cell. Rae *et al.* [43] discovered that the copper chaperone for the superoxide dismutase gene is necessary for expression of an active, copper-bound form of superoxide dismutase, SOD, *in vivo*, in spite of the high affinity of SOD for copper and the high intracellular concentrations of both, SOD and copper. In the course of this investigation, the authors made the remarkable discovery that the upper limit of so-called "free pools" of copper was far less than a single atom per cell. Until recently, it had been commonly believed that metal ions were in equilibrium with metalloproteins, however, their results suggest that there is a significant overcapacity for chelation of copper in the cell and there must be multiple processes that bind the copper and prevent it from ever being randomly available. The implications of this finding are profound, especially if applicable to other physiologically important transition metals. This discovery has wide implications on the mechanisms of intracellular formation of free radicals by means of Fenton chemistry (see below).

The absorption of copper in the human body is a complex process and depends on various factors and dietary components. About 40% of ingested Cu is absorbed in the small intestine, very small amounts are absorbed in the stomach [102]. Copper is absorbed mainly through so-called amino acid transport system, mostly through histidine, methionine, and also cysteine. Copper absorbed from the small intestine is transported in the blood bound predominantly to serum albumin [103]. Copper taken up by the liver may be stored in hepatocytes (predominantly bound to metallothionenin), secreted into plasma, or excreted in bile. About 80% of the copper circulating in the blood is an integral part of ceruloplasmin (an enzyme containing six copper atoms both in cupric Cu(II) or in cuprous Cu(I) state).

Homeostatic regulation of copper is the primary mechanism for regulating the body’s copper content. Homeostatic regulation involves copper absorption and excretion. The major excretory route of copper stored in liver is *via* biliary pathway (~80%). Loss through faeces in adults is about 3 mg/day [104]. There are also minor losses of copper through sweat (~ 70 µg/day) and urin/e (~ 30 µg/day), representing only 3% of the daily copper intake. The recommended daily copper intake in adults is 0.9 mg/day [102].

Until recently, very little was known about the detailed mechanism of how trace amounts of dietary copper are assimilated by intestinal absorptive cells to enter the body. Cu transport at the cell surface and the delivery of Cu to intracellular compartments are critical events for a wide variety of biological processes. The components that orchestrate intracellular Cu trafficking and their roles in Cu homeostasis have been elucidated by the studies of model microorganisms and by the characterization of the molecular basis of Cu-related genetic diseases, including Menkes disease and Wilson disease [98]. However, little is known

about the mechanisms for Cu uptake at the plasma membrane and the consequences of defects in this process in mammals.

Ironically, insights into copper metabolism initially came from experiments aimed at understanding how cells take up iron [98, 105]. Dancis and co-workers were trying to identify components of the high-affinity iron uptake system in yeast cells [105]. One of the first genes they found encoded a putative transmembrane transport protein that, to their surprise had no affinity for iron, but rather transported copper. This protein supplies the metal for a multicopper ferroxidase needed for iron transport and was designated Ctr1p. Recently, three groups independently confirmed these results on mammalian cells [99, 106, 107]. For the sake of completeness we note that in fact two possible candidate proteins responsible for the absorption of dietary copper have emerged; the divalent metal transporter 1 (DMT1), also known as natural resistance associated macrophage protein 2 (Nramp2) and divalent cation transporter 1 (DCT1), and the recently discovered high-affinity copper transporter 1 (Ctr1) (Fig. (**2**)). Here we briefly discuss recent discoveries about the possible role of the high-affinity copper transporter Ctr1 as an intestinal copper transporter and its role in body copper homeostasis.

As already mentioned above, three groups recently confirmed that the mouse *Ctr1* gene encodes a component of the copper transport machinery. The results also show that mice completely deficient in *Ctr1* exhibit profound growth and developmental defects and die *in utero* in mid-gestation. These results demonstrate a crucial role for Cu acquisition through Ctr1 protein transporter. The Ctr1 is therefore most probably the primary route for copper uptake in mammals. Furthermore, it has been proposed that human Ctr1 (hCtr1) contains three transmembrane domains and that the *N*-terminus of hCtr1, which contains several putative copper-binding sites, is localized extracellulary, whereas the C-terminus is exposed to the cytosol [108]. Further studies also indicate that multiple regions in the N-terminus are essential for the self-interaction of protein. Protein Ctr1 spans the membrane at least six times, permitting formation of a channel, which is consistent with its proposed role as a copper transporter [108]. The energy coupling mechanism is not known for this protein. Since copper is largely present as Cu(II) in the diet, it must first be reduced to Cu(I) to be transported *via* Ctr1. There are numerous mechanisms potentially capable of reducing copper including endogenous plasma membrane reductases as well as dietary components such as ascorbate (Fig. (**2**)). However, it is generally accepted that dietary reducing agents inhibit copper absorption. Once copper is reduced it is known that Ctr1 takes up Cu(I) across the plasma membrane by an energy independent mechanism that is stimulated by extracellular acidic pH and high K^+ concentrations [106]. It has been speculated [106] that Ctr1 also transports another metals, such as nickel and zinc, both neighbours to copper in the Periodic table.

The human genetic disorders, X-linked Menkes disease and autosomal recessive Wilson disease highlight the importance of intact cellular transport mechanisms. These two diseases are caused by mutations in distinct genes encoding copper-transporting P-type ATPases [109, 110]. The protein encoded by the Wilson disease gene (*WND; ATP7B*) has a 56% overall identity to that of the Menkes disease gene (*MNK; ATP7A*). The patterns of *MNK* and *WND* gene expression in the adult are markedly different and correlate with the distinct clinical manifestations of the disorders. The Menkes gene is ubiquitously expressed in adult tissues, with little or no expression in liver. Mutations in this gene lead to defective cellular export of copper. In Menkes disease, copper is significantly accumulated in some tissues (kidney and intestinal mucosa), leading to failure of copper delivery to other tissues, resulting in systematic copper insufficiency. Clinical manifestations include progressive neurologic degeneration (seizures), growth failure, hypopigmentation, arterial aneurysms and skeletal defects. Deficiencies in cuproenzyme activity in multiple tissues, e.g. lysyl oxidase (cross-linking of elastin and collagen), cytochrome *c* oxidase (mitochondrial transport of electrons), superoxide dismutase (free radical detoxification), tyrosinase (pigmentation) and dopamine (β-amyloid), are possibly responsible for many of the clinical features [102].

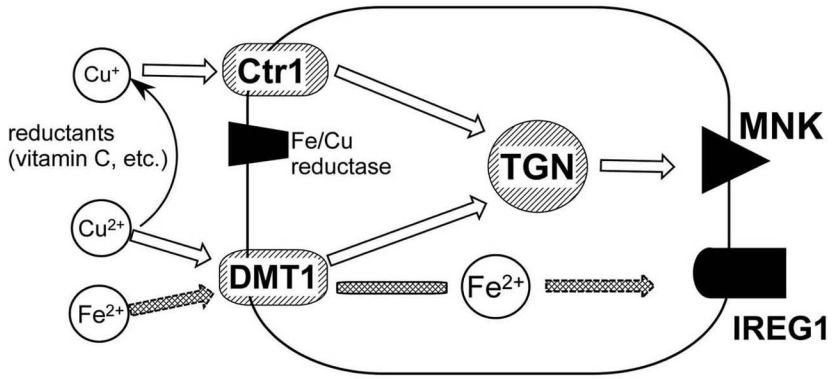


Fig. (2). A simplified model for the absorption of copper by intestinal enterocytes. Copper is present in the diet as Cu^{2+}, and could be absorbed across the apical membrane of enterocytes as either Cu^{2+} *via* DMT1 (using a common pathway with iron) or reduced (by endogenous biological reductases and dietary components such as vitamin C) to Cu^+ prior to uptake by high-affinity copper transporter, Ctr1. Absorbed copper is bound by intracellular chaperones and delivered to the transgolgi network (TGN) and is ultimately exported from the cell *via* the Menkes ATPase (MNK). Iron is also absorbed *via* DMT1, and is released across the basolateral membrane of enterocytes *via* its own specific transporter, IREG1. Both transport processes are coupled to the proton gradient that exists across the enterocyte apical membrane.

Copper and Free Radicals

One of the most accepted explanations for copper-induced cellular toxicity comes from the assumption that copper ions are prone to participate in the formation of reactive oxygen species (ROS) [102]. Cupric and cuprous copper ions can act in oxidation and reduction reactions. The cupric ion (Cu(II)), in the presence of biological reductants such as ascorbic acid or GSH, can be reduced to cuprous ion (Cu(I)) which is capable of catalyzing the formation of reactive hydroxyl radicals ($^{\bullet}OH$) through the decomposition of hydrogen peroxide (H_2O_2) *via* Fenton reaction [111]

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^- \quad (13)$$

The hydroxyl radical is extremely reactive and can further react with practically any biological molecules in the near vicinity *via* hydrogen abstraction leaving behind carbon-centered radical e.g. form a lipid radical from unsaturated fatty acids [112]. Experimental studies confirmed that copper is also capable of inducing DNA strand breaks and oxidation of bases *via* oxygen free radicals [113]. Copper in both oxidation states (cupric or cuprous) was more active that iron in enhancing DNA breakage induced by the genotoxic benzene metabolite 1,2,4-benzenetriol. DNA damage occurred mainly by a site-specific Fenton reaction [113]. When considering copper as a metal participating in Fenton chemistry one should bear in mind a recent discovery that upper limit of "free" pools of copper is far less than a single ion per cell [43]. This suggests a significant overcapacity for chelation of copper in the cell (see also above).

Some authors studied the interaction of copper on DNA in solutions irradiated by γ-radiation which revealed that copper causes dose-dependent changes in DNA conformation in oligonucleotides; no conformation changes were observed in the absence of copper. Several other studies confirmed the protective effect of antioxidants against copper-induced changes in DNA conformations induced by γ-radiation (see below) [114].

A series of studies exploring the role of copper on low-density lipoprotein (LDL) oxidation were conducted. LDL oxidation has several consequences including promotion of atherogenesis by enhancing the transformation of macrophages into foam cells and by developing vasoconstrictor and prothrombotic properties [115]. Whilst *in vivo* the relevance of the initiation of LDL oxidation by copper ions is not yet clear, *in vitro* studies clearly demonstrated LDL oxidation induced by copper [116]. *In vitro* studies of LDL oxidation are realized by incubating the system with copper ions. Samples possessing atherosclerotic lesions contained either copper or iron ions capable of catalyzing free radical formation. In addition to studies on the oxidation of LDL, it is also known that high-density lipoprotein (HDL) is susceptible to oxidation. These studies are very important because oxidation of HDL significantly affects their cardioprotective properties [117]. It is assumed that HDL is even more prone to copper-induced oxidative damage than LDL due to an increase in the so called α-tocopherol-mediated peroxidation model, in which polyunsaturated fatty acid oxidation is initiated by the α-tocopheroxyl radical, generated during the reduction of Cu(II) to Cu(I) by α-tocopherol [118]. Peroxidation of lipids can further be achieved by generation of hydroxyl radicals *via* Cu(I) catalyzed Fenton chemistry. Dose-dependent oxidative damage to HDL was observed in the studies of incubating copper with HDL [118].

It has been reported that not only free ions of copper but also an enzyme ceruloplasmin may be involved in LDL oxidation by serving as a source of unbound copper [119]. Nitric oxide and superoxide react by forming peroxynitrate, a cytotoxic radical which attacks proteins and lipids. Attack of a protein–cerulopasmin may lead to release of copper ions which may in turn contribute to LDL oxidation [119].

Superoxide dismutases (SODs) are normally involved in the protection of cells from oxygen toxicity, therefore the role of SOD in DNA damage was also investigated. Of interest were recent experimental studies exploring the effect of copper deficiency on oxidative DNA damage in Jurkat T-lymphocytes [120]. The results have shown that copper deficiency increased cellular susceptibility to oxidative damage. This observation can be explained by an orchestrated action of cellular antioxidant defence mechanisms; copper depletion leads to decreased capability of cells to produce SOD, thus increasing their propensity to oxidative damage.

However, several papers have reported that the overexpression of CuZn-SOD causes oxidative damage to cells. A mechanism by which an excess of SODs accelerates oxidative stress was investigated [121]. The presence of CuZn-SOD, Mn-SOD or Mn(II) enhanced the frequency of DNA damage induced by hydrogen peroxide and Cu(II), and altered the site specificity of the latter: H_2O_2 induced Cu(II)-dependent DNA damage with a high frequency at the 5'-guanine of poly G sequences; when SODs were added, the frequency of cleavages at the thymine and cytosine residues increased. SODs also enhanced the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine by H_2O_2 and Cu(II). It was concluded that overexpression of SODs may increase carcinogenic risks.

Of particular interest were results obtained from an investigation of the role of intracellular iron, copper, and calcium in hydrogen peroxide-induced DNA damage using cultured Jurkat cells [122]. These results demonstrate the involvement of intracellular redox-active Fe ions in H_2O_2-induced single strand breaks (SSB) formation. This conclusion was based on the ability of a number of Fe chelators to reduce the levels of H_2O_2-induced SSB. In contrast, specific copper chelators were ineffective at preventing H_2O_2-induced DNA damage, indicating that Fe represents the main redox-active transition metal inside the cell. These results are consistent with recent findings indicating that intracellular free copper is limited to less than one free copper ion per cell and suggest that a pool of free copper ions is not used in the physiological activation of metalloenzymes and therefore intracellular free copper is undetectable (see above) [110]. On the other hand intracellular free iron was detected in micromolar concentrations [123]. In this paper the authors also proposed that intracellular Ca(II) changes are involved in this process as well. The exact role of Ca(II) and its relation to intracellular transition metal ions, in particular iron, however, needs to be further investigated.

The studies of reproductive toxicity and the carcinogenicity of metals, performed on human protamine

HP2(1-15) a peptide modeling the *N*-terminal amino acid sequence of human protamine HP2, were presented by Liang *et al*. [124] and Bal *et al*. [125]. Human protamine (HP2) has an amino acid motif at its N-terminus that can serve as a heavy metal trap, especially for Ni(II) and Cu(II). The circular dichroism spectra of Cu(II) and Ni(II) complexes of HP2(1-15) indicate that the N-terminal metal binding affects the overall conformation of the peptide that, in turn, may alter interaction of HP2 with DNA. These results imply that HP2 is a likely target for the toxic metals Cu(II) and also Ni(II). The effects of HP2(1-15) on Cu(II)- and also Ni(II)-mediated DNA oxidation by H_2O_2 were investigated in detail using the circular plasmid pUC19 DNA as a target, and the single/double-strand breaks and production of oxidised DNA bases, as end points. The results revealed that HP2 is capable of binding Ni(II) and Cu(II) and, in this way, attenuating the mediation of oxidative DNA damage by copper(II), (but not nickel(II)). These effects may be mechanistically involved in the reproductive toxicity and carcinogenicity of metals.

Copper and Neurodegenerative Disorders

Since copper is an integral part of many proteins necessary for neurological functioning, the role of copper in oxidative mediated stress in neurodegenerative diseases was intensively studied. The role of copper has been implicated in neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease and amyotrophic lateral sclerosis [126].

The direct evidence supporting increased oxidative stress in AD include (i) increased brain Cu, Fe, Al, and Hg in AD, capable of stimulating free radical generation; (ii) increased lipid peroxidation and decreased polyunsaturated fatty acids in the AD brain, and increased 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid; (iii) increased protein and DNA oxidation in the AD brain; (iv) diminished energy metabolism and decreased cytochrome c oxidase in the brain in AD; (v) advanced glycation end products (AGE), malondialdehyde, carbonyls, peroxynitrite, heme oxygenase-1 and SOD-1 in neurofibrillary tangles [127]. Very important aspect of AD is Amyloid β peptide (Aβ). Individuals with genetic alterations in one of the genes that code three transmembrane proteins – amyloid precursor protein, presenilin-1 and presenilin-2 deposit large amounts of the amyloid fragment Aβ(1-42). Amyloid β peptides (Aβ) have been shown to play an important role in the pathogenesis of Alzheimer's disease *via* generation of free radicals [127]. Elevated production of amyloid-β (Aβ), as a preventive antioxidant for brain lipoproteins under the action of increased oxidative stress and neurotoxicity in aging, is postulated to represent a major event in the development of Alzheimer's disease [127]. Copper binds to Aβ with a high affinity *via* histidine (His13, His14, His6) and tyrosine (Tyr10) residues and copper in abnormally high concentrations and markers indicating oxidative stress have also been found in amyloid plaques. In addition to Cu(II), Aβ also binds Zn(II) and Fe(III) *in vitro* and the amounts of these metals are also markedly elevated in the neocortex and especially enriched in amyloid plaque deposits in individuals with Alzheimer's disease [127]. Zn(II) precipitates Aβ *in vitro* and Cu(II) interaction with Aβ promotes its neurotoxicity which correlates with the metal reduction [Cu(II) → Cu(I)] and the generation of hydrogen peroxide [128]. Cu(II) promotes the neurotoxicity of Aβ with the greatest effect for Aβ (1-42) > Aβ (1-40), corresponding to the capacity to reduce Cu(II) to Cu(I), respectively and form hydrogen peroxide. The copper complex of Aβ (1-42) has a highly positive reduction potential, characteristic of strongly reducing cupro-proteins [129].

Dikalov *et al*. [130] recently reported that neurotoxic forms of Amyloidβ, Aβ (1-42), Aβ (1-40), and also Aβ (25-35) stimulated copper-mediated oxidation of ascorbate, whereas nontoxic Aβ (40-1) did not. Based on this study it was concluded that toxic Aβ peptides stimulate copper-mediated oxidation of ascorbate ($AscH^-$) and generation of hydroxyl radicals and therefore cupric-amyloid peptide-stimulated free radical generation may be involved in the pathogenesis of Alzheimer's disease. This can be described by the following set of equations.

$$A\beta\text{-Cu(II)} + AscH^- \leftrightarrow A\beta\text{-Cu(I)} + Asc^{\bullet -} + H^+$$

$$A\beta\text{-Cu(II)} + Asc^{\bullet -} \leftrightarrow A\beta\text{-Cu(I)} + Asc \quad (14)$$

$$A\beta\text{-Cu(I)} + H_2O_2 \rightarrow A\beta\text{-Cu(II)} + {}^{\bullet}OH + OH^- \text{ (Fenton)}$$

$$A\beta\text{-Cu(I)} + O_2 \leftrightarrow A\beta\text{-Cu(II)} + O_2^{\bullet -}$$

In the presence of oxygen or H_2O_2, Cu(I) may catalyze free radical oxidation of the peptide *via* the Fenton reaction.

As mentioned above, it has been shown using EPR spectroscopy that the *N*-terminal residues of His 13, His14, His6 and Tyr10 are involved in the complexation of Cu in Aβ. Huang *et al*. [129] recently proposed that *N*-terminally complexed Cu(II) is reduced by electrons originating from the C-terminal methionine (Met35) residues according to reaction.

$$MetS + A\beta\text{-Cu(II)} \leftrightarrow MetS^{\bullet +} + A\beta\text{-Cu(I)} \quad (15)$$

forming the sulphide radical of Met35 ($MetS^{\bullet +}$) and reducing Cu(II). While thermodynamic calculations based on the reduction potentials of the Cu(II)/Cu(I) and Met/ $MetS^{\bullet +}$ couples show that the reaction (15) is rather unfavorable, electron transfer between MetS and Aβ-Cu(II) may be accelerated by the subsequent exorgenic reaction of deprotonation of $MetS^{\bullet +}$, leaving behind the 4-methylbenzyl radical, thus making the reaction (15) viable *in vivo* [131]. The sulphide radical $MetS^{\bullet +}$ may also undergo very fast reactions with e.g. superoxide radical anion, originating from the reaction (14). This reaction leads to the formation of Met-sulphoxide (MetO) which has been isolated from AD senile plagues

$$MetS^{\bullet +} + O_2^{\bullet -} \xrightarrow{Met} 2MetO \quad (16)$$

In concluding, methionin35 is strongly related to the pathogenesis of AD, since it represents the residue in Aβ most susceptible to oxidation *in vivo*. It has been proposed that Met35 oxidation to Met-sulphoxide reduced toxic and pro-apoptotic effects of the amyloid beta protein fragment on isolated mitochondria [131].

Recently Apolipoprotein E (apoE), a lipid transport molecule, which has been linked to the pathogenesis of Alzheimer's disease has been found to be subject to free radical attack and a direct correlation exists between Apo E peroxidation and Alzheimer's disease [127].

The neurodegenerative status of cells may influence the interaction of copper with dopamine. It was demonstrated [132] that copper-induced neurotoxicity is related to the formation of Cu-dopamine complex followed by dopamine oxidation to aminochrome.

Studies exploring the etiology of Parkinson's disease were also of interest. One of the novel neurotoxin-salsolinol (SAL, dopaminergic catechol tetrahydroisoquiniline) interacts with calf thymus DNA and Cu(II) resulting in substantial oxidative DNA damage as determined by 8-OH-dG formation. In the presence of GSH and catalase the neurotoxic properties of SAL are inhibited, which indicates that SAL undergoes a redox cycling cycle with the participation of Cu(II) followed by production of free radicals, mainly hydroxyl radicals [133].

Copper-Induced Toxicity and Vitamins E, C and Selenium

The combination of ascorbate, transition metal ions, and hydrogen peroxide is an efficient hydroxyl radical generating system called "the Udenfriend system" [134]. Although the pro-oxidant role of ascorbate in this system has been well characterized *in vitro*, it is uncertain whether ascorbate also acts as a pro-oxidant under physiological conditions. To resolve the uncertainty as to whether ascorbate also acts as a pro-oxidant in the presence of copper and iron under physiological conditions, human plasma, as a representative biological fluid, was used. The results have shown that even in the presence of redox-active iron or copper and H_2O_2, ascorbate acts as an antioxidant that prevents lipid peroxidation and does not promote protein oxidation in human plasma *in vitro* [93].

Burkitt [116], in the so-called tocopherol-mediated peroxidation model (in which polyunsaturated fatty acid oxidation is initiated by the α-tocopheroxyl radical, generated during the reduction of Cu(II) to Cu(I) by α-tocopherol), provided experimental evidence for the role of the hydroxyl radicals in oxidative modifications of LDL.

Several studies dealing with the protecting effect of antioxidants against oxidation of LDL and HDL were presented. Homocysteine, an atherogenic amino acid is known to promote copper and iron-dependent oxidation of low-density lipoprotein (LDL). Alul *et al.* [135] investigated whether vitamin C could protect LDL from homocysteine-mediated oxidation. It was shown that vitamin C protected LDL from oxidation as evidenced by an increased lag time preceding lipid diene formation, decreased thiobarbituric acid-reactive substances accumulation and decreased lipoprotein anodic electrophoretic mobility. Near-maximal protection was observed at vitamin C concentrations similar to those in human blood (50-100 μM). Partial protection was observed even at lower concentrations (5-10 μM).

In addition to studies on LDL, HDL is also susceptible to oxidation, which affects their cardioprotective properties. Although several studies have reported inhibition of HDL copper-mediated oxidation by vitamin E, none has determined the potential protective effect of vitamin C. Very recent results demonstrate that vitamin C inhibits lipid oxidation in HDL and preserves the antioxidant activity associated with this lipoprotein fraction [136].

An interesting mechanism for the inhibition of copper-induced LDL oxidation by vitamin C has been suggested by Retsky *et al.* [137]. These authors concluded that copper-incubation of LDL with ascorbic acid or dehydro-ascorbic acid led to the time- and concentration-dependent release of up to 70% of bound Cu, which was associated with the inhibition of LDL oxidation. Incubation of LDL with Cu and ascorbic acid or dehydro-ascorbic acid also led to the time-dependent formation of 2-oxo-histidine, an oxidised derivative of histidine with a low affinity for Cu. Thus inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation.

The effect of selenium on copper-induced oxidation of LDL was studied by *in vivo* EPR spin trapping study [138]. In this study copper-mediated free radical toxicity has shown lipid derived radicals in vitamin E and selenium-deficient rats. These findings support the proposal that dietary selenium and vitamin E can protect against lipid peroxidation and copper toxicity.

The role of vitamin C in radiation-induced DNA damage in presence and absence of copper was also investigated [139]. The results indicate that while vitamin C can act an antioxidant to protect DNA damage from ionizing radiation, in the presence of copper it acts as a reducing agent to induce DNA damage. These effects are important in assessing the role of vitamin C, in the presence of mineral supplements or radioprotective therapeutic agents, particularly in patients with abnormally high tissue copper levels.

Copper-induced regulation of transcription factors was also studied. Mattie and Freedman [140] tested the hypothesis that metal- and oxidative stress-responsive signal transduction pathways mediate the cellular and molecular responses associated with copper exposure. Using transient transfection assays on COS-7 cells and mouse metallothionein-I (MT-I) or rat NAD(P)H oxidoreductase 1-based reporter genes they demonstrated that copper activates transcription *via* metal and antioxidant response elements. Simultaneously with copper exposures is observed a decrease in the level of total glutathione and an increase in oxidised glutathione. Depletion of glutathione, before copper exposure, increases metal- and oxidative stress-inducible transcription and cytotoxicity. Pretreatment with the reactive oxygen scavengers aspirin or vitamin E provides partial protection against copper toxicity and reduces inducible transcription. Experiments using signal transduction inhibitors and a metal transcription factor (MTF)-1 null cell line demonstrate that copper-inducible MT-I transcription is regulated by protein kinase C and mitogen- activated protein kinase signalling pathways and requires MTF-1. The results of these studies indicate that copper activates transcription through both metal- and oxidative stress-responsive signal transduction pathways.

The investigation of copper-induced hepatoxicity resulted in a dose-dependent elevation in heat shock protein 70 (hsp70) expression [141]. At high concentrations of copper (200 μM $CuSO_4$) there was no effect of copper on hepatoxicity at 24 h, whereas longer exposures (48 h) resulted in increased lactate dehydrogenase (LDH) leakage and apoptosis. Vitamin C inhibited this copper-induced apoptosis implying a role for reactive oxygen species in

copper toxicity. However, no parallel inhibition of either LDH leakage or hsp70 protein expression was observed with vitamin C suggesting that at least two independent mechanisms are involved in the cellular response to copper. These results suggest that hepatoxicity of copper includes impairment of hsp70 response to subsequent stressors and/or signals, which is crucial for protecting cells from proteotoxicity.

A study to examine the ability of aspirin to inhibit ROS-mediated DNA damage in the presence of copper was also reported [142]. Hydrogen peroxide (H_2O_2) plus copper(II) and hydroquinone (HQ) plus copper(II) were used to cause oxidative DNA strand breaks in phiX-174 plasmid DNA. The results demonstrated that the presence of aspirin resulted in a marked inhibition of oxidative DNA damage induced by either H_2O_2/Cu(II) or HQ/Cu(II). Aspirin was also found to be much more potent than the hydroxyl radical scavengers, mannitol and dimethyl sulfoxide, in protecting against the H_2O_2/Cu(II)-mediated DNA strand breaks. It was observed that aspirin did not alter the Cu(II)/Cu(I) redox cycle in either the H_2O_2/Cu(II) or HQ/Cu(II) system.

Pourahmad and O'Brien [143] have shown that when isolated hepatocytes are incubated with Cu there is an immediate, rapid, increase in ROS production. ROS formation and Cu-induced cytotoxicity are prevented by the ROS scavengers dimethyl sulfoxide, mannitol, catalase, or SOD, as well as by desferoxamine, *N*,*N*-diphenylphenylenediamine or α-tocopherol succinate. These studies suggest that Cu-induced cytotoxicity may occur as a result of mitochondrial ROS formation and is independent of cytosolic ROS formation due to redox cycling.

The effect of copper ions on the free radical-scavenging properties of reduced glutathione was investigated [144]. The results support the formation of a copper-glutathione complex which is stable in the presence of some copper-chelators, lacks all thiol reactivity, but fully conserves the free-radical scavenging properties of GSH.

High concentrations of the trace metal zinc have previously been shown to provide transient protection of cells from apoptotic death [145]. Copper-induced toxicity (100 μM) in human neurons in culture (NT2-N) resulted in significant apoptotic neuronal death. However, addition of high concentrations of zinc (700 μM) to Cu-treated cells resulted in neuronal viability that was not different from untreated controls after 24 h. Thus, this work explored the ability of Zn to protect human neurons in culture (NT2-N) from Cu-mediated death and tested the hypothesis that the tumour-suppressor protein p53 plays a role in Cu-induced neuronal death and is part of the mechanism of Zn protection.

CHROMIUM

Chromium is the 24th element of the Periodic table. Chromium exists in a series of oxidation states with a valence from -2 to +6; the most important stable states are 0 (elemental metal), +3 (trivalent), and +6 (hexavalent). Trivalent (Cr[III]) and hexavalent (Cr[VI]) compounds are thought to be the most biologically significant. Cr(III) is an essential dietary mineral in low doses. It is required to potentiate insulin and for the normal glucose metabolism [146]. Chromium deficiency has been associated with impaired glucose tolerance, fasting hyperglycemia, glucosuria, elevated percent body fat, decreased lean body mass, maturity-onset diabetes, cardiovascular disease, decreased sperm count, and impaired fertility [147]. The US National Academy of Science has established a safe and adequate daily intake for chromium in adults of 50–200 micrograms per day (μg/day) [148]. Cr (III) is found in most fresh foods and drinking water. Dietary sources rich in chromium include bread, cereals, spices, fresh vegetables, meats, and fish. Other significant sources of chromium are mineral supplements, brewer's yeast, and beer. Of interest were recent comparative studies on two Cr(III) popular dietary supplements; chromium(III)-picolinate and chromium(III)-niacin [149]. This study revealed that Cr(III)-picolinate produces significantly more oxidative stress and DNA damage. Studies have implicated the toxicity of chromium picolinate in renal impairment, skin blisters, anemia, haemolysis, tissue edema, liver dysfunction, neuronal cell injury, depletion of antioxidant enzymes (SOD, GPx, and GSH) and DNA damage. Oxidative stress has therefore been proposed as a major pathway of Cr(III)-picolinate induced toxicity. Niacin-bound chromium(III) has been demonstrated to be more bioavailable and efficacious with almost no toxicity. This study demonstrates that the toxicity of chromium(III) compounds is largely dependent on the ligand [149].

Chromium carcinogenicity was first identified over a century ago and Cr(VI) compounds were amongst the earliest chemicals to be classified as carcinogens [150]. Chromium is used in three basic industries: metallurgical, chemical, and refractory (heat-resistant applications). Occupational exposure to Cr(VI)-containing compounds is known to induce lung toxicity and increased incidence of respiratory-system cancers [151-153].

After entering the body from an exogenous source, Cr(III) binds directly to transferrin, an iron-transporting protein in the plasma. In contrast, Cr(VI) is rapidly taken up by erythrocytes after absorption and reduced to Cr(III) inside the cell. Regardless of the source, Cr(III) is widely distributed in the body and accounts for most of the chromium in plasma or tissues. The greatest uptake of Cr(III) as a protein complex is *via* bone marrow, lungs, lymph nodes, spleen, kidney, and liver, the highest being in the lungs [149]. Excretion of chromium occurs primarily *via* urine, with no major retention in organs. Approximately 10% of an absorbed dose is eliminated by biliary excretion, with smaller amounts excreted in hair, nails, milk, and sweat. Clearance from plasma is generally rapid (within hours), whereas elimination from tissues is slower (with a half-life of several days). Cr(VI) administered to volunteers is eliminated more rapidly than Cr (III).

While Cr(VI)-induced carcinogenicity is well established, numerous discussions regarding the actual species most likely to be responsible for cellular damage have been conducted [154]. All chromates, Cr(VI) can actively enter the cells through channels for the transfer of the isoelectric and isostructural anions, such as SO_4^{2-} and HPO_4^{2-} channels [146]. Insoluble chromates are absorbed by cells *via* phagocytosis. Until recently, transport of chromium through

the cell membrane concerned exclusively the Cr(VI) species. However, very recent models also considered the uptake of reduced Cr species generated by extracellular redox mechanisms [155]. Certain extracellulary generated Cr(V) and Cr(III) complexes also have high permeabilities through the cell membrane and therefore such species have to be taken into account when describing the complex model of chromium carcinogenicity [156].

Chromium, Biological Reductants and Free Radicals and Activation of Transcription Factors

Chromium(VI) alone does not react with DNA *in vitro*, or isolated nuclei. However, once inside the cell, in the presence of cellular reductants, it causes a wide variety of DNA lesions including Cr–DNA adducts, DNA–protein crosslinks, DNA–DNA crosslinks, and oxidative damage [157]. Within the cell glutathione rapidly forms a complex with Cr(VI), followed by a slow reduction of Cr(VI) to yield Cr(V) (reaction 1, Fig. (**3**)) [156]. Thus, glutathione can act as an intramolecular stabiliser of Cr(VI) *via* the formation of a thiolate ester. Once formed, Cr(V) species were found to alter the DNA conformation. In addition to GSH, a number of *in vitro* studies have confirmed that various other substances were capable of reducing Cr(VI). These include ascorbate, cystein [158, 159], lipoic acid, NAD(P)H, fructose, ribose and others [160, 161]. It may be more appropriate to think of the small-molecule Cr(VI) reducing agents, such as glutathione and ascorbate, as detoxifying agents, where the ultimate genotoxic agents are species formed between Cr(V) and diolato or sugar-like molecules. Some Cr(V)–sugar species are very stable at physiological pH values, with an EPR signal detectable after 48 h of the initiation of the reaction [162]. Among the most probable candidates for *in vivo* reduction of Cr(VI) substances is GSH and also ascorbate, especially because of their occurrence within cells. Kinetically, ascorbate is reported to be even more favored in reduction of Cr(VI) compared to GSH in the rat lung [163]. *In vivo* reduction of Cr(VI) was also reported by Liu *et al*. [164]. Using a specially designed EPR spectrometer for *in vivo* detection of free radicals, this group was able to show that Cr(V) intermediates, generated as a result of one-electron reduction, were detected in liver and also in the blood. Liver autopsies confirmed the *in vivo* findings. While pretreatment of the animals with ascorbate and GSH decreased formation of Cr(V), pretreatment with NAD(P)H augmented it. Based on these studies it was suggested that the *in vivo* one-electron reductant of Cr(VI) is most probably NAD(P)H flavoenzymes (reaction 2, Fig. (**3**)).

Several *in vitro* studies of the reaction of Cr(VI) with GSH were conducted. Using an EPR spin trapping technique it was possible to demonstrate the formation of Cr(V) species (most probably the Cr(V)-glutathione complex) and the glutathione-derived thiyl radical ($GS^{\bullet}$) (reaction 1, Fig. (**3**)) [165]. Once formed Cr(V) can react *via* Fenton reaction (reaction 3, Fig. (**3**)) with H_2O_2 forming the hydroxyl radical capable of causing DNA damage [159]. In addition to the cellular damaging effect of the $GS^{\bullet}$ radical, it can further react with other thiol molecules in oxygenated tissues to give the superoxide radical (reactions 4 and 5, Fig. (**3**)). Superoxide can further reduce Cr(VI) to Cr(V) (reaction 6) which can then catalyze the decomposition of H_2O_2 thus creating the DNA damaging hydroxyl radical (reaction 7, Fig. (**3**)). Cr(V) can also be reduced by cellular reductants (e.g. ascorbate, GSH) to Cr(IV) (reaction 8, Fig. (**3**)), again participating in Fenton chemistry generating hydroxyl radical (reaction 9, Fig. (**3**)).

A reaction of Cr(VI) with ascorbate generated a mixture of both Cr(V) and Cr(IV) intermediates; addition of H_2O_2 led to competitive Fenton reactions with both Cr(V) and Cr(IV) species generating hydroxyl radicals [166]. Fenton chemistry and the redox couple Cr(III)/Cr(II) has been studied by Ozawa and Hanaki [167] who demonstrated that Cr(III) can be reduced to Cr(II) by the biological reductants L-cystein and NAD(P)H. In turn, the newly formed Cr(II) reacts with hydrogen peroxide to produce hydroxyl radical, detected by both EPR spectroscopy and HPLC. Shi *et al.* [168] investigated free radical generation from hydrogen peroxide and lipid hydroperoxides in the presence of Cr(III) using spin trapping EPR spectroscopy. At physiological pH, incubation of Cr(III) with H_2O_2 resulted in generation of hydroxyl radical. Diethylenetriamine pentaacetic acid (DPTA) substantially reduced generation of hydroxyl radicals; L-cystein, glutathione, and NADPH exhibited no significant effect. These results indicate that Cr(III) is capable of producing free radicals from both hydrogen peroxide and lipid peroxides. Sugden *et al.* [169] also reported mutagenic properties of Cr(III) complexes consistent with the ability of Cr(III) complexes to serve as cyclic electron donors in Fenton chemistry. These data are in agreement with the

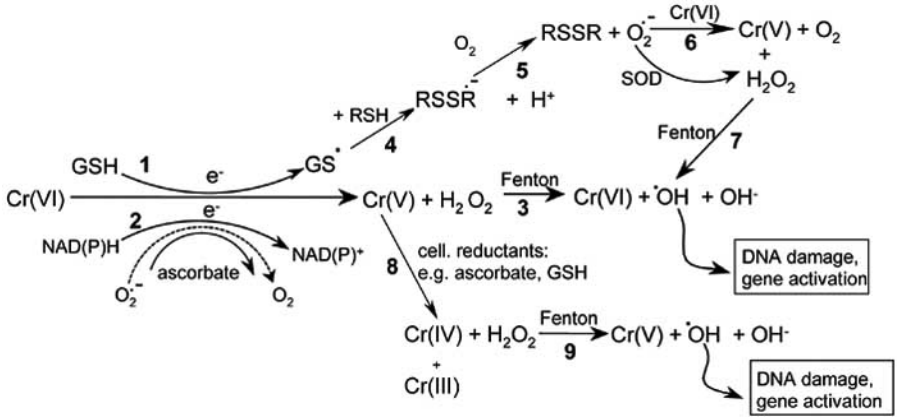


Fig. (3). Biological reductants of Cr(VI) and its reactions (For more details see the text).

studies of Cr(III)-picolinate toxicity (see above), indicating that the bioavailability *vs.* toxicity of Cr(III) compounds strongly depends on fine tuning by the ligand.

There has been much effort devoted to identification of the intermediates responsible for the induction of strand breaks during reactions of chromium(VI) with biological reductants. The results obtained by Stearns and Wetterhahn [166] suggest that it is not a single type of species that universally produces the DNA strand breaks observed in different chromium(VI) systems and that the reactivity of intermediates will depend on the experimental conditions, e.g. the concentration of chromium(VI), the presence of biological reductants, pH and others.

A mixture of Cr(VI) and ascorbate, both with and without H_2O_2, was tested for DNA strand breaking [170]. As detected by agarose cell electrophoresis, DNA strand breaks occurred when the DNA was incubated with Cr(VI) and ascorbate. The amount of damage was concentration-dependent with respect to both reactants and was significantly increased when H_2O_2 was introduced into the reaction mixture. The $^{\bullet}OH$ generated is able to react with guanine residues creating a variety of radical adducts, the most studied being 8-hydroxy-deoxyguanosine (8-OH-dG). It has been well established that the 8-OH-dG adduct is a good marker of oxidative damage [33] implicated in ROS-induced toxicity and carcinogenicity.

The effect of Cr(VI) on the growth, survival, and mode of cell death in normal human lung fibroblasts (HLF cells) in the presence of ascorbate and tocopherol was examined [171]. The predominant cellular response to Cr(VI) was growth arrest. It was found that Cr(VI) caused up to 20% of HLF cells to undergo apoptosis, and documented apoptotic morphology and the phagocytosis of apoptotic bodies by neighbouring cells. p53 levels increased 4- to 6-fold in chromium-treated cells. Pretreatment with vitamin C did not affect the p53 induction observed after chromium treatment and neither vitamin had any effect on Cr-DNA adduct formation.

An increase in lipid peroxidation of 1.8 and 2.2-fold occurred in rat hepatic mitochondria and microsomes, respectively, 48 h after oral administration of 25 mg sodium dichromate, Cr(VI), while increases of 1.2 and 1.4-fold, respectively, were observed after 895 mg chromium chloride hexahydrate, Cr(III). The urinary excretion of malondialdehyde, formaldehyde, acetaldehyde, and acetone were determined at 0-96 h after Cr administration. In Cr(VI)-treated rats, the excretion of all four lipid metabolites were 1.7- and 3.0-fold greater than for Cr(III)-treated animals [172]. The protective effect of vitamin E on chromium(VI)-induced cytotoxicity and lipid peroxidation in primary cultures of rat hepatocytes was investigated by Susa *et al.* [173]. Pretreatment of primary cultures of rat hepatocytes with α-tocopherol succinate (vitamin E) for 20 h prior to exposure to $K_2Cr_2O_7$ resulted in a marked decrease of chromium (VI)-induced cytotoxicity. These results indicate that the protective effect of vitamin E against chromium(VI)-induced cytotoxicity as well as lipid peroxidation (monitored by malondialdehyde formation) may be associated rather with the level of nonenzymatic antioxidants than the activity of enzymatic antioxidants (including glutathione reductase, superoxide dismutase, and catalase).

A series of detailed studies advocating a Cr(III)-dependent pathway in Cr(VI) carcinogenicity and mutagenicity was presented by Zhitkovich and his group who presented the evidence that Cr(III)-DNA adducts play the dominant role in the mutagenicity caused by the metabolism of Cr(VI) by a biological reducing agent [174]. Several further studies from the same laboratory disproved the existence and genotoxic/mutagenic effect of the Cr(V) species and the hydroxyl radical. Reduction of carcinogenic Cr(VI) by physiological concentrations of vitamin C has been shown to generate ascorbate-Cr(III)-DNA crosslinks, binary Cr(III)-DNA adducts, both potential sources of oxidative DNA damage by intermediate reaction products [175]. The results show that Cr-DNA adducts are responsible for both the mutagenicity and genotoxicity of Cr(VI). A lack of any significant production of the hydroxyl radical and Cr(V)-peroxo complexes in Cr(VI)-ascorbate reactions was confirmed. While ascorbate-Cr(III)-DNA cross-links were much more mutagenic, the Cr(VI)-induced DNA adducts were only highly genotoxic, but not mutagenic under either normal or SOS-induced conditions. The Cr(VI)-induced mutational spectrum consisted of an approximately equal number of deletions and G/C-targeted point mutations (51% G/C → T/A and 30% G/C → A/T). Lower toxicity and high mutagenicity of ascorbate-Cr(III) -DNA adducts in human cells may result from the recruitment of an error-prone bypass DNA polymerase(s) to the stalled replication forks. Further experiments [176] from the same laboratory demonstrated that in addition to reduction of Cr(VI) to DNA-reactive Cr(III), vitamin C contributes to the genotoxicity of Cr(VI) *via* a direct chemical modification of DNA. The absence of ascorbate in A549 and other human cultured cells indicates that cells maintained under the usual *in vitro* conditions lack the most important reducing agent for Cr(VI) and would primarily display slow thiol-dependent activation of Cr(VI). Again, similar to previous study, no evidence was found for the involvement of Cr(V) or Cr(IV) intermediates in the formation of either binary or ternary DNA adducts. These findings are in agreement with studies exploring the toxicity of Cr(III)-picolinate (see above) indicating that the toxicity of chromium(III) compounds is largely dependent on the type of ligand.

The effect of trivalent chromium Cr(III) was estimated through DNA-protein crosslinks (DPC) – a promising biomarker of exposure to chromium. DPC quantification was carried out in lymphocytes of a group of tannery workers exposed to trivalent chromium, a small group of manual metal arc stainless steel welders exposed to hexavalent chromium and a control group [177]. The results indicate a significant increase in the formation of DPC in tannery workers compared with controls. Urinary chromium was increased in both groups, with a greater increase observed in tanners compared with controls. It was concluded that chronic occupational exposure to trivalent chromium can lead to a detectable increase in lymphocyte DNA damage which correlates with a significant exposure of the cells to the metal. The same group in another study speculated that bulky DPC could also have a significant promutagenic effect [178]. Another study on Cr(III)-mutagenicity has shown that not only cysteine, but also glutamic acid and histidine were the major amino acids crosslinked to DNA in chromate-treated cells [179].

Carcinogenic Cr(VI) compounds were previously found to induce amino acidiglutathion-Cr(III)-DNA crosslinks with the site of adduction on the phosphate backbone [180]. Utilizing the pSP189 shuttle vector plasmid it was found that these ternary DNA adducts were mutagenic in human fibroblasts. The Cr(III)-glutathione adduct was the most potent in this assay, followed by Cr(III)-His and Cr(III)-Cys adducts. Single base substitutions at the G:C base pairs were the predominant type of mutations for all Cr(III) adducts. Cr(III), Cr(III)-Cys and Cr(III)-His adducts induced G:C → A:T transitions and G:C → T:A transversions with almost equal frequency, whereas the Cr(III)-glutathione mutational spectrum was dominated by G:C → T:A transversions. These studies of Cr(III)-induced phosphotriesters demonstrate the importance of a Cr(III)-dependent pathway in Cr(VI) carcinogenicity.

Cr(VI) was also found to induce activation of NF-κB in Jurkat cells [181]. Activation of NF-κB was attributed to hydroxyl radicals generated by the Cr(V)/Cr(IV)-mediated Fenton reaction. In addition, activity of another AP-1 transcription factor is stimulated by the Cr(VI) species. The induction of AP-1 by Cr(VI) is associated with phosphorylation of MAP kinase p38 and JNK, but not extracellular-signal-regulated kinase (ERK). Interestingly, aspirin was found to inhibit the activation of both AP-1 and NF-κB induced by Cr(VI). The induction of AP-1 and NF-κB by Cr(VI) was attenuated by inhibition of p38 and IkB kinase (IKK), respectively. These results suggest that Cr(VI)-mediated generation of radicals as a result of the Fenton reaction may serve as upstream signal initiating the activation of both AP-1 and NF-κB, whereas p38 and JNK act as a downstream executive kinase for the activation of AP-1 and NF-κB respectively.

The protein p53 is tumour suppressor protein which plays an important role in protecting cells from tumourigenetic alterations. For the majority of cancers it was found that cells contained mutations in the p53 gene. This transcription factor can be activated by a variety of stimuli, involving oxidative stress, radiation and others. While SOD, which converts $O_2^{\bullet-}$ to H_2O_2, was found to increase p53 activity; catalase, a scavenger of H_2O_2, inhibited p53 activation. In addition aspirin, a scavenger of $^{\bullet}OH$, suppressed activation of p53. Increased formation of $^{\bullet}OH$ enhanced p53 activation in A549 cells through Cr(VI) reduction by NAD(P)H followed by Cr(V) catalyzed decomposition of H_2O_2. Wang and Shi [182] also studied the mechanism of Cr(VI)-induced p53 activation. They found that the activation of p53 was at the protein level instead of the transcriptional level. In response to Cr(VI) treatment, protein p53 becomes phosphorylated and acetylated at Ser15 and Lys383, respectively.

COBALT

Cobalt is the 27th element of the Periodic table. The most common oxidation numbers of cobalt are +3 [Co(III)], and +2 [Co(II)] which form a number of organic and inorganic salts [183]. Cobalt is an element that occurs naturally in many different chemical forms throughout our environment. It is a natural earth element and is present in trace amounts in soil, plants and in our diets. In pure form it is a steel-grey to black shiny hard metal. Cobalt usually occurs in association with other metals such as copper, manganese, nickel and arsenic. Small amounts are found in most rocks, soil, surface and underground water, plants and animals. Cobalt is also released to the environment from burning coal and oil, from automotive/airplane exhausts and from industrial processes that use the metal or its compounds. Cobalt and its salts are used in a variety of processes e.g. as a paint drier, as an ingredient of colored pigments and others. Some radioactive isotopes of cobalt, such as Cobalt 60, are used in treating patients in nuclear medicine and in research. Natural cobalt is air stable for a few days but it is more stable in water and soil.

The discovery, in 1948, that Vitamin B12 contains 4% cobalt proved this element was essential to man although it was known to be essential for ruminant animals much earlier [184]. Even before 1935 it was known that a lack of cobalt, from deficiency in the soil, produced a wasting disease in animals.

Cobalt is considered an essential element since the body cannot produce it. It is readily absorbed in the small intestine, but the retained cobalt serves no physiological function since human tissues cannot synthesize B12 in the intestine by *E. coli* [184]. Most of the consumed cobalt is excreted in the urine with very little being retained, mainly in the liver and kidneys. Cobalt's only known function is its integral part of Vitamin B12. A cobalt deficiency has never been produced in humans. Signs and symptoms of one are actually those of a B12 deficiency. Interestingly, recent studies show that cobalt may be linked with iodine in the formation of thyroid hormones [185].

Cobalt has also been used as a treatment for anemia, because it causes red blood cells to be produced [186]. The toxicity of cobalt is relatively low compared to many other metals in soil, however it is toxic and carcinogenic at higher concentrations [187]. Effects on the lungs, including asthma, pneumonia, and wheezing, have been found in workers who breathed high levels of cobalt in the air. Epidemiological studies revealed that workers exposed to cobalt in an electrochemical plant producing cobalt and workers exposed to cobalt-containing hard metal compounds exhibited a significantly higher risk of developing lung cancer [187].

Cobalt and Free Radicals

Experimental studies confirmed that cobalt can not only interfere with DNA repair processes [188] but can also cause direct induction of DNA damage [188], DNA-protein crosslinking, and sister-chromatid exchange [187]. Co(II) compounds have been shown to have a carcinogenic effect in animal studies [189] and also enhance the frequency of UV induced mutations and sister-chromatid exchanges in V79 Chinese hamster cells [190].

While the mechanism of cobalt-induced toxicity and carcinogenicity is not fully understood, it is well established that cobalt-mediated free radical generation contributes to the toxicity and carcinogenicity of cobalt [190].

Hanna *et al.* [191] performed EPR spin trapping studies to generate oxygen free radicals from the reaction of hydrogen peroxide with various Co(II) complexes under physiological

conditions. The superoxide radical was generated from the reaction of H_2O_2 with Co(II), but was inhibited when Co(II) was chelated with adenosine 5'-diphosphate or citrate. An EDTA Co(II) complex also prevented detectablefree-radical formation when H_2O_2 was added, but visible absorbance data indicated oxidation of the Co(II) to Co(III) in this case. Nitrilotriacetate cobalt(II) complex catalyzed decomposition of H_2O_2 accompanied by the formation of hydroxyl radicals and slow oxidation of Co(II).

Another detailed EPR study by Leonard *et al.* [187] revealed that, in the presence of SOD, cobalt in suspension (cobalt metal particles) is able to react with dissolved oxygen to generate $^{\bullet}OH$, as monitored by EPR spin trapping experiments. EPR spin trapping experiments with cobalt (as metal particles) with the spin trap DMPO alone in the presence of oxygen indicated generation of the radical intermediate Co(I)-$OO^{\bullet}$ species exclusively.

$$Co + O_2 \rightarrow Co(I) + O_2^{\bullet -} \rightarrow Co(I)\text{-}OO^{\bullet} \quad (17)$$

indicating that the reaction

$$Co(0) + H_2O_2 \not\rightarrow Co(I) + {}^{\bullet}OH + OH^- \quad (18)$$

does not take place. In the presence of SOD, the enzyme catalyzes the decomposition of Co(I)-$OO^{\bullet}$ species to H_2O_2 and Co(I)

$$Co(I)\text{-}OO^{\bullet} \xrightarrow{SOD} H_2O_2 + Co(I) \quad (19)$$

where H_2O_2 is produced from $O_2^{\bullet -}$ *via* a dismutation reaction and $O_2^{\bullet -}$ by one-electron reduction of molecular dioxygen catalyzed by Co (reaction 17). Based on the EPR evidence of $^{\bullet}OH$ formation it was proposed that Co(I) participates in the Co(I)-mediated Fenton reaction [187]

$$Co(I) + H_2O_2 \rightarrow Co(II) + {}^{\bullet}OH + OH^- \text{ (Fenton)} \quad (20)$$

This study also involved EPR experiments with cobalt(II) in the presence of various chelators [187]. It was proposed that, in the presence of the appropriate chelators, the reactivity of Co(II) toward H_2O_2 can be tuned. The EPR spin trapping studies have shown that GSH, anserine (both biological antioxidants) and Gly±Gly±His enhance the Co-mediated $^{\bullet}OH$ generation [187] according to Co(II)-mediated Fenton chemistry

$$[Co(II)\text{-chelate}] + H_2O_2 \rightarrow [Co(III)\text{-chelate}] + {}^{\bullet}OH + OH^- \text{ (Fenton)} \quad (21)$$

These experiments lead to the conclusion that under suitable conditions, (e.g presence of cobalt[II]), these peptides may function as pro-oxidants. Since GSH is the major non-protein thiol in cellular systems, (with the concentrations up to 10 mM [192]) and that anserine has been reported to be present in the range of 1-20 mM in the skeletal muscles of many vertebrates [193], the results evoke serious concerns about the deleterious effects of these low-molecular cobalt(II) chelates. The switch of GSH, anserine and other chelators from antioxidants to pro-oxidants mediated by cobalt(II) (and other metals too) may explain cellular damage due to the presence of cobalt(II) species.

Co(II) plus H_2O_2 was found to induced DNA cleavage at all bases with a preference for G > T, C >> A. EPR experiments showed that Co(II) reacts with H_2O_2 forming not only $^{\bullet}OH$, but also singlet oxygen ($^1O_2$) especially in the presence of chelators [194]. The study of reactive oxygen species formation in hepatocytes has shown that lysosomes are sites of cytotoxic ROS formation in the presence of Co(II) [195].

Recently, mitochondrial DNA damage and a hypoxic response induced by $CoCl_2$ in rat neuronal PC12 cells was reported [196]. This was the first study which provided evidence, that in the presence of $CoCl_2$, mtDNA damage as well as alterations in cell morphology occurred that appear consistent with induction by excessive radical species. Marked changes in levels of proteins indicative of cellular responses to hypoxia and to DNA damage were also observed; hypoxia-inducible factor 1 alpha (HIF-1 alpha), p53, p21 and PCNA were modulated. However, it was not possible to differentiate which changes are exclusively attributable to either hypoxia or to DNA damage.

Cobalt (Co(II)) mimic hypoxia was used as a tool to study the role of oxygen sensing and signalling cascades in the regulation of hypoxia-inducible gene expression by producing oxidative stress [197]. It was found that the exposure of A549 cells to Co(II) (and also Ni(II)) produced oxidative stress, and although Co(II) was a more potent producer of ROS than Ni(II), both metals equally increased the expression of Cap43, a hypoxia-regulated gene. The free radical scavenger 2-mercaptoethanol completely suppressed ROS generation by $CoCl_2$ and $NiCl_2$, but did not diminish the induced Cap43 gene expression. It was concluded that ROS are produced during the exposure of cells to metals that mimic hypoxia, but the formation of ROS was not involved in the activation of HIF-1-dependent genes.

It was also investigated whether mitochondria act as oxygen sensors during hypoxia and whether hypoxia and cobalt activate transcription by increasing the generation of ROS [198]. The results show that wild-type Hep3B cells increase ROS generation during hypoxia or $CoCl_2$ incubation. The results also revealed that hypoxia activates transcription *via* a mitochondria-dependent signalling process involving increased ROS, whereas $CoCl_2$ activates transcription by stimulating ROS generation *via* a mitochondria-independent mechanism.

In another study of cobalt and hypoxia-inducible transcription factors it was found that, unlike cobalt, hypoxia did not stimulate 15-kilobase pair mouse ho-1 promoter (pHO15luc) expression and did not increase stress-response elements (StREs) binding activity, indicating distinct mechanisms for heme oxygenase-1 (ho-1) gene activation by cobalt and hypoxia in Chinese hamster ovary cells [199]. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types, including also cobalt, was studied. Instillation of 125 μg ultrafine cobalt (UFCo) particles induced a significant influx of neutrophils at both 4 and 18 h post-instillation [200]. Accompanying the influx of neutrophils was an increase in the macrophage inflammatory protein-2 (MIP-2) (at 4 h) and an increase in γ-glutamyl transpeptidase (at 18 h) in bronchoalveolar lavage fluid (BAL). A role for reactive oxygen species in mediating ultrafine inflammation was further strengthened by the ability of the antioxidants N-acetylcysteine (NAC) and glutathione monoethyl ester (GSHme) to block the particle induced release of tumour necrosis factor-alpha (TNF-alpha) from alveolar macrophages *in vitro*.

Some metalloporphyrins possess radical scavenging properties. The cytoprotective effect of redox-active metalloporphyrins (e.g., CoTBAP) is generally attributed to their ability to scavenge reactive oxygen and nitrogen species. In the study by Joseph and Kalyanaraman [201] the pro- and anti-apoptotic potentials of different metalloporphyrins containing also cobalt exposed to doxorubicin were evaluated. We note that doxorubicin is an anticancer drug that forms superoxide and hydrogen peroxide *via* redox-cycling of doxorubicin semiquinone in the presence of molecular oxygen. The study concluded that several metalloporphyrins, including CoTBAP, can inhibit apoptosis either by inducing heme oxygenase-1 and antiapoptotic protein signalling or by scavenging reactive oxygen species [201].

In the study of cobalt chloride-induced apoptosis in PC12 cells it was observed that a significant increase of the DNA-binding activity of AP-1 occurred in response to $CoCl_2$ and this increase was blocked by antioxidants, showing that $CoCl_2$-induced apoptosis is accompanied by ROS-activated AP-1 [202]. This study was proved to be useful as an *in vitro* model for studies of molecular mechanisms in ROS-linked neuronal disorders.

VANADIUM

Vanadium is the 23rd element of the Periodic table. Vanadium may be beneficial and possibly essential in humans, but certainly essential for some living organisms [203-206]. The usual oxidation states of the vanadium are V(III), V(IV) and V(V). The vanadium ions play a role in biology as counterions for various proteins, DNA and RNA.

Vanadium is widely distributed on Earth, however, its role as a micronutrient in humans is not yet established. Humans are exposed to vanadium mainly through the polluted atmosphere from combustion products of vanadium-bearing fuel oils, fumes and dust. Food contains a very low content of vanadium, usually below 1 ng/g. Vanadium enters the organism by inhalation, skin and gastrointestinal tract and accumulates mainly in the liver, kidney, spleen, bones and to a lesser extent also in lungs [207-209].

The studies of uptake of vanadate into tunicates revealed that the majority of the vanadium is taken up as vanadium(V) from seawater. Since the vanadium ions inside the blood cells are in an oxidation state of III or IV, the aqueous vanadium has been reduced. This is not a simple process since the V(V)/V(IV) and V(IV)/V(III) redox couples, in strongly acidic solutions, are 1.00 and 0.337 V (vs NHE), respectively [203].

Vanadium(V), dominant at the cellular level at physiological pH, enters the cell through the anion transport mechanism [210, 211]. Inside the cell vanadate(V) is reduced to vanadyl(IV) by biological reductants, such as intracellular glutathione or ascorbic acid. For example vanadium was found to exists in the oxidation state III in cells; this is bioenergetically unfavorable and incompatible with physiological conditions. It has been suggested that reduction involves NADPH in the tunicate [212].

Reduced vanadyl(IV) is bound to protein. Experiments on erythrocytes indicate a two-phase mechanism of vanadium entrance into a cell. The first phase involves relatively fast equilibration of vanadate(V) by transport *via* the anion channels and the slower phase, reduction of vanadate(V) to vanadium(IV). Vanadium bound to vanadium-binding proteins (vanabins) in the cytoplasm may facilitate the transport and accumulation of vanadium in the vacuoles. The fact that vanabins bind V(IV) more strongly than V(V) suggests that reduction takes place before the vanadium ion binds to the protein. The oxidation states of reduced vanadium involves both V(IV) and/or V(III) [213].

Vanadium in cells is distributed in the nucleus and the supernatant fractions [214]. Almost 90% of vanadium is in the supernatant fractions, the rest is in the mitochondria, microsomes and the nuclear and cell debris. Vanadyl and vanadate is bound to carboxyl and amino groups of the aminoacids of proteins and phosphates [215]. Brain cells contain high levels of ascorbate which also bind to V(IV).

Vanadium compounds may exert a variety of toxic effects depending on the nature of species, dose, route and duration of administration. Short-term treatment (< 4 weeks) of vanadium salts in humans caused mild gastrointestinal disturbance [216]. Long-term treatment (> 5 months) can cause anorexia, loss of weight and abdominal pain.

Animal studies revealed various toxic effects induced by vanadium compounds. The most affected organs, as documented by histopathological alterations, were liver and kidney. Intraperitoneal injections of rats with orthovanadate revealed nephrotoxicity [217]. The effect of vanadium on the reproductive and developmental functions in rats has also been well established [218]. Acute toxicity of vanadium compounds in animals involves dehydration, reduction of body weight, loss of appetite, renal tubular necrosis and pulmonary hemorrhage.

Vanadium oxides are usually more toxic than vanadium salts and vanadium(V) is usually more toxic than vanadium(IV) compounds. While potential mutagenic effects of vanadium are also related to the ability of vanadium generate reactive oxygen species, cancer induction has not yet been satisfactorily confirmed.

Vanadium and Signalling Pathways

A study of the role of vanadate on cell cycle control and the involvement of reactive oxygen species in these vanadate-mediated responses in a human lung epithelial cell line, A549, was conducted [219]. Vanadate stimulated in A549 cells the generation of hydroxyl radicals, H_2O_2, and the superoxide anion. ROS generation involved the reduction of molecular oxygen by both a flavoenzyme-containing NADPH complex and the mitochondria electron transport chain. Superoxide *via* dismutation reaction forms H_2O_2 which then reacts with vanadium(IV) through a Fenton reaction (see reaction 25 below) generating the hydroxyl radical. The ROS generated by vanadate induced G(2)/M phase arrest in both a time-and dose-dependent manner as determined by measuring DNA content. The results demonstrate that, among the ROS, H_2O_2 is the species responsible for vanadate-induced G2/M phase arrest. Several regulatory pathways were also involved in this process including activation of p21, an increase of Chk1 expression and inhibition of Cdc25C, which results in phosphorylation

of Cdc2 and possible inactivation of cyclin B-1/Cdc2 complex.

The role of ROS in the vanadate-induced activation of AP-1 was investigated in mouse epidermal JB6P+ cells [220]. EPR measurements confirmed that JB6 P+ cells are able to reduce vanadate to vanadium(IV) in the presence of NADPH. The results show a concentration-dependent transactivation of AP-1, superoxide dismutase and catalase inhibited AP-1 activation induced by vanadate, indicating the involvement of the superoxide radical, hydroxyl radical and/or H_2O_2 in the mechanism of vanadate-induced AP-1 activation. However, sodium formate, a specific $^{\bullet}OH$ scavenger, did not alter vanadate-induced AP-1 activation, suggesting a minimal role for the $^{\bullet}OH$ radical.

Vanadium compounds activate many key effectorproteins of the signalling pathways including AP-1, MEK-1, ERK-1, JNK-1, PI-3K and NF-κB [221]. Activation of these pathways is linked to the formation of reactive oxygen species and DNA damage. Activation of signalling pathways is mediated through the phosphorylation of tyrosines, dependent on the activity of cytosolic protein tyrosine phosphates and phosphokinases that regulate phosphorylation and dephosphorylation of proteins critical for signal transduction.

The very recent results [222] have shown that vanadate is able to increase phosphatidylinositil-3 kinase (PI-3K) activity through phosphorylation. PI-3K activated p70 S6 kinase (p70S6K), which phosphated protein S6, and promoted S phase entry.

Vanadate was found to induce expression of hypoxia-inducible factor 1 alpha (HIF-1) and vascular endothelial growth factor through the phosphatidylinositol 3-kinase/Akt pathway and reactive oxygen species [223]. It was found that ROS were directly involved in vanadate-induced expression of HIF-1 and vascular endothelial growth factor (VEGF) in DU145 cells. The major species of ROS responsible for the induction of HIF-1 and VEGF expression was H_2O_2. These results suggest that the expression of HIF-1 and VEGF induced by vanadate through PI-3K/Akt may be an important signalling pathway in the vanadate-induced carcinogenesis, and ROS may play an important role in this mechanism.

Another study also demonstrated that H_2O_2 plays an important role in vanadium-induced NFAT transactivation in mouse embryo fibroblast PW cells or mouse epidermal Cl 41 cells [224]. Pretreatment of cells with nifedipine, a calcium channel blocker or with cyclosporin A, a pharmacological inhibitor of the phosphatase calcineurin show that vanadium induces NFAT activation not only through a calcium-dependent and cyclosporin A-sensitive pathway, but also through H_2O_2 generation.

Recent reports have shown that vanadium salts administrated to mouse induced apoptosis *via* the production of hydrogen peroxide and other ROS which in turn activates the tumour suppressor gene *p53*, involved in cell regulation, DNA repair and apoptosis [225].

Vanadium and Reactive Oxygen Species

Vanadium, as a transition metal element which occurs in various oxidative states, may participate in the reactions involving formation of free radicals [203]. As mentioned above, vanadium(V) in plasma is rapidly reduced to vanadium(IV) by plasmatic, both enzymatic (e.g. NADPH) and non-enzymatic (ascorbic acid) antioxidants and then transported and bound to plasma proteins. The following reactions may take place inside the cell.

$$V(V) + NADPH \rightarrow V(IV) + NADP^{+} + H^{+} \quad (22)$$

$$V(IV) + O_2 \rightarrow V(V) + O_2^{\bullet -} \quad (23)$$

$$V(V) + O_2^{\bullet -} \rightarrow [V(IV)\text{-}OO^{\bullet}] \quad (24)$$

leading to formation of peroxovanadyl $[V(IV)\text{-}OO^{\bullet}]$ and vanadyl hydroperoxide $[V(IV)\text{-}OH^{\bullet}]$. The superoxide generated is further converted, by dismutation reaction with SOD, into H_2O_2. *In vitro* EPR studies have confirmed that one-electron reduction of vanadium(V) mediated by non-enzymatic ascorbate together with phosphate may represent an important vanadium(V) reduction pathway *in vivo* [226]. It has been proposed that the resulting reactive species generated by vanadium(IV) from H_2O_2 and lipid hydroperoxide *via* a Fenton-like reaction may play a significant role in the mechanism of vanadium(V)-induced cellular injury [227].

Under physiological conditions (pH~7), in the presence of V(IV)/V(V) and H_2O_2 the following reaction producing free radicals may take place (Fenton reaction).

$$V(IV) + H_2O_2 \rightarrow V(V) + OH^{-} + {}^{\bullet}OH \quad (25)$$

Formation of reactive $^{\bullet}OH$ may in turn produce the superoxide radical which is depleted *via* dismutase (SOD) to oxygen and H_2O_2 which further enters the cycle in the reaction. It has been proposed that the resulting reactive species generated by vanadium(IV) from H_2O_2 and lipid hydroperoxide *via* a Fenton-like reaction may play a significant role in the mechanism of vanadium(V)-induced cellular injury. *In vitro* incubation of vanadium(IV) with molecular oxygen dependent 2'-deoxyguanosine (dG) or with DNA in the presence of H_2O_2 resulted in enhanced 8-hydroxyl-2'deoxyguanosine (8-OH-dG) formation and substantial DNA strand breaks [228]. Reaction of vanadium(IV) with t-butyl hydroperoxide generated hydroperoxide-derived free radicals, which caused 8-OH-dG formation from dG and DNA strand breaks.

Similar conclusions were drawn from the study of vanadium(IV)-mediated free radical generation and related 2'-deoxyguanosine hydroxylation and DNA damage [229]. Vanadium(IV) caused molecular oxygen dependent dG hydroxylation to form 8-OH-dG. Catalase and formate inhibited the 8-OH-dG formation, while superoxide dismutase enhanced it. Vanadium(IV) also caused molecular oxygen-dependent DNA strand breaks in a pattern similar to that observed for dG hydroxylation. Incubation of vanadium(IV) with dG or with DNA in the presence of H_2O_2 resulted in an enhanced 8-OH-dG formation and substantial DNA double strand breaks.

A mouse model study was used to investigate vanadium(V)-induced apoptosis, lung inflammation and the role of ROS [230]. Vanadium(V) caused a rapid influx of polymorphonuclear leukocytes into the pulmonary airspace with a peak inflammatory response. During this period the number of apoptotic lung cells (predominantly neutrophils) increased considerably. EPR experiments showed the

formation of ROS, including the superoxide radical, H_2O_2, and reactive $^{\bullet}OH$ radicals which were confirmed by inhibition with free radical scavengers. These results indicate that multiple oxidative species are involved in vanadium(V)-induced lung inflammation and apoptosis, and that H_2O_2 plays a major role in this process.

Spermicidal activity of metallocene complexes containing vanadium(IV) in humans was thoroughly studied. Several oxovanadium(IV) complexes with 1,10-phenanthroline, 2,2 '-bipyridyl, 5 '-bromo-2 '-hydroxyacetophenone and others have shown [231] unprecedented evidence that the spermicidal and apoptosis-inducing activities of vanadium(IV) complexes are determined by the oxidation state of vanadium as well as the geometry of the complexes. Because of its rapid and potent sperm-immobilizing activity, the bromo-hydroxyacetophenone complex, [VO(Br,OH-acph)(2)], may be useful as a contraceptive agent.

In another study of spermicidal activity [232], computer-assisted sperm analysis was used to evaluate the spermicidal activity of 8 metallocene dihalides containing various metal atoms. Whereas the metallocene complexes containing titanium, zirconium, molybdenum, and hafnodium were inactive, all 12 vanadocene complexes elicited potent spermicidal activity at nano up to micromolar concentrations. The sperm-immobilizing activity of the vanadocene complexes was rapid and irreversible, since the treated sperm underwent apoptosis as determined by the flow cytometric annexin vanadium binding assay, DNA nick end-labelling, and confocal laser scanning microscopy. The results provide clear evidence that metallocene complexes containing vanadium(IV), especially vanadocene diselenocyanate, may be useful as contraceptive agents.

V**anadium as an Therapeutic Agent**

Vanadium compounds may exert preventive effects against chemical carcinogenesis in animals, by modifying xenobiotic enzymes, thus inhibiting carcinogenic active metabolites. Research on the activity of antitumour metal compounds received strong attention after the discovery of the anticancer effects of *cis*-$PtCl_2(NH_3)_2$ (*cis*-platinum). Research on vanadium initiated the findings of English *et al*. [233] who discovered the role of metal as an inhibitor of terminal differentiation of murine erythroleukemia cells. Vanadium may also exert inhibitory effects on cancer cell metastatic potential through modulation of cellular adhesive molecules and reverse antineoplastic drug resistance [234]. Due to its relatively low-toxicity, vanadium was established as an effective non-platinum, metal antitumour agent. However, many problems remain which require both basic and applied research in this area.

The role of oxidative stress in the etiology of diabetes has recently been proposed to be involved in both the origin of the disease and increasing secondary complications. The role of oxidative stress caused by glucose toxicity and the resulting production of free radicals, especially in the pancreas, has been proposed to be a major cause of the development of insulin resistance in both type 1 [235] and type 2 diabetics [236]. The experimental results suggest that antioxidant effects of the oral administration of vanadate have been established in diabetic rats and support the hypothesis that changes in cellular GSH metabolism are connected to the insulin-enhancing properties of transition metal complexes. The insulin-like effect of vanadium compounds on cells [237] and diabetic animals [238] has been known for more than 20 years. Diabetic patients frequently have both abnormal glucose and lipid metabolism, which can be normalized by treatment with insulin. Studies testing compounds in animal model systems [239] and in human beings [240] show that simple vanadium salts and V-complexes alleviate the symptoms of diabetes. Initially, the vanadium salts were believed to be able to substitute for insulin; however, currently their effects are most commonly attributed to enhancing insulin action [241]. Specifically, vanadate is generally believed to exert its insulin enhancing effect through competitive inhibition of regulatory protein phosphatases, with a major candidate being phosphatase 1B (pp1B). This phosphatase is the first phosphatase in the insulin regulatory cascade and is particularly sensitive to inhibition by vanadium-compounds.

NICKEL

Nickel is the 28th element of the Periodic table. It is a silver-white metal found in several oxidation states, ranging from -1 to + 4 [242]. However, the +2 oxidation state [Ni(II)] is the most common form of nickel in biosystems. Chemical and physical forces (e.g., erosion, leaching, precipitation) constantly redistribute nickel between land, water, and air. Depending on the soil type and pH, nickel is highly mobile in soil. At pH > 6.7, most nickel exists as insoluble hydroxides, whereas at pH < 6.5, the compounds are relatively soluble. The range of nickel concentrations in plants averages 0.05-5 mg Ni/kg dry weight with concentrations above 50 mg Ni/kg dry weight being toxic for most plants [243]. Based on laboratory studies, nickel probably does not accumulate in fish and there is little evidence for the biomagnification of nickel in the food chain [244]. Occupational exposure occurs in mining, alloy production, electroplating, refining and welding. Epidemiological studies revealed an increased risk of respiratory tract and nasal cancers in miners and workers in nickel refineries [245].

Solubilized Ni(II) ions in aqueous media at physiological pH are hydrated to the greenish hexahydrated $[Ni(H_2O)_6]^{2+}$. Nickel forms not only mononuclear complexes, but also polynuclear complexes [246]. The inorganic polynuclear nickel species have no direct biological significance but they can help understand the complex structure of nickel bioinorganic species.

Nickel Homeostasis, Essentiality and Toxicity

Nickel can enter body *via* inhalation, ingestion and dermal absorption. The amount of nickel absorbed by the gastrointestinal tract depends on the type of nickel species in the food, the content and the absorptive capacity. Normally, only 1-2% of ingested nickel is absorbed. The daily intake of nickel has been estimated to be in the range 35 – 300 μg per day [247]. The chemical form of nickel determines the route by which nickel enter the cells. Soluble nickel, for example nickel carbonyl is fat soluble and can freely cross cell

membranes, most probably by diffusion or through calcium channels. Some authors in fact [248] suggested absorption of nickel by transmembrane diffusion, whilst others proposed absorption of Ni(II) *via* Ca(II) channels [249].

Calcium is maintained at a very high gradient (~10^4) between the extracellular and intracellular compartments of cells and is recognized as an important intracellular second messenger. Several studies related toxic and carcinogenic effect of nickel with changes in calcium metabolism. Soluble nickel compounds are known to enter the cell through the calcium ionophore channel ionomycin, which increases the uptake of nickel by a factor of 4-5 [249]. Nickel is known to be a calcium channel blocker [250], thus the decrease in transport of Ca(II) to intracellular space is compensated by increase of free Ca(II) from intracellular stores. These changes of intracellular concentrations of Ca(II) have been shown to signal gene expression changes associated with cell growth, differentiation and apoptosis [251]. The mechanism of nickel invoked release of stored intracellular calcium involves a cell surface receptor [252].

Absorption of nickel carbonyl during inhalation has been confirmed by numerous studies [253]. On the other hand, insoluble nickel particles enter the vertebrate cells by phagocytosis [254] as documented in the work of Heck and Costa in which they found that crystalline NiS was phagocytized by cultured Syrian hamster embryo fibroblasts [255].

The main transport protein of nickel in blood is albumin, although a nickel containing ([[Alpha].sub.2]-macroglobulin, called nickeloplasmin, also transports nickel. Following exposure to nickel carbonyl, the highest concentrations of nickel appear in the lung, brain, kidney, liver, and adrenals. The biological half-life of nickel oxide in the lung depends, in part, on particle size and ranged from 11-21 months in animal studies [256]. Nickel is not a cumulative metal, it is excreted well *via* urine and feces. The urinary excretion of nickel is rapid and the elimination appears to follow first-order kinetics without evidence of dose-dependent excretion of nickel. Following absorption, the kidney is the primary route of elimination. Excretion of nickel also occurs in the saliva and sweat, which may contribute significantly to the elimination of nickel in hot environments.

In experimental animals nickel compounds induce tumours at all sites of application. Many studies documented the induction of malignant tumours after the intramuscular administration, inhalation, and intraperitoneal, intrarenal, intratesticular, intraocular and subcutaneous injections [257, 258]. Of interest are experimental data suggesting a much higher cytotoxicity and genotoxicity associated with insoluble nickel compounds. In fact while exposure to crystalline Ni_3S_2 resulted in high cytostolic and nuclear nickel content [259], on the other hand, water soluble nickel compounds entering the cell through calcium channels showed very low nuclear and cytosolic nickel content [260]. The toxicity of Ni_3S_2 was recently also confirmed in the study of Kawanishi *et al*. [261]. In this study, various nickel compounds were used to map the DNA strand breaks in cultured cells. Among nickel compounds involving Ni_3S_2, NiO (black), NiO (green), and $NiSO_4$, only Ni_3S_2, which is highly carcinogenic, induced lesions of both double- and single-stranded DNA in cultured human cells. Only Ni_3S_2 treatment of cultured HeLa cells induced a 1.5-fold increase in 8-hydroxy-2'-deoxyguanosine (8-OH-dG) compared with a control. Other compounds in this series had no effect. Histological studies showed that all the nickel compounds used induced inflammation in the lungs of rats. Nitroxy oxide generation in RAW 264.7 cells stimulated with lipopolysaccharide was enhanced by all the nickel compounds under study. Based on the data obtained two mechanisms for nickel-induced oxidative DNA damage have been proposed: (i) all the nickel compounds used induced indirect damage through inflammation, and (ii) Ni_3S_2 also showed direct oxidative DNA damage through H_2O_2 formation. This double action may explain the relatively high carcinogenic risk of Ni_3S_2. This implies that a high content of nickel and its clearance from tissue is directly proportional to nickel carcinogenic activity.

Nickel-Induced Activation of Transcription Factors

NF-κB is an important transcription factor in both apoptosis and the inflammatory process and was found to be activated by nickel. For example nickel-induced allergic response and skin hypersensitivity are connected with activation of NF-κB [262].

Also the ATF-1 transcription factor was found to be activated in nickel treated cells. The ATF-1 transcription factor belongs to an ATF/CREB family that was originally identified as a target of the cAMP signalling pathway [263]. As described above, nickel is known to affect calcium homeostasis. An increase of intracellular calcium after nickel administration in turn leads to activation of a protein kinase cascade that mediates ATF/CREB phosphorylation. Some studies suggest that the activation of the ATF-1 transcription factor by nickel played the role of a negative regulator of TSP I [264]. The TSP I protein is a potential regulator of tumour development *in vivo* since the increased levels of this protein suppress growth of blood vessels into the tumour body. Thus, the diminution of TSP I expression in tumours enhances angiogenesis which in turn stimulates the growth of tumours.

The hypoxia-inducible factor I (HIF-1) was found to be elevated in nickel treated cells [265]. HIF-1 is very sensitive to hypoxia stimulus and precise regulation of oxygen homeostasis. HIF-1 is composed of two bHLH proteins, HIF-1α and HIF-1β. HIF-1α is expressed and HIF-1β accumulated only in hypoxic cells [266]. One explanation of the nickel-induced activation of the HIF-1 transcription factor is based on the assumption that nickel replaces iron in the oxygen carrier, Fe(II)-hybrid hemoglobin. Substitution of iron by nickel switches signal to permanent hypoxia, which in turn activates the HIF-1 factor [267]. HIF-1 is also involved in the regulation of numerous genes involving also glucose transport and glycolysis [266]. Several experiments on various cells confirmed activated glucose metabolisms and glycolysis after nickel exposure [268].

P53 is an important tumour suppressor gene and transcription factor involved in the regulation of apoptosis and cell proliferation. The *p53* gene was reported to be mutated in human kidney epithelial cells exposed to nickel [269]. Mutations in *p53* are where most of the transformations were observed, *e.g.* these are the common

genetic alterations found in human cancers [270], however, several conflicting results have been reported. Maehle *et al.* [269] found altered *p53* gene structure and expression in human epithelial cells after exposure to nickel, however, in contrast, a low incidence of point mutations were detected in the *p53* tumour suppressor gene from nickel induced rat renal tumours [271]. These studies raise questions as to whether *p53* mutations are really involved in nickel-induced transformation.

Oxidative Stress and Nickel-Induced Carcinogenicity

Experimental data suggests that oxidative stress may be important in nickel-induced carcinogenesis, however a direct correlation between the ability of nickel to produce oxidative stress and carcinogenicity is not yet fully understood. The controversy of the role of oxidative stress in nickel carcinogenesis is documented in experiments with animals. Whilst administration of nickel, but also cobalt, induced tumour formation, the administration of copper and iron (both known to produce strong oxidative stress) failed to induce tumours [272]. This suggests that oxidative stress may not be as important in nickel carcinogenicity.

A further problem appeared in studies of carcinogenic activity of nickel compounds in various animals. While exposure to insoluble Ni_3S_2 induced carcinomas in 30% of the rats exposed, a similar exposure did not cause lung neoplasms in mice [272]. However, inflammation and lung fibrosis were observed in both sets of animals. The genetic differences between animals responsible for the different activity of enzymes involving the protective activity against oxidative stress seems to play an important role in nickel-induced carcinogenicity.

Nickel produces rather low, but measurable levels of free radicals in cells [274]. Fluorescent methods revealed that both, soluble $NiCl_2$ and insoluble Ni_3S_2 evoked formation of free radicals [275]. Many studies also revealed depletion of glutathione (GSH), representing another marker of oxidative stress [276].

The oxidative effect in human lymphocytes after acute nickel treatment was evaluated [277]. The levels of intracellular reactive oxygen species, lipid peroxidation and hydroxyl radicals and also the potential effects of antioxidants were examined. The level of hydroxyl radical in the Ni-treated group was much higher than in control. Also the levels of thiobarbituric acid-reactive substances (TBARS) in human lymphocytes *in vitro* in a concentration-dependent manner was detected. Catalase partially reduced the $NiCl_2$-induced elevation of oxidants, whereas superoxide dismutase (SOD) enhanced the level of oxidants and TBARS. Both $NiCl_2$-induced lipid peroxidation was prevented significantly by glutathione (GSH) and mannitol. $NiCl_2$-induced increase in generation of hydroxyl radical was prevented significantly by catalase, GSH and mannitol, but not by SOD. These results suggest that $NiCl_2$-induced lymphocyte toxicity may be mediated by oxygen radical intermediates. Catalase, GSH and mannitol each provides protection against the oxidative stress induced by Ni.

In another study, nickel chloride was found to induce lipid peroxidation in the plasma of human blood *in vitro* in a concentration-dependent and time-dependent manner [278]. The hydroxyl radical production increased in a concentration-dependent manner after Ni treatment for 1 h. Furthermore, a decreasing trend in α-tocopherol levels in plasma was observed after Ni treatment. Concurrent incubation with gluthathione (GSH), catechin (CTCH), and mannitol decreased lipid peroxidation and reduced hydroxyl radical formation induced by Ni, but an exacerbation of the decrease of α-tocopherol levels in plasma occurred with catechin.

A study to examine the effects of nickel on human platelets function was also undertaken. Ni significantly inhibited the function of platelet aggregation induced by collagen in a concentration-dependent manner [279]. The phenomenon of lipid peroxidation was involved as Ni significantly increased malondialdehyde (MDA) levels with a reduction in platelet reduced glutathione (GSH) and α-tocopherol content. Treatment with ascorbic acid significantly lowered the levels of MDA and increased the content of α-tocopherol and reduced GSH. The results show that Ni is toxic as evidenced by lipid peroxidative damage and inhibition of human platelet aggregation, but that ascorbic acid provides protection, at least partially, against this metal.

In contrast to the above reports, Shi *et al.* [280] suggested a pro-oxidant effect of GSH in the presence nickel. They reported that the reaction between Ni(II) with t-butyl hydroperoxide in the presence of glutathione (GSH) resulted in damaged DNA and that 8-hydroxy-2'-deoxyguanosine (8-OH-dG) adduct from 2'-deoxyguanosine (dG) and from dG residues in calf thymus DNA at physiological pH was detected. No significant amounts of 8-OH-dG was generated in the absence of GSH, indicating an important role of GSH in enhancing the reactivity of Ni(II) toward lipid hydroperoxide to oxidise dG or dG residues in DNA. Because GSH is ubiquitously present in cellular systems at relatively high concentration, and the exposure of cells to Ni(II) results in the generation of lipid hydroperoxides, the 8-OH-dG generation and DNA double-strand breaks caused by the reaction of Ni(II) with lipid hydroperoxides in the presence of GSH may be an important mechanism in Ni(II)-induced carcinogenesis. The inhibitory effect of chelators suggests a possible prevention strategy against Ni(II)-induced toxicity and carcinogenesis.

The role of free radicals in the effect of ultrafine nickel particles was studied by Dick *et al.* [281]. Ni causes significant increases in inflammatory markers, as well as inducing a significant depletion of supercoiled plasmid DNA, indicative of hydroxyl radical generation. The role of free radicals and reactive oxygen species in mediating ultrafine nickel inflammation was further strengthened by the ability of the antioxidants N-acetylcysteine (NAC) and glutathione monoethyl ester (GSHme) to block the particle induced release of tumour necrosis factor-alpha (TNF-alpha) from alveolar macrophages *in vitro*.

Nickel has been shown to inhibit DNA repair in a way that may play a role in its toxicity. Since nickel treatment increases cellular reactive oxygen species (ROS), Lynn *et al.* [282] investigated the involvement of ROS in nickel inhibition of DNA repair. Inhibition of glutathione synthesis or catalase activity increased the enhancing effect of nickel on the cytotoxicity of ultraviolet (UV) light. Inhibition of

catalase and glutathione peroxidase activity also enhanced the retardation effect of nickel on the rejoining of DNA strand breaks accumulated by hydroxyurea plus cytosine-β-D-arabinofuranoside in UV-irradiated cells. In addition, glutathione could completely recover the inhibition by nickel or H_2O_2 alone but only partially recover the inhibition by nickel plus H_2O_2. Therefore, it has been proposed that nickel may bind to DNA-repair enzymes and generate oxygen-free radicals which cause protein degradation *in situ*. This irreversible damage to the proteins involved in DNA repair, replication, recombination, and transcription could be important for the toxic effects of nickel.

Several studies were devoted to the role of nickel(II) in Fenton chemistry. The role of nickel(II) Fenton reactions in oxidative DNA damage was investigated by Lloyd and Phillips [283]. Experiments confirmed the formation of putative intrastrand cross-links, 8-hydroxydeoxyguanosine (8-OHdG) and single- and double-strand breaks. The nickel(II) Fenton reaction generated exclusively open-circular DNA, and this was completely inhibited by the addition of 25 μM EDTA.

In another report Dally and Hartwig [284] investigated the potential of nickel(II) to induce DNA lesions in human HeLa cells. As indicators of oxidative DNA damage, the frequencies of DNA strand breaks and of lesions recognized by the bacterial formamidopyrimidine-DNA glycosylase (Fpg protein), including 7,8-dihydro-8-oxoguanine (8-hydroxyguanine), a pre-mutagenic DNA base modification were reported. Nickel(II) caused a slight increase in DNA strand breaks at 250 μM and higher, while the frequency of Fpg-sensitive sites was enhanced only at the cytotoxic concentration of 750 μM. The repair of oxidative DNA lesions induced by visible light was reduced at 50 μM and at 100 μM nickel(II) for Fpg-sensitive sites and DNA strand breaks, respectively. The removal of both types of lesions was almost completely blocked at 250 μM nickel(II). An impaired repair of the observed lesions might well explain the carcinogenic action of nickel(II).

CADMIUM

Cadmium is the 48th element and a member of group 12 in the Periodic table of elements. The most common oxidation number of cadmium is +2. Cadmium is is a heavy metal; roughly 13,000 tons of cadmium is produced, worldwide, each year for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings and alloys. The toxicity of cadmium relates to smelting where the main route of exposure is through the lungs. In contaminated areas, there is evidence to suggest increased body burdens of cadmium among a proportion of the exposed population, with some evidence of increased urinary excretion of β-2-microglobulin and some loss of bone density among people with the highest urinary cadmium concentrations [285, 286]. There are also areas where extensive mining operations have led to contamination, particularly affecting the soil. Soluble cadmium salts accumulate and result in toxicity to the kidney, liver, lungs, brain, testes, heart, and central nervous system. Cadmium is listed by the US Environmental Protection Agency as one of 126 priority pollutants. In most studies, the half-life in humans is estimated to be between 15 and 20 years [287]. Cadmium can cause osteoporosis, anemia, non-hypertrophic emphysema, irreversible renal tubular injury, eosinophilia, anosmia and chronic rhinitis. Cadmium is a potent human carcinogen and has been associated with cancers of the lung, prostate, pancreas, and kidney. Because of its carcinogenic properties, cadmium has been classified as a #1 category human carcinogen by the International Agency for Research on Cancer of USA [288].

Cadmium, Genes and Proteins

Failure in the regulation of gene expression is regarded as a major factor in a multi-stage model of chemical carcinogenesis. In cadmium-related carcinogenicity, the following genes are induced: (i) Immediate early response genes (IEGs). IEGs are protooncogenes and encode transcription factors and play a significant role in chemical carcinogenesis by influencing the expression of target genes including those controlling cell growth and division. They are involved in cell proliferation and differentiation. These genes are often found to be overexpressed in tumours. The most frequently studied IEGs are c-*fos*, c-*jun* and c-*myc* [289-291]. Significant cadmium-induced overexpression of IEGs constitute mitogenic growth signals stimulating cell proliferation and they are regarded as major players in the promotion stage in a multi-stage model of carcinogenesis [292]. (ii) Further target in cadmium-induced carcinogenicity studies have been found to be the induction of expression of several stress response genes such as those for encoding metallothionein (MT) synthesis, genes for encoding heat-shock proteins (HSPs), genes involved in oxidative stress response, genes involved in the synthesis of glutathione (GSH) and related genes. For example lack of expression of the MT protein, under basal and cadmium-stimulated conditions, has been regarded as one of the major underlying causes of tissue susceptibility to cadmium toxicity and carcinogenicity. Experimental data on lung carcinogenesis in mice and rats have shown that a high abundance of MT provides protection against cadmium-induced lung carcinogenesis. (iii) *In vitro* results demonstrate that cadmium has also been found to influence the activity of several transcription factors. It has been found that cadmium is a powerful inducer of c-*fos* and c-*jun* and this has been considered as a major mechanism for cadmium-induced cell transformation and tumourigenesis [293-294]. (iv) Very recently, the effect of cadmium on the expression of genes regulating translation has also been reported [295]. These results might indicate that cell transformation and tumourigenesis induced by cadmium may, in part, be mediated through the overexpression of translation factors.

It should be noted that, in addition to the above genes affected by cadmium, recent progress in experimental techniques, such as differential display and microarray analysis has led to the identification of a large number of genes exhibiting alterations in expression in response to exposure to cadmium. Details can be found elsewhere [296].

Cadmium itself is unable to generate free radials directly however, *via* indirect mechanisms (see below), it can cause free radical-induced damage to the gene expression. It has been reported that cadmium can cause activation of cellular protein kinases (protein kinase C), which result in enhanced

phosphorylation of transcription factors and consequently lead to the transcriptional activation of target gene expression. These conclusions are based on experiments involving inhibitors that are regarded as specific for various kinases [297].

New findings in the explanation of cadmium-induced carcinogenicity have recently been reported [298]. It has been reported that E-cadherin, a transmembrane Ca(II)-binding glycoprotein playing an important role in cell-cell adhesion, can bind cadmium to Ca(II)-binding regions, changing the glycoprotein conformation [299]. The disruption of cell-cell adhesion caused by cadmium binding to the E-cadherin could play an important role in the tumour induction and promotion. Calcium was found to inhibit colorectal carcinogenesis, and thus may act as an anticarcinogen [300].

Cadmium and Apoptosis

Several *in vivo* and *in vitro* experiments employing cadmium confirmed programmed cell death (apoptosis) of a variety of organs in rat models [301]. Cadmium-induced apoptosis was observed in the testes, however not in the prostate [302]. The induction of apoptosis in testes was inversely correlated with expression of tumour suppressor gene p53. This could be interpreted as an experimental support for cadmium-induced tumourigenesis.

The mechanism of cadmium induced apoptosis does not necessarily contribute to the protection against malignant transformation. Several experiments confirmed that only a 60 % fraction of cells were committed to apoptosis, while the remainder of the cells stayed viable and exhibited increased production of metallothionein [303]. It has been proposed that cadmium-adaptation inhibits cell apoptosis, which in turn may allow the overproduction of critical mutations.

Cadmium and DNA Repair Mechanism

The results indicate that cadmium primarily causes toxicity by deactivating an essential DNA repair activity [304]. The direct inhibition of DNA mismatch repair by cadmium provides a molecular mechanism for cadmium toxicity. There exist many mutation avoidance systems that correct damaged DNA. These include direct damage reversal, base excision repair, nucleotide excision repair, double-strand-break repair and mismatch repair (MMR). Cadmium seems to inactivate only one of them—the MMR system. During the past decade, results from human systems and yeast have showed that genetic disruption of MMR by mutations in the MutS homolog (MSH) family of proteins leads to substantial increases in genome instability and greatly increases the incidence of several kinds of human cancer. Jin *et al.* [305] found that cadmium-induced inhibition of MMR in human cell extracts leaves about 20–50% of DNA mismatches unrepaired. Inhibition of MMR leads to the propagation of cellular errors, thus the toxic effects of cadmium can be amplified in cells by creating mutations in genes that induce further faulty functions. As cadmium binds to protein sites with high occupancy when it has multiple protein ligands and typically replaces zinc, these results suggest an unidentified zinc site for MMR function that is specifically disrupted by cadmium.

Cadmium, Free Radicals, Vitamins C and E, Aspirin

Cadmium itself is unable to generate free radicals directly, however, indirect generation of various radicals involving the superoxide radical, hydroxyl radical and nitric oxide has been reported. [306]. Some experiments also confirmed the generation of non-radical hydrogen peroxide which itself in turn may be a significant source of radicals *via* Fenton chemistry [307].

Various studies have been made on the cadmium-induced testicular toxicity in rat models. Yang and coworkers reported significantly higher contents of malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) in exposed groups [308]. Glutathione was found to scavenge intracellular oxygen radicals either directly or *via* the GSH peroxidase/GSH system. The activity of superoxide dismutase (SOD) in the tested animals was lowered. This study also revealed that the number of cells with DNA single strand breaks and the levels of cellular DNA damage was significantly higher in exposed groups than in controls.

Increase in lipid peroxidation with increased MDA levels has been observed in a cadmium-induced experimental group of wistar albino rats [309]. SOD enzyme levels were also found to be significantly lower in the experimental group and glutathione levels in this group were found to be decreased relative to the control group values.

Recent studies [310] using the human-hamster hybrid A(L) cell mutation assay showed that, at low concentrations of cadmium, attenuated removal of hydrogen peroxide was observed. This suggests that cadmium is involved in the inhibition of the systems involved in H_2O_2 removal (glutathione/glutathione reductase, catalase, and ascorbate peroxidase), resulting in H_2O_2 accumulation.

An interesting mechanism explaining the indirect role of cadmium in free radical generation was presented some years ago [311]. In this mechanism it was proposed that cadmium can replace iron and copper in various cytoplasmic and membrane proteins (e.g. ferritin, apoferritin), thus increasing the amount of unbound free or chelated copper and iron ions participating in oxidative stress *via* Fenton reactions [312]. These results support very recent findings by Watjen and Beyersmann [313]. Displacement of copper and iron by cadmium can explain the enhanced cadmium-induced toxicity, because copper, displaced from its binding site, is able to catalyze breakdown of hydrogen peroxide *via* the Fenton reaction.

Sen Gupta *et al.* [314] reported that vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. Supplementation with vitamin C and/or vitamin E reduced the level of testicular ROS and restored normal testicular function in Cd-exposed rats. Similarly, the protective role of vitamin E against cadmium-induced toxicity in rat models were reported by Ognjanovic *et al.* [315] who studied the effect of acute exposure to cadmium on the blood antioxidant defence system, lipid peroxide concentration and hematological parameters. They also assessed the possible protective role of vitamin E. Red blood cell counts, hematocrit value and hemoglobin concentration were significantly decreased in the blood of Cd-treated rats. Intoxication with cadmium was also followed by significantly increased lipid peroxide

concentrations. The authors also observed an increased activity of antioxidant defence enzymes: copper-zinc containing superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase as well as concentrations of non-enzymatic components of the antioxidant defence system: reduced glutathione, vitamin C and vitamin E. Pretreatment with vitamin E showed a protective role on the toxic effects of cadmium on the hematological values, lipid peroxide concentration as well as on enzymatic and non-enzymatic components of antioxidant defence system.

In contrast to the above reports, *in vitro* experiments on cadmium carcinogenicity reported by Hossain and Huq [316] have shown that, in the presence of antioxidants such as cysteine, glutathione and ascorbate (especially cysteine and ascorbate), DNA damage is found to be greater than expected for the combined effects of the antioxidant and Cd(II). The increased DNA damage is believed to be due to the formation of reactive oxygen species. These results suggest that Cd(II) binds covalently with DNA (possibly at G, A and T centres) and can form intrastrand bifunctional AT adducts but not the GG adducts. These results are consistent with the theory that cadmium can displace iron and copper from various cytoplasmic and membrane proteins and also in agreement with the recently expressed concerns over the potentially deleterious effects of transition metal ion (copper and iron)-mediated pro-oxidant effect of ascorbate [85].

The study of Beytut and coworkers [317] demonstrated the effectiveness of vitamin E in reducing oxidative stress in Cd-treated rabbits. The effect of dietary vitamin E intake on lipid peroxidation as measured by the production of thiobarbituric acid reactive substances (TBARS) was assessed. The results suggest that reduction in the increase in TBARS due to Cd toxicity may be an important factor in the action of vitamin E.

The effect of Cd on pituitary hormone secretion has also been studied, in particular the mechanisms that induce this misregulation [318]. Cd(II) concentrations above 10 μM were found to be cytotoxic for pituitary cells. The experimental results showed that Cd(II)-treated cells undergo apoptosis. The antioxidants N-acetyl-cysteine and Trolox (a water soluble derivative of vitamin E), but not ascorbic acid, reversed both Cd(II)-mediated cytotoxicity and the inhibition of prolactin release, supporting the involvement of oxidative stress in the mechanism of Cd(II) action. This work demonstrates that Cd(II) is cytotoxic for anterior pituitary cells, and that this effect is due to an induction of apoptosis, and that it can be reversed by antioxidants, mainly by vitamin E.

Casalino *et al*. [319] proposed that the toxic effect of cadmium is due to imidazole residue binding of SOD His-74 which is essential in the decomposition of hydrogen peroxide. Cadmium inhibition of liver mitochondrial MnSOD activity was completely removed by Mn(II) ions, suggesting that the reduced effectiveness of this enzyme is probably due to the substitution of cadmium for manganese. These authors also observed antioxidant capacity of Mn(II) ions, since they were able to normalize the increased TBARS levels occurring when liver mitochondria were exposed to cadmium.

Ulusu *et al*. [320] studied the protective role of selenium against Cd-toxicity. One group of rabbits was fed a selenium-deficient diet, a second group was fed a selenium-rich diet, and the control group was fed a normal diet. The brain and liver tissues of these groups were investigated for the *in vitro* inhibitory effects of Cd(II) on glutathione reductase activity. For the liver, the percentage inhibition of glutathione reductase by 40 nmol/mg protein of Cd(II) was similar for the selenium-deficient and control groups, but significantly lower in the selenium-rich group. This study demonstrates the protective effect of dietary selenium against inhibition of glutathione reductase by Cd ions.

The effect of aspirin and vitamin E against copper and cadmium toxicity was investigated on COS-7 cells [321]. Exposure to cadmium significantly reduces the viability of cells. Of interest were the findings that aspirin protected cells from both copper- and cadmium-induced toxicity. A combination of aspirin and vitamin E increases cell viability in copper-exposed cells in a clearly additive manner, while in cadmium-exposed cells the effects are slightly additive. These results suggest that aspirin and vitamin E can protect cells from metal-induced toxicity. Differences in the protective effects of aspirin and vitamin E on copper versus cadmium-induced toxicity may be due to either alternative mechanisms of metal toxicity or antioxidant activity.

The protective role of melatonin, an effective antioxidant and free radical scavenger, against cadmium was studied [322]. Melatonin slightly, but not significantly, reduced cadmium-induced lipid peroxidation in the testes. It is concluded that cadmium toxicity, at least with respect to the resulting lipid peroxidation, is reduced by the administration of melatonin.

Oteiza *et al.* [323] evaluated the protective role of zinc in cadmium toxicity. The results support the concept that zinc deficiency increases the susceptibility of testes to cadmium-mediated free radical damage.

ARSENIC

Arsenic is the 33rd element of the Periodic table of elements. The most common oxidation numbers of arsenic are +5, +3, and -3. Arsenic can form both inorganic and organic compounds in the environment and human body. Inorganic arsenic includes arsenite (As(III)) and arsenate (As(V)). The inorganic arsenics can be either methylated (monomethylarsonic acid, MMA) or dimethylarsinic acid (DMA) *in vivo*.

Arsenic is a well-documented carcinogen in a number of studies [324]. Exposure to arsenic is linked with a risk of developing tumours of the lung, skin, liver, bladder, and kidney [324]. The most common source of arsenic is drinking water. The concentration of arsenite in drinking water is in the range 0.01 – 4 mg/l [325]. Besides carsinogenic effects of arsenic, arsenic compounds can be used as a medicine to treat acute promyelotic leukaemia (APL) [326, 327]. Recent *in vivo* studies indicate that methylated forms of arsenic may serve as co-carcinogens or tumour promoters [327, 328]. Both methylated and dimethylated arsenicals that contain arsenic in the trivalent oxidation state have been identified as intermediates in the

metabolic pathway. These compounds have been detected in human cells cultured in the presence of inorganic arsenic and in the urine of individuals who were chronically exposed to inorganic arsenic. Methylated and dimethylated arsenicals that contain arsenic in the trivalent oxidation state are more cytotoxic, more genotoxic and more potent inhibitors of the activity of some enzymes than arsenicals that contain arsenic in the pentavalent oxidation state. Hence, it is reasonable to describe the methylation of arsenic as a pathway for its activation, not as a mode of detoxification [329]. While arsenite can react with the sulfhydryl groups of proteins and inhibits many biochemical pathways, arsenate is a phosphate analogue which interferes with phosphorylation reactions. The trivalent arsenite is biologically significantly more active than the pentavalent arsenate. This includes the ability to induce gene amplification in mammalian cells [330]. Since absorbed arsenate is mostly reduced to arsenite in blood, the effect of arsenite and arsenate appears to be very similar.

The mechanisms by which arsenic causes human cancers are not well understood. Arsenic is an atypical carcinogen since it is classified neither in the initiator nor the promoter categories of carcinogenic agents [331]. Thus, arsenic probably does not act as a classical carcinogen, but rather enhances the carcinogenic action of other carcinogens.

In an attempt to explore the carcinogenic effect of arsenic, several animals were studied including monkey, dog and others. However, to date, no satisfactory animal model of arsenic-induced cancer has been developed. To overcome the absence of an acceptable animal model, normal human cells such as normal human keratinocytes, which represent a primary *in vivo* target of arsenite, provide a relevant and reasonable *in vitro* model to study the molecular mechanisms of arsenic carcinogenicity. Such studies are essential for risk assessment and establishment of exposure limits.

Many studies confirmed the generation of free radicals during arsenic metabolism in cells [332]. Oxidative stress has been linked with the development of arsenic related diseases including cancers. In addition to reactive oxygen species (ROS), also reactive nitrogen species (RNS) are thought to be directly involved in oxidative damage to lipids, proteins and DNA in cells exposed to arsenic. Many recent studies have provided experimental evidence that arsenic-induced generation of free radicals can cause cell damage and death through activation of oxidative sensitive signalling pathways [332].

Arsenic-Induced Regulation of Transcription Factors

Various investigations have shown that exposure to arsenic is able to activate cellular transcription factors in response to exposure. Regulation and activation of transcription factors that can bind specifically to certain sequences of DNA is an important component in mediating cellular responses to stimuli. The transcription factors include NF-κB, AP-1 and p53 which were discussed above. While some metals appear to affect all three major MAP kinases, involving ERK, JNK and p38, other metals may activate only one or two of the MAP kinases. Several studies have shown that exposure of JB6 cells to arsenic induced phosphorylation and activation of ERKs and JNKs [333]. Based on these studies Bode and Dong [327] hypothesised that the induction of ERKs by arsenic may promote the carcinogenic effect of arsenic, while induction of JNKs by arsenic may enhance its apoptotic activity and therefore its anti-carcinogenic effect.

Huang *et al*. also found that the transcriptional factor AP-1 plays a critical role in tumour promotion [334]. Cavigelli *et al.* [335] reported that arsenite-induced JNKs and p38 kinase activation in HeLa cells was accompanied by increased AP-1 activity and enhanced c-jun and c-fos gene expression.

Some studies proposed activation of protein kinase C (PKC) that can result in activation of MAPKs pathways. The translocation of PKC from the cytosol to the membrane appears to be a critical step for its activation [336]. Some experiments demonstrated that PKC mediates arsenite-induced AP-1 activation in JB6 cells *via* ERKs, JNKs and p38 kinases. In contrast other studies using myeloid leukemia cells have shown no effect on apoptosis induced by arsenic [337]. Taken together the research findings indicate that the effects associated with arsenic exposure appear to be mediated through MAPK pathways and possibly through PKC. The activated signalling pathways lead to an induction of AP-1 transactivation and an increase in AP-1 DNA binding activity.

Activation of nuclear factor kappa B (NF-κB) (for more general description, see above) is a well-known phenomenon and is linked to MAPK signalling pathways, especially p38 kinase. Arsenic has been shown to have varying effects on NF-κB activity and DNA binding, including activation [338], inhibition of TNFα-induced activation [339] as well as no effect at all [335]. 500 μM concentration of arsenite was reported to prevent TNFα-induced NF-κB activation by directly blocking the activity of the IkB kinase complex (IKK), which resulted in an inhibition of the phosphorylation and degradation of inhibitory kappa B alpha (IkBα) [340]. Recent studies using low concentrations of arsenite (up to 5 μM) was linked to cell proliferation whereas concentrations greater that 50 μM caused cell death [341]. Kapahi *et al*. [339] concluded that an arsenite concentration greater than 12 μM inhibits TNF-α induced NF-κB and also IKK activation through reaction with critical cystein (Cys-179) in the activation loop of the IKKα and IKKβ. Replacing Cys-179 by alanine suppressed inhibition. While the role of MAP kinase pathways in the modulation of arsenic's effect on NF-κB is not unequivocal, experimental results from various laboratories indicate that IKK mediation and phosphorylation and degradation of IkBα is almost certainly affected by arsenic exposure.

The effect of arsenic on p53, the protein crucial for the induction of apoptosis (programmed cell death), is not fully understood. The problem is that various studies have reported conflicting results spanning no effect of arsenic on p53, induced p53 phosphorylation and decrease of p53 expression. For example, Huang *et al.* [342] reported no effect for various concentrations of arsenic on the *p53*-dependent transcription in *p53* promoter transfected in C141 JB6 cells, suggesting no involvement of p53 in the arsenic-induced apoptosis. In contrast, other groups have reported overexpression of *p53* gene in patients suffering of arsenic-related skin disease. In addition a high incidence (28 – 55

%) of *p53* gene mutations was observed in these patients. In conclusion, the role of p53 in arsenic-stimulated cellular effects is not yet clear. The experimental results suggest both p53-dependent and p53-independent induction of apoptosis, and also both an increased and decreased expression of the protein.

Arsenic and Free Radicals

Arsenic-mediated generation of reactive oxygen species involves generation of superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), the peroxyl radical ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$) [343], hydrogen peroxide (H_2O_2), dimethylarsinic peroxyl radicals ($[(CH_3)_2AsOO^{\bullet}]$) and also the dimethylarsinic radical $[(CH_3)_2As^{\bullet}]$ [344]. The exact mechanism responsible for the generation of all these reactive species is not yet clear, but some studies proposed the formation of intermediary arsine species [345].

Arsenite was found to generate detectable levels of superoxide in U937 cells at concentrations of 1–10 μM [346], human-hamster hybrid cells at 50 μM [347] and human vascular smooth muscle cells (VSMC) at 7–16 μM [348]. Also superoxide and hydrogen peroxide formation in vascular endothelial cells [349] as well as in human keratinocyte cell line (HaCat) was detected by EPR spin trapping, with DMPO, and EPR spectroscopy [350].

The arsenite-induced apoptosis *via* the formation of hydrogen peroxide was observed in NB4 cells [351] and also in CHO-K1 cells [352]. The results of sodium arsenite-induced cell death in CHO-K1 (Chinese hamster ovary K1) cells [353] lead to a working hypothesis that arsenite-induced apoptosis in CHO-K1 cells is triggered by the generation of hydrogen peroxide, followed by a copper-mediated Fenton reaction that catalyzes the production of $^{\bullet}OH$ radicals, which then selectively activates protein kinase through de novo synthesis of macromolecules.

In a recent study by Samikkannu *et al.* [354] it was shown that arsenite can inhibit pyruvate dehydrogenase (PDH) activity through binding to vicinal dithiols in both the pure enzyme and tissue extract. As_2O_3 increased H_2O_2 levels in HL60 cells, but this was not observed for phenylarsine oxide. Mitochondrial respiration inhibitors suppressed the As_2O_3-induced H_2O_2 production and As_2O_3 inhibition of PDH activity. Treatment with H_2O_2 plus Fenton metals also decreased the PDH activity in HL60 cells. Therefore, it seems that As_2O_3 elevates H_2O_2 production in mitochondria and this may produce the hydroxyl radical through the Fenton reaction which then results in oxidative damage to the protein of PDH. These results suggest that arsenite may cause protein oxidation to deactivate an enzyme and this can occur at a much lower concentration than arsenite binding directly to the critical thiols.

Another route to produce H_2O_2 was explained by the oxidation of As(III) to As(V) which, under physiological conditions, results in the formation of H_2O_2.

$$H_3AsO_3 + H_2O + O_2 \rightarrow H_3AsO_4 + H_2O_2 \ (\Delta_rG = -40.82 \text{ kcal/mol}) \quad (26)$$

The above reaction is spontaneous and exergonic with an estimated standard reaction free energy change for H_2O_2 formation of –40.82 kcal/mol (–170.87 J/mol). H_3AsO_3 (arsenious acid) has the ability to generate a dioxygenated complex because it is a Lewis acid that may accept unpaired electrons that could originate either from water or from oxygen.

In recent studies on the mechanism of arsenite toxicity in the brain it was reported that some of its effects have been traced to the generation of the hydroxyl radical [355]. The time-evolution of the formation of the hydroxyl radical in the striatum of both female and male rats who underwent a direct infusion of different concentrations of arsenite was investigated. The treatment with arsenite induced significant increases of hydroxyl radical formation. These results support the participation of hydroxyl radicals in arsenic-induced disturbances in the central nervous system.

In addition to oxygen species, arsenic exposure can also initiate the generation of nitrogen species [332]. $NO^{\bullet}$ is a messenger molecule that plays an important role in the immune response, neurotransmittion and vasodilation. Production of $NO^{\bullet}$ is normally catalyzed by NO synthases. Several conflicting reports concerning arsenic-induced production of $NO^{\bullet}$ have been published. One concluded that there was no cadmium-induced increase in $NO^{\bullet}$ generation in hepatocytes and human liver cells, which inhibited inducible NO synthase gene expression in cytokine-stimulated human liver cells and hepatocytes [356]. In a second report, arsenite was found to inhibit inducible NO synthase gene expression in rat pulmonary artery smooth muscle cells [357]. Similarly, a third study with low levels of arsenite (< 5 μM) reported no change in intracellular concentration of Ca(II) as well as no $NO^{\bullet}$ generation as detected by EPR spectroscopy [358].

However, a series of several other studies have documented the inverse effect of arsenic-induced $NO^{\bullet}$ formation. At concentrations of arsenite greater than 20 μM, Lynn *et al.* [359] observed an arsenite concentration- and time-dependent increase of nitrite levels in the medium of arsenite-treated CHO-K1 cells. The increase in nitrite levels has been related to increased $NO^{\bullet}$ production in these cells. It appears that the stimulation of $NO^{\bullet}$ production by arsenite is through activation of endothelial NO synthase (eNOS) by calcium [360]. Nitric oxide seems to be involved in arsenite-induced DNA damage and pyrimidine excision inhibition [361].

Arsenic-Induced Oxidative Damage to DNA

Schwerdtle *et al.* [362] compared the induction of oxidative DNA damage by arsenite and its methylated metabolites in cultured human cells as well as in isolated PM2 DNA, by measuring the frequency of DNA strand breaks and of lesions recognized by the bacterial formamidopyrimidine-DNA glycosylase (Fpg). It was found out that only dimethylarsinous [DMA(III)] (at concentrations greater than or equal to10 μM) generated DNA strand breaks in isolated PM2 DNA. In HeLa S3 cells, short-term incubation (0.5-3 h) with doses as low as 10 nM arsenite induced a high frequency of Fpg-sensitive sites, whereas the induction of oxidative DNA damage after 18 h incubation was rather low. Both monomethylarsonous [MMA(III)] and dimethylarsinic [DMA(V)] generated DNA strand breaks in a

concentration-dependent manner. These results show that very low physiologically relevant doses of arsenite and the methylated metabolites induce high levels of oxidative DNA damage in cultured human cells. Thus it was proposed that biomethylation of inorganic arsenic may be involved in inorganic arsenic-induced genotoxicity/carcinogenicity.

A similar conclusion based on EPR spin trapping spectroscopy was reached by Nesnow *et al*. [363]. They showed that reactive ROS were intermediates in the DNA-damaging activity of MMA(III) and DMA(III). Using the phiX174 DNA nicking assay they found that the ROS inhibitors Tiron, melatonin, and the vitamin E analogue Trolox inhibited the DNA-nicking activities of both MMA(III) and DMA(III) at low micromolar concentrations. The use of the DMPO spin trap revealed formation of reactive hydroxyl free radical. These data are consistent with the conclusions that the DNA-damaging activity of DMA(III) is an indirect genotoxic effect mediated by ROS-formed concomitantly with the oxidation of DMA(III) to DMA(V). Thus, for inorganic arsenic, it may be concluded that oxidative methylation followed by reduction to trivalency may be an activation, rather than a detoxification pathway. This would be particularly true for arsenate. Based on the current literature data it may be concluded that reduction from the pentavalent state to the trivalent state of inorganic arsenic may be even more important than methylation of arsenic in forming toxic and carcinogenic arsenic species.

A good biomarker of oxidative stress to DNA is 8-Hydroxyl-2-deoxyguanosine (8-OH-dG), one of the major products of ROS induced DNA damage. Increased levels of 8-OHdG have been observed in animal models, cellular and human tissues exposed to arsenic. Matsui *et al.* [364] studied the levels of 8-OHdG by immunohistochemistry using N45.1 monoclonal antibody in 28 cases of arsenic-related skin neoplasms and arsenic keratosis as well as in 11 cases of arsenic-unrelated Bowen's diseases. The frequency of 8-OHdG positive cases was significantly higher in arsenic-related skin neoplasms (22 of 28; 78%) than in arsenic-unrelated Bowen's disease (one of 11; 9%). Wanibuchi *et al.* [365] observed that the formation of 8-OHdG was significantly increased in male F344 rat liver after administration of DMA for 8 weeks. In another animal model, Wei *et al.* [366] observed a significant increase in urinary 8-OHdG levels induced by DMA for 3–9 h.

Another study has shown that catalase, and inhibitors of calcium, nitric oxide synthase, superoxide dismutase, and myeloperoxidase, could moderate arsenite-induced DNA damage. It was concluded that arsenite induces DNA adducts through calcium-mediated production of peroxynitrite, hypochlorous acid, and hydroxyl radicals [367]. Arsenic species have been found to cause release of iron from ferritin. The *in vitro* effects of four different species of arsenic (arsenate, arsenite, monomethylarsonic acid, and dimethylarsinic acid) in mobilizing iron from horse spleen ferritin under aerobic and anaerobic conditions were investigated [368]. The results indicate that exogenous methylated arsenic species and endogenous ascorbic acid can cause (i) the release of iron from ferritin, (ii) the iron-dependent formation of reactive oxygen species, and (iii) DNA damage. This reactive oxygen species pathway could be a mechanism of action of arsenic carcinogenesis in humans.

Arsenic-induced strand breaks were detected in a concentration-dependent manner in murine keratinocyte line JB6. These observations indicate that a large proportion of arsenite-induced DNA strand breaks come from excision of oxidative DNA adducts and DNA-protein cross-links, confirming that arsenite is able to induce a high level of oxidative DNA adduct and DNA protein cross-links [327].

Nitric oxide synthase inhibitors, N^{ω}-nitro-L-arginine methyl ester and s-methyl-L-thiocitrulline, had no apparent effects on arsenite-induced DNA strand breaks. However, SOD, catalase, diphenylene iodinium, DMSO, d-mannitol, and pyruvate effectively reduced arsenite-induced DNA strand breaks. This suggests that ROS, but not nitric oxide, are involved in arsenite induced DNA strand breaks [369]. DNA single strand breakage is an obligatory trigger for the activation of poly (ADP-ribose) polymerase (PARP), which can result in the depletion of ATP and cell death. DNA strand breaks are also known to cause chromosomal rearrangements.

Arsenic and Membrane Lipids and Proteins

Experimental studies have shown that that exposure to arsenite results in lipid peroxidation. The recent results of an epidemiological study of chronic exposure of Chinese residents to arsenic containing drinking water indicated that, although the activity for superoxide dismutase (SOD) in blood did not differ significantly between the group exposed and low-exposure group (control), the mean serum level of lipid peroxides (LPO) was significantly higher among the high-exposed group [370]. Those in the high-arsenic-exposure group had mean blood nonprotein sulfhydryl (NPSH) levels 58% lower than those in the low-exposure group, providing evidence that chronic exposure to arsenic from drinking water in humans results in the induction of oxidative stress, as indicated by the reduction in NPSH and the increase in LPO.

The effect of arsenite, arsenate on human erythrocyte membrane proteins and lipids was assessed by Zhang *et al.* [371]. It was shown that the arsenite species are bound to the membrane from cytosol. In contrast, arsenate bound rapidly from the outside, followed by releasing and re-binding. The binding to the membrane *via* sulfhydryl was indicated by the decrease of the sulfhydryl level of membrane proteins. The binding of arsenite and arsenate to the membrane also induced changes in the fluidity of membrane lipids and leaves a negative charge density on the outer surface of the membrane.

Twelve weeks of arsenic exposure increase the formation of oxidised glutathione (GSSG) and promote malondialdehyde production in both liver and brain samples [372]. In addition to a significant reduction in δ-aminolevulinic acid dehydratase activity and GSH levels, a marked elevation in MDA production may also contribute to arsenic-induced oxidative stress.

Interesting results on the role of lipid peroxidation in the mechanism of arsenic toxicity was observed in female rats pretreated with either N-acetylcysteine (NAC, a glutathione [GSH] inducer) or buthionine sulfoximine (BSO, a GSH depletor) [373, 374]. The results showed that arsenic decreased GSH levels and increased lipid peroxidation in the

liver, kidney, and heart, with a larger effect at 18.2 mg/kg than at 14.8 mg/kg. In the liver of rats treated with arsenic, pretreatment with NAC increased the levels of GSH and decreased lipid peroxidation. In kidney and heart, NAC pretreatment protected the tissues against arsenic-induced depletion of GSH levels, but the same degree of protection was not found for lipid peroxidation induction. BSO had an additive effect with arsenic in lowering the levels of GSH in the liver and kidney, but an inverse correlation was observed between GSH levels and lipid peroxidation in the liver. The arsenic content in the tissues of rats pretreated with NAC was lower than in rats treated only with arsenic. In rats with depleted levels of GSH (BSO-pretreated rats), a shift in arsenic tissue distribution was found, with higher levels in skin and lower levels in kidney. A clear tendency for a positive correlation between arsenic concentration and lipid peroxidation levels was found in liver, kidney, and heart.

An interesting medical application employing arsenic containing lipids has been reported. Lipids containing aresenic or phosphorus have been shown to be an alternative to viral vector-mediated gene delivery into *in vitro* and *in vivo* model applications [375]. It was demonstrated that arsenic substitution on the polar domain of cationic phosphonolipids results in a significant increase in the ability to transfer DNA into various cells for both *in vitro* and *in vivo* assays and decrease the cellular toxicity.

Arsenic Toxicity and Glutathione

Oxidative stress is an imbalance between free radical generation and the antioxidant defense system. Many reports evidenced a decrease in the levels of antioxidants after exposure to arsenic. Decreased antioxidant levels in plasma from individuals exposed to arsenic in Taiwan have been reported by Wu *et al.* [376] who showed that there was a significant inverse correlation between plasma antioxidant capacity and arsenic concentration in whole blood.

Several papers have reported decreased levels of GSH after exposure to arsenic. It was reported that one hour after exposure to arsenic (15.86 mg/kg body wt), the GSH concentration was significantly decreased in the liver of male Wistar rats [377]. After 6 months exposure to arsenic (3.2 mg/l), hepatic GSH and the enzymes glucose-6-phosphate dehydrogenase and GPx were significantly lowered in mice. Oral administration of orpiment (50 mg/kg body wt) caused

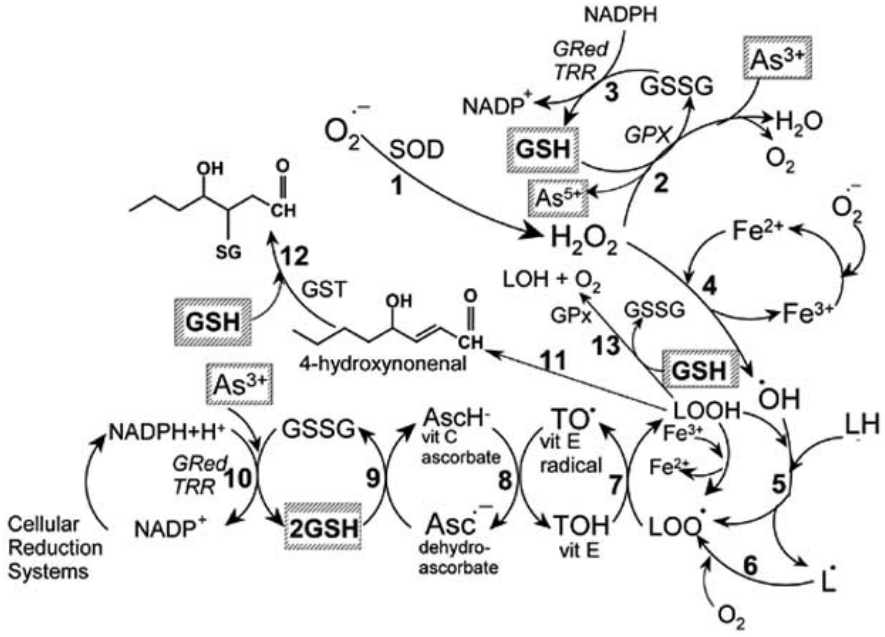


Fig. (4). Arsenic toxicity and the various pathways of glutathione (GSH) in the management of oxidative stress. (Note, that equations are not balanced). Reaction 1: superoxide is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction 2: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) that requires GSH as the electron donor. The oxidation of As(III) to As(V) under physiological conditions results in the formation of H_2O_2 (backward reaction 2). Reaction 3: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) that uses NADPH as the electron donor. Reaction 4: Some transition metals (e.g. Fe^{2+}, Cu^{+} and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction 5: Hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centered lipid radical ($L^{\cdot}$). Reaction 6: Lipid radical ($L^{\cdot}$) can further interact with molecular oxygen to give a lipid peroxyl radical ($LOO^{\cdot}$). Reaction 7: Lipid peroxyl radical ($LOO^{\cdot}$) are reduced within the membrane with reduced form of vitamin E (TOH) resulting in the formation of a lipid hydroperoxide and radical of vitamin E ($TO^{\cdot}$). Reaction 8: The regeneration of Vitamin E by Vitamin C: The Vitamin E radical ($TO^{\cdot}$) is reduced back to vitamin E (TOH) by ascorbic acid (physiological form of ascorbate is ascorbate monoanion, $AscH^{-}$) leaving behind ascorbyl radical ($Asc^{\cdot -}$). Reaction 9: The Regeneration of vitamin C by GSH: Oxidised vitamin C, (acsorbyl radical, $Asc^{\cdot -}$) is reduced by GSH. Reaction 10: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) that uses NADPH as the electron donor. (see also reaction 3). Reaction 11: Lipid hydroperoxides can break down into aldehydes, such as the strong oxidant 4-hydroxynonenal. Reaction 12: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct. Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor.

significant decreases in GSH levels (47%) in rat liver microsomes [378]. Based on these studies, the following conclusions can be drawn: (i) GSH plays an important role in maintaining cellular redox status and its GSH level is considered a significant marker of oxidative stress. (ii) Glutathione levels are highly correlated with the cellular redox status induced by arsenic. (iii) Generally, there are three pathways that arsenic can decrease cellular levels of GSH. In the first pathway GSH possibly acts as an electron donor for the reduction of pentavalent to trivalent arsenicals. Secondly, arsenite has high affinity to GSH. The third pathway involves oxidation of GSH by arsenic-induced generation of free radicals. Taken together, exposure to arsenite is likely to cause depletion of GSH level. The various roles for GSH in the management of oxidative stress are outlined in Fig. (**4**).

The effect of ascorbic acid and α-tocopherol on arsenic-induced oxidative damage, antioxidant status and on xenobiotic metabolizing systems in arsenic-exposed rat liver and kidney microsomes [379] was studied. Arsenic exposure increases oxidative damage to lipids and proteins and decreases the levels of antioxidants and the activities of xenobiotic metabolizing enzymes. Co-administration of ascorbic acid and α-tocopherol to arsenic-exposed rats resulted in a reduction in the levels of lipid peroxidation, protein carbonyls and hydrogen peroxide and an elevation in the levels of reduced glutathione, ascorbic acid and α-tocopherol. The results provide evidence that ascorbic acid and α-tocopherol supplementation can improve the arsenic-induced altered microsomal functions in liver and kidney. Similarly a significant reversed dose-response relationship with arsenic-related ischemic heart disease was also observed for serum level of α- and β-carotene [380].

Therapeutic Effect of Arsenic in Cancer Treatment

As mentioned at the beginning of this section, in addition to its carcinogenic effects, arsenic compounds can be used as a medicine to treat acute promyelotic leukemia (APL). Using leukemia cells with genetic alterations in mitochondrial DNA and biochemical approaches, it was demonstrated [381] that As_2O_3, a clinically active anti-leukemia agent, inhibits mitochondrial respiratory function, increases free radical generation, and enhances the activity of superoxide generating agents against cultured leukemia cells and primary leukemia cells isolated from patients. This novel mechanism of action could provide a biochemical basis for developing new drug combination strategies using As_2O_3 to enhance the activity of anticancer agents by promoting generation of free radicals. The dual role of arsenic to act both as a carcinogen and also as a valuable therapeutic tool in cancer treatment may be presented as an arsenic paradox.

Of interest were recently published results on malignant APL-derived NB4 cells that can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system [382]. It was shown that the intracellular glutathione (GSH) content had a decisive effect on As_2O_3-induced apoptosis. As_2O_3-induced apoptosis was not enhanced by ascorbic acid in normal cells, suggesting that the combination of ascorbic acid and As_2O_3 may be selectively toxic to some malignant cells. It was concluded that As_2O_3 alone or administered with ascorbic acid may provide a novel therapy for lymphoma.

MERCURY

Mercury is the 80th element of the Periodic table of elements. Mercury is unique in that it is found in nature in several chemical and physical forms. At room temperature, elemental (or metallic) mercury exists as a liquid with a high vapor pressure and consequently is released into the environment as mercury vapor. Mercury also exists as a cation with an oxidation state of +1 (mercurous) or 2+ (mercuric). Of the organic forms of mercury, methylmercury is the most frequently encountered compound in the environment. It is formed mainly as the result of methylation of inorganic (mercuric) forms of mercury by microorganisms in soil and water.

In the environment, humans and animals are exposed to numerous chemical forms of mercury, including elemental mercury vapor (Hg), inorganic mercurous (Hg(I)), mercuric (Hg(II)) and organic mercuric compounds [383]. Environmental mercury is ubiquitous and consequently it is practically impossible for humans to avoid exposure to some form of mercury.

All forms have toxic effects in a number of organs, especially in the kidneys [384]. Elemental, inorganic, and organic forms of mercury exhibit toxicologic characteristics including neurotoxicity, nephrotoxicity, and gastrointestinal toxicity with ulceration and hemorrhage. Organic mercuric compounds possess a lesser degree of nephrotoxicity. Within the kidney, the *pars recta* of the proximal tubule is the most vulnerable segment of the nephron to the toxic effects of mercury [384]. The toxicological activity of mercurous and mercuric ions in the kidney is driven largely by the molecular interactions that occur at critical nucleophilic sites in, and closely target, cells. Therefore when considering the biological activity of mercuric ions in humans or other mammals, one must take into account the bonding properties of these ions. Mercuric ions have a greater affinity to bond to reduced sulfur atoms, especially those on endogenous thiol-containing molecules, such as glutathione, cysteine, metallothionein, homocysteine, *N*-acetylcysteine, and albumin [385]. The affinity constant for mercury bonding to thiolate anions is of the order of $10^{15} – 10^{20}$. For comparison, the affinity constants for mercury bonding to oxygen- (e.g. carbonyl) or nitrogen (e.g. amino)-containing ligands are about 12 orders of magnitude lower. Therefore the biological effects of inorganic or organic mercury is related to their interactions with sulfhydryl-containing residues. Molecular interactions of mercury with sulfhydryl groups in molecules of albumin, metallothionein, glutathione, and cysteine have been implicated in mechanisms involved in the proximal tubular uptake, accumulation, transport, and toxicity of mercuric ions [386].

The majority of the mercury present in plasma is bound to albumin and other large proteins. It is known that the organic anion transport system does not transport mercuric conjugates of proteins into proximal tubular epithelial cells. However, mercuric conjugates of low-molecular-weight ligands are the most likely species of mercury taken up at the basolateral membrane by the organic anion transporter [387].

Two conjugates, glutathione and/or cystein, have been implicated in the basolateral transport of Hg conjugates. Molecules of glutathione have a net negative charge at physiological pH. Because of this charge and its size, glutathione has been postulated to be substrate at the site of the organic anion transporter. On the other hand, cysteine has a net neutral charge at physiological pH, however, it also has been proposed to be highly relevant to consider that inorganic or organic mercuric conjugates of cysteine are transportable species at the site of the organic anion transporter. The relevance of this comes from studies in which organic *S*-conjugates of cysteine have been shown to be taken up at the basolateral membrane of proximal tubular cells by a mechanism consistent with the activity of the organic anion transporter [384].

In addition to the currently accepted models of mercuric conjugates of glutathione and cysteine being primarily involved in the luminal and basolateral uptake of inorganic mercury along the proximal tubule (after exposure to mercuric chloride), it is clear that other thiols, especially homologues of cysteine, such as homocysteine and *N*-acetylcysteine, can significantly influence the manner in which inorganic mercury is being handled in the kidney [388, 389].

Once inorganic mercuric ions gain entry into proximal tubular cells, it appears that they distribute throughout all intracellular pools [390, 391]. The cytosolic fraction was found to contain the greatest content of mercury. Interestingly, the relative specific content of mercury was shown to increase to the greatest extent in the lysosomal fraction when rats were made proteinuric with an aminoglycoside or when rats were treated chronically with mercuric chloride [392]. Increases in the lysosomal content of mercury may reflect the fusion of primary lysosomes with cytosolic vesicles containing complexes of inorganic mercury bound to proteins.

One of the main intracellular effects of mercury is the induction and binding to metallothionein [393]. Metallothioneins are small intracellular proteins with an approximate molecular weight of 6 to 7 kDa. They contain numerous cystein residues and have the capacity to bind various metals, including inorganic mercury, cadmium, zinc, copper, silver, and platinum. The administration of a single, daily, nontoxic dose of mercuric chloride over several days has been shown to cause a near doubling in the concentration of metallothionein in the renal cortex or outer stripe of the outer medulla in rats [394]. Enhanced synthesis of metallothionein in the kidney has also been demonstrated in rats exposed to mercury vapor over the course of several days [395]. Mercury vapor is converted into inorganic mercury, which is recovered predominantly (~ 98%) in the kidney, suggesting that the induction of metallothionein in the kidneys after exposure to elemental mercury may actually be mediated by inorganic mercury.

Mercury, Oxidative Stress and Antioxidants

Several *in vivo* and *in vitro* studies suggested that exposure of experimental animals to inorganic or organic forms of mercury is accompanied by the induction of oxidative stress. The high affinity of mercuric ions for binding to thiols naturally suggests that following depletion of intracellular thiols (especially glutathione) either directly or indirectly causes, or predisposes, proximal tubular cells to oxidative stress. Lund *et al*. [396] have demonstrated that the administration of mercury as Hg(II) in rats resulted in glutathione depletion and increased formation of H_2O_2 and lipid peroxidation in kidney mitochondria.

The *in vitro* effects of Hg(II) on hydrogen peroxide production by rat kidney mitochondria, a principal intracellular target of Hg(II) was also studied by Lund *et al*. [397] in mitochondria supplemented with a respiratory chain substrate (succinate or malate/glutamate) and an electron transport inhibitor (antimycin A (AA) or rotenone). Hg(II) increased H_2O_2 formation approximately 4-fold at the uniquinone-cytochrome b region (AA-inhibited) and 2-fold at the NADH dehydrogenase region (rotenone-inhibited). These results suggest that Hg(II), at low concentrations, depletes mitchondrial GSH and enhances H_2O_2 formation in kidney mitochondria under conditions of impaired respiratory chain electron transport. The increased H_2O_2 formation by Hg(II) may lead to oxidative tissue damage, such as lipid peroxidation, observed in mercury-induced nephrotoxicity.

The effects of mercuric chloride on lipid peroxidation (LPO), glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione (GSH) levels in different organs of mice (CD-1) were evaluated by Mahboob *et al*. [398]. These results indicated that Hg treatment enhanced lipid peroxidation in all tissues, but showed a significant enhancement only in kidney, testis and epididymus, suggesting that these organs were more susceptible to Hg toxicity. It was proposed that the increase in antioxidant enzyme levels in testis could be a mechanism protecting the cells against reactive oxygen species.

The effects of methylmercuric chloride on the rate of oxygen uptake were determined in purified cultures of oligodendrocytes, astrocytes, and cerebral cortical and cerebellar granular neurons obtained from embryonic and neonatal rat brains [399]. The results of this study revealed that mitochondria may be the earliest target of MeHg neurotoxicity and that the mitochondrial electron transport chain is the most likely site where an excess of reactive oxygen species are generated in the brain to induce oxidative stress in MeHg poisoning.

Fukino *et al*. reported [400] that, in addition to glutathione levels, the levels of other cellular antioxidants, including vitamin C and E have been reported to be depleted in the kidneys of rats treated with mercuric chloride. The correlation between mercuric-chloride toxicity and level of zinc was also investigated [401]. The effect of the administration of mercuric chloride on zinc deficiency in rats has been studied in the kidney of rats fed with either zinc-deficient (low-zinc, < 1.5 ppm) or zinc-supplemented diet (80 ppm) for 10 days. It was concluded that depletion of zinc affects various protective mechanisms of the organism and thus increases susceptibility to the toxic effects of mercuric chloride.

Aposhian *et al*. [402] tested the hypothesis as to whether GSH, vitamin C, or lipoic acid, alone or in combination with DMPS (2,3-dimercapto-1-propanesulfonate) or DMSA (meso-2,3-dimercaptosuccinic acid), would decrease brain mercury in young rats. The results show that none of these

regimens reduced the mercury content in the brain. Although DMPS or DMSA was effective in reducing kidney mercury concentrations, GSH, vitamin C, lipoic acid alone, or in combination were not. It was concluded that the palliative effect, if any, of GSH, vitamin C, or lipoic acid for the treatment of mercury toxicity due to mercury vapor exposure does not involve mercury mobilization from the brain and kidney.

The protective effect of vitamin E against mercuric chloride reproductive toxicity was investigated in male mice [403]. Animals given vitamin E with mercuric chloride had lower concentrations of mercury in the testis and epidimyis. Permitting animals to recover for 45 days after mercuric chloride treatment resulted in partial recovery of sperm and biochemical parameters. Vitamin E co-treatment has been shown to have a protective role against mercury-induced male reproductive toxicity.

The administration of mercuric chloride to male Sprague-Dawley rats was reported to cause marked decreases in the activity of superoxide dismutase, catalase, glutathione peroxidase, and glutathione disulfide reductase in the renal cortex [404]. Suppressed activities of these enzymes would be expected to enhance the susceptibility of renal epithelial cells to oxidative injury.

Some ambiguities have been addressed concerning the mechanism of mercury-induced oxidative injury. To resolve the problem as to whether mercury itself causes oxidative damage or that it makes cells more sensitive to species producing oxidative stress, several studies have been undertaken. It was found out that inorganic mercury can enhance the ability of other reagents to induce lipid peroxidation [404]. Mercury induced H_2O_2 production and lipid peroxidation was investigated *in vitro* in rat kidney [405], depending upon the supply of mercury and coupling site specificity, variable increases in the production of H_2O_2 were observed. Concentrations of 12 nmol mercury/mg protein completely depleted the content of glutathione in 30 min which suggests that depletion of mitochondrial glutathione is responsible for the intramitochondrial oxidative stress.

Mercury and Calcium

The importance of maintaining appropriate intracellular concentrations of calcium for proper cellular function is well documented. Inorganic mercury has been found to affect calcium homeostasis [406]. It was suggested that higher concentrations of inorganic mercury cause nonlethal effects in renal cells associated with redistribution of intracellular stores of calcium. The toxic effects of inorganic mercury are associated with changes in permeability of the plasma membrane. While treatment of cells with low concentrations (2.5–10 mM) of inorganic mercury produced 2- to 10-fold increases in the intracellular content of calcium, exposure of cells to higher concentrations (25–100 mM) produced an initial, rapid, 10- to 12-fold increase in intracellular calcium. The levels of calcium returned quickly to about twice those in control cells. Cytotoxicity was associated with this phase of increase in intracellular calcium and was dependent on the presence of extracellular calcium. The cytosolic content of calcium increased mainly from nonmitochondrial intracellular stores, presumably derived from the endoplasmic reticulum. The subsequent decrease in intracellular calcium may be due to buffering processes such as uptake through the microsomal Ca(II), Mg(II)-ATPase or through the mitochondrial uniporter.

The importance of cytosolic free calcium level ([Ca(II)]i) in lymphocyte activation was investigated through changes in [Ca(II)]i in T cells caused by two mercury compounds, methyl-mercury (MeHg) and inorganic mercury ($HgCl_2$), both showing immunomodulatory and immunotoxic properties [407]. Using fura-2 as fluorescent Ca(II) indicator it was shown that MeHg and $HgCl_2$ exert their effects on [Ca(II)]i in different ways: MeHg-induced increases in [Ca(II)]i are due to influx from outside the cells as well as to mobilization from intracellular stores, possibly the endoplasmic reticulum, and, to a minor extent, the mitochondria. On contrary $HgCl_2$ causes only Ca(II) influx from the extracellular medium.

Another study demonstrated that exposure of human neutrophils to $HgCl_2$ dose-dependently impairs chemoattractant-stimulated motility. Long-term exposure to Hg(II) yields a rapid influx of extracellular Ca(II) followed by leakage of cytosolic fluorophores, as assessed using fura-2 and ratio imaging microscopy [408]. The inhibition on motility was partly reversible, since pre-treated neutrophils placed in an Hg(II)-free environment displayed higher migration rates. The Hg(II)-induced fluxes were prevented by addition of small-sized polyethylene glycols, which also dose-dependently inhibited neutrophil transmigration. This study suggested an inverse effect of Hg(II) and small-sized polyethylene glycols on membrane permeability, while both impaired neutrophil cell motility.

Study of Kuo *et al.* [409] revealed that in addition to MeHg increasing [Ca(II)]i in the mouse peritoneal neutrophil, MeHg also potently decreased nitric oxide (NO) production and also the protein and mRNA levels of NO synthase induced by lipopolysaccharide. Both the L-type calcium channel blocker verapamil (1 μM) and H-89 (10 μM) can antagonize the inhibitory effect of MeHg (10 μM) on NO production. These findings lead to the conclusion that MeHg inhibits NO production mediated at least in part by Ca(II)-activated adenylate cyclase-cAMP-protein kinase A pathway.

Mercury and Cardiovascular Death

Nearly ten years ago, based on clinical trials undertaken in Finland [410], it was concluded that a high intake of mercury from nonfatty freshwater fish and the consequent accumulation of mercury in the body are associated with an excess risk of acute myocardial infarction as well as death from coronary heart disease, cardiovascular disease. The increased risk may be due to the promotion of lipid peroxidation by mercury. In agreement with the conclusions of this trial were two recent epidemiological studies exploring possible participation of mercury in myocardial infarction [411]. This trial was related to the fish intake, because fish is a major source of exposure to mercury. It is assumed that the mercury content of fish may counteract the beneficial effects of its n-3 fatty acids. In a case-control study conducted in eight European countries and Israel, the joint

association of mercury levels in toenail clippings and docosahexaenoic acid (C22: 6n-3, or DHA) levels in adipose tissue were evaluated and compared with the risk of a first myocardial infarction among men (n = 684). The controls were 724 selected men. The results led to the conclusion that the toenail mercury levels were directly associated with the risk of myocardial infarction, and the adipose-tissue DHA level was inversely associated with the risk. Thus a high mercury content may diminish the cardioprotective effect of fish intake. A similar trial was conducted in order to investigate the association between the serum n-3 end-product fatty acids docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and eicosapentaenoic acid and the risk of acute coronary events in middle-aged men (n = 1871) in Finland [412]. It was found that men ranked in the highest fifth of DHA+DPA levels who had a low hair content of mercury (less than or equal to 2.0 μg/g) had a 67% reduced risk (P=0.016) of acute coronary events compared with men in the lowest fifth who had a high hair content of mercury (>2.0 μg/g). There was no association between the proportion of eicosapentaenoic acid and the risk of acute coronary events. Based on these findings, and the previous study, it may be concluded that the assumption that fish oil-derived fatty acids reduce the risk of acute coronary events has a rational basis, however, a high mercury content in fish could attenuate this protective effect.

Mercury and Stress Proteins

It is well established that various stimuli, including toxic chemicals enhance the synthesis of a class of proteins known as stress proteins. This large superfamily of proteins, collectively referred to as stress proteins, include heat-shock proteins (hsps) and glucose-regulated proteins (grps). This particular stress-protein response has evolved as a cellular strategy to protect, repair, and chaperone other essential cellular proteins.

An enhanced *de novo* synthesis of 24-, 70-, and 90-kD, 70- and 90-kD, and 70-kD proteins was observed in chick embryos exposed to mercury [413]. The study to evaluate the differential expression of four hsps in the renal cortex and medulla during experimental nephrotoxic injury in Male Sprague-Dawley rats using $HgCl_2$ was conducted by Goering *et al*. [414]. In whole kidney, Hg(II) induced a time- and dose-related accumulation of hsp72 and grp94. Accumulation of hsp72 was predominantly localized in the cortex and not the medulla, while grp94 accumulated primarily in the medulla but not the cortex. The high, constitutive expression of hsp73 did not change as a result of Hg(II) exposure, and it was equally localized in both the cortex and medulla. Hsp90 was not detected in kidneys of control or Hg-treated rats.

The results demonstrate that expression of specific stress proteins in rat kidney exhibits regional heterogeneity in response to Hg(II) exposure, and a positive correlation exists between accumulation of some stress proteins and acute renal cell injury. While the role of accumulation of hsps and other stress proteins *in vivo* prior to, or concurrent with, nephrotoxicity remains to be completely understood, these stress proteins may be part of a cellular defense response to nephrotoxicants. It has also been proposed that renal tubular epithelial cells that do not, or are unable to, express stress proteins, such as hsp72, may be more susceptible to nephrotoxicity.

ZINC

Zinc is the 30th element of the Periodic table of elements. The most common and most stable oxidation number of zinc is +2 [Zn(II)]. Zinc is a ubiquitous trace element found in plants and animals. The adult human body contains approximately 1.5 to 2.5 grams of zinc, present in all organs, tissues, fluids and secretions. The level of free intracellular Zn(II) is as low as 0.5nM, as estimated from measurements of the zinc-specific ^{19}F-NMR signal of a fluorinated metal chelating probe [415]. About 90% of total body zinc is found in skeletal muscle and bone, with much smaller amounts in the liver, gastrointestinal tract, skin, kidney, brain, lung, prostate and other organs [416].

Zinc plays as essential role in cell membrane integrity, it helps manage insulin action and blood glucose concentration and has an essential role in the development and maintenance of the body's immune system. Zinc is also required for bone and teeth mineralization, normal taste and wound healing. Zinc is a component of more than 70 different enzymes that function in many aspects of cellular metabolism, involving metabolism of proteins, lipids and carbohydrates [417]. The functions of zinc comprise the stabilization of conformation in transcription factors. Zinc also has been found to modulate cellular signal transduction processes and even to function as a modulator of synaptic neurotransmission in the case of the zinc-containing neurons in the forebrain.

Zinc deficiency is related to poor dietary zinc intake, excessive dietary phytate intake, chronic illness or over-supplementation with iron or copper [418]. Incidence of zinc deficiency in well-nourished humans is unknown due to difficulties in sufficiently diagnosing zinc deficiency and the diversity of its metabolic roles. Symptoms of zinc deficiency include poor growth and development, appetite loss, dermatitis, hypogonadism, alopecia, reduced taste acuity, delayed wound healing, impaired reproduction and poor immune function [419]. Severe zinc deficiency is rare and usually caused by genetic or acquired conditions. Acrodermatitis enteropathica is an autosomal recessive inherited condition of severe zinc deficiency. Zinc deficiency or chelation caused impaired availability of growth hormones and also led to decreased secretion of growth hormone from the pituitary gland in rats [420].

Prasad and coworkers [421] found, in the early 60s, that low zinc levels in blood were causally related to a rare condition of dwarfism, testicular retardation, and susceptibility to infections in a group of patients who, although not genetically related, were alike in having a diet that produced zinc deficiency. Since then zinc is considered to be an essential component of the human diet. In general, about 20% of zinc consumed from a usual mixed diet is absorbed. Unlike other trace elements, zinc does not accumulate in the body to form permanent stores. Processing of certain foods, mainly heat treatment may affect amount of zinc content that is available for absorption.

Acute zinc toxicity is rare but has been reported. Acute zinc toxicity has occurred as a result of food and beverage intake contaminated with zinc from galvanized containers [422]. Intakes of 2 g or more of zinc sulfate can cause GI irritation. Other acute toxicity symptoms include metallic taste, nausea, vomiting, lethargy, fatigue, diarrhea, muscle pain and fever. Chronic zinc toxicity (100-300 mg/day) is more common than acute toxicity and may retard immune function. This type of toxicity occurs from self-supplementation or prolonged use of oral zinc supplements for medicinal purposes. Excess zinc may also effect copper and iron status and lower plasma HDL concentrations. Zinc is excreted mostly in feces (12–15 mg/day) and lesser amounts (0.5 mg/day) are eliminated in urine. Hyperzincuria occurs in active hepatic disease, sickle cell anemia, and chronic renal disease [423].

Zinc Homeostasis

Since there is increasing evidence that zinc ions are involved as inter- and intra-cellular messengers, the homeostasis of zinc has to be controlled tightly [424]. Cells have to take up zinc actively to secure the supply for structural and catalytic functions of zinc proteins even under conditions of very low environmental concentrations of this element. Elevated cellular concentrations of zinc are toxic, and accumulation of free zinc ions has to be avoided by pumping them out of the cell into storing vesicles (zincosomes) or by binding them tightly to metallothionenin. Transport of Zn(II) in eukaryotes is mediated by a family of transmembrane proteins termed ZIP (Zinc-Iron related transporter Proteins). ZIP proteins are located in the plasma membrane and form an eight transmembrane domain channel [425]. These channels are involved in the regulation of intracellular zinc level. The efflux of Zn(II) *via* plasma membranes is exerted by specific zinc transporters when the level of intracellular zinc is exceeded or when zinc has to be transported through cells.

Zinc and Signalling, Proliferation and Differentiation of Cells

Zinc is involved in extracellular signal recognition, second messenger metabolism, protein phosphorylation and dephosphorylation and also the activity of transcription factors.

Zinc interaction with second messenger metabolism has been studied [424]. This involves interference of zinc with different aspects of calcium regulation and cyclic nucleotide metabolism. Free calcium is one of the major second messengers in the regulation of many physiological processes and both cell proliferation and cell death. Electrical stimulation of heart cells evoked influx of Zn(II) through voltage-dependent Ca(II) channels [426]. It was also reported that in hepatocytes elevated zinc increased intracellular Ca(II) by stimulation of hormone-sensitive intracellular calcium stores [427].

Zinc also interacts with the metabolism of cyclic nucleotides. Zinc has been shown to mediate second messenger cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by modulating cyclic nucleotide phosphodiesterase (PDE) activities. PDE is activated at lower zinc concentrations (up to 1 μM) and inhibited at higher zinc concentrations (above 1 μM). Zinc was also found to affect cellular cGMP concentration. Elevation of zinc results in a raise in cGMP which deactivated further import of zinc [428].

Zinc is also known to regulate protein kinase C (PKC) and cause translocation of PKC to cytoskeleton. Elevated extracellular zinc concentrations stimulated protein tyrosine phosphorylation and MAPK (mitogen-activated protein kinases) activity in cells [429]. A synergistic effect of zinc and calcium on MAP kinases, DNA synthesis and mitogenic signalling was detected in NIH3T3 cells [430].

All these experiments suggest that zinc stimulates mitogenic signalling. Receptor tyrosine kinases seem to be stimulated by extracellular zinc in murine fibrobalsts and in human bronchial epithelial cells [429].

From the analysis of gene sequencing it has been estimated that zinc is an element of more than a thousand transcription factors containing zinc finger domains [431]. Cells grown in media containing a low content of Zn (0.5 or 5.0 μM) were characterized by high levels of oxidative stress, however, cells grown in the 50 μM Zn media had normal levels of oxidative stress. The level of oxidative stress caused by zinc deficiency induced changes in the intracellular redox status of the cell that triggered the activation of oxidant-sensitive transcription factors. Whereas levels of activated AP-1 was markedly higher in the 0.5 and 5 μM group, the NF-κB binding activity was lower in the 0.5 and 5 μM Zn cells than in controls [432]. The most characteristic zinc-activated transcription factor is the metal response element-binding transcription factor-1 (MTF-1) which induces the metallothionein and zinc transporter ZnT-1 genes in response to cellular zinc. MTF-1 is composed of six finger structures and the binding of zinc to first low-affinity site results in translocation of MTF-1 from the cytoplasm to the nucleus [433, 434].

Zinc is not only a structural element of proteins involved in cell proliferation but it is also involved in growth regulation. Zinc deprivation by chelation or starvation causes cells to die, in many cell types by programmed cell death (apoptosis) [434]. On the other hand, if the extracellular concentration of zinc exceeds the capacity of the zinc homeostasis, it becomes cytotoxic and resulting enhanced intracellular zinc concentration trigger the activation of apoptosis [424]. At very high concentration of zinc the predominant form of cell death is necrosis [435].

Zinc and its Antioxidant and Immune Functions

The mechanism of antioxidant action of zinc can be divided into chronic and acute effects. Whereas chronic exposure of an organism to zinc on a long-term basis results in enhanced synthesis of metallothioneins, chronic zinc deprivation generally results in increased sensitivity to some oxidative stress [436].

Zinc is a redox inert metal and does not participate in oxidation-reduction reactions. The acute effect of zinc's function as an antioxidant was first proposed late 80's and involves two distinct mechanisms: (i) the protection of

sulfhydryl groups of proteins and enzymes against free radical attack, or oxidation, and (ii) reduction of •OH formation from H_2O_2 through the prevention of free radical formation or in other words antagonism of redox-active transition metals, such as iron and copper [419].

As mentioned above chronic administration of zinc is an example of the indirect effect of zinc. The beneficial effects of long-term administration of zinc can be linked to the induction of some other species that serves as the ultimate antioxidants, the most effective seems to be metallothioneins. The metallothioneins are metal-binding proteins (6000–7000 kDa) containing 60–68 amino acid residues. About 25–30% of all aminoacids are cysteine, containing no aromatic amino acids or disulfide bonds and therefore can effectively bind 5–7 g zinc (mol/protein) [436]. Recent studies proposed that the metallothioneins represent a connection between cellular zinc and the redox state of the cell. Under conditions of high oxidative stress, changes in the cellular redox state result in release of zinc from metallothionein as a result of sulfide/disulfide exchange [437].

Zinc deficiency has been associated with higher than normal levels of tissue oxidative damage including, increased lipid [438], protein and DNA oxidation [432]. Several experiments on animals confirmed that chronic or long-term deprivation of zinc makes an organism more susceptible to oxidative stress-induced injury. Zinc deficiency effects, linked with formation of ROS, has been documented by hyperoxic lung damage [439], conjugated dienes and malondialdehyde formation in liver microsomes [440], carbon-centered free radical production in lung microsomes [441], induced lipid peroxidation in liver microsomes and mitochondria [442], sensitivity to copper-mediated lipoprotein oxidation [443] and galactosamine-induced hepatitis in rats [444].

The effects of zinc deficiency on oxidative damage to testes proteins, lipids and DNA, in rats was also investigated [445]. Testes from rats fed a low zinc diet had lower glutamine synthetase activity, lower Fe(II)-stimulated 2-thiobarbituric acid-reactive substances (TEARS) production, higher protein carbonyl concentrations, and higher 8-oxo-2'-deoxyguanosine levels (8-OH-dG). Testes iron concentrations were higher in the zinc-deficient rats. The oxidative damage observed was explained as a consequence of increased reactive oxygen species generation secondary to tissue iron accumulation and/or reductions in zinc-dependent antioxidant processes.

As already mentioned above, the acute antioxidant effect of zinc is described by two distinct mechanisms: (i) the protection of sulfhydryl groups of proteins and enzymes against free radical attack; (ii) that zinc functions as an antioxidant through the prevention or antagonism of redox active transition metals. Sulfhydryl stabilization means the protection of sulfhydryl groups of certain enzymes from oxidation. The most studied enzyme was δ-aminolevulinate dehydratase which catalyzes the formation of the pyrrole porphobilinogen from two molecules of δ-aminolevulinic acid. Based on these studies Gibbs *et al*. [446] suggested three structural possibilities which may explain the stabilization of sulfhydryl groups. The first model considered direct binding of zinc to the sulfhydryl groups, second model assume binding of zinc to a binding site close to the sulfhydryl groups and finally the third assumed binding of zinc to another site of the protein resulting in a conformational change of the protein. Any of these models result in a decreased reactivity of sulfhydryl groups. Zinc was found to protect the following sulfhydryl-containing proteins: dihydroorotase [447], DNA zinc-binding proteins (zinc fingers) [448], alanyl tRNA synthetase [449], class I tRNA synthetases [450], 5-Enolpyruvylshikimate-3-phosphate synthase [451], *E. coli* DNA topoisomerase I [452] and protein farnesyltransferase [453].

The second mechanism involves antagonism of zinc in redox active transition metals. It is well established that in the process of protein oxidation, oxidative modifications occur predominantly around the metal binding site. This is an example of site-specific reactions [454]. The pool of redox-active transition metals (copper, iron) is associated with certain cellular components at which the cyclic production of ·OH occur. Two potential mechanisms that would antagonize (or prevent) the formation of ·OH or possibly relocate the site of formation to one less critical have been proposed. The first process involves removal or "pull" of the metal from its binding site through the use of a high-affinity ligand-chelator. The second process is consider to "push" the redox metal off of its binding site through replacement by chemically (isostructurally) similar redox-inactive metal (e.g. copper by zinc). The displaced redox metal can then be washed out of the cell, reducing thus bioavailability of the metal to participate in ·OH formation *via* the Fenton reaction. Antagonism of free radical formation was demonstrated by EPR spin-trapped ·OH from iron and cysteine in the presence of zinc, suggesting that competitive reaction between two metals for the thiol amino acid interfered with transfer of electrons to oxygen [455]. Zinc also antagonized iron-mediated xanthine/xanthine oxidase-induced peroxidation of erythrocyte membranes [456]. Antagonism of radical formation by zinc was reported in copper-iron ascorbate-induced DNA strand breaks [457], superoxide and ·OH from xanthine oxidase and NADPH oxidase [436], Fe(III)-ascorbate-induced methemoglobin formation in red blood cells [458] and other systems [436].

Zinc also has significant cardioprotective effects. *In vitro* and *in vivo* studies demonstrated that zinc has an inhibitory effect on isoproteronol-induced cardiac oxidative injury [459]. A series of detailed studies describing the cardioprotective effects of zinc-bishistidinate in several *in vitro* and *in vivo* models of cardiac ischemic injury has been published [437]. Several studies provided unequivocal evidence documenting the presence of various reactive oxygen intermediates in tissue, blood or perfusates from postischemic organs. The effect of radicals in ischemic and postischemic injury was decreased by zinc [460]. The improvements in postischemic function is associated with decreased levels of ·OH and alterations in cardiac copper content (increased excretion) and provides a reasonable basis for the theory that zinc is cardioprotective as a result of inhibition of transition metal–mediated oxidative stress.

Zinc also acts as an antioxidant in the central nervous system, particularly the brain [461]. Compared to other soft tissues, the human brain contains significant amounts of zinc. Among the essential trace elements, zinc is second only to iron in total concentration in the brain. Zinc

deficiency has been proposed to lead to nervous system disorders, including mental disturbances, loss of sensory acuity, and impaired cognitive and psychological function. Oxidative stress is associated with the development and progression of several different neuropathological diseases, including Alzheimer's disease and Parkinson's disease [462]; see below.

Some studies examined the role of zinc in maintaining the integrity of the blood brain barrier (BBB), which is the highly specialized blood vessel system of the central nervous system that serves to protect the brain by excluding toxic agents and other foreign compounds. The high polyunsaturated fatty acid content of the BBB membrane is very susceptible to free radical attack. Therefore the effect of zinc as protective antioxidant was investigated. Using magnetic resonance imaging, Noceworthy and Bray [463] have demonstrated that zinc deficiency in rats dramatically increases the permeability, or leakiness, of the BBB. It was also observed that when zinc deficiency is accompanied by oxidative stress, as might occur during a bacterial or viral infection, BBB permeability increases dramatically. These findings led these authors to hypothesize that under normal conditions, zinc protects the BBB against oxidative stress through its antioxidant properties and prevent the development of neurological disorders.

The role zinc is closely linked with the immune system. Zinc specifically interacts with components of the immune system and the important role of zinc as an essential trace element for immune function has already been well established [464]. Decreased zinc absorption rapidly leads to immune deficiency and to the development of life threatening infections [465], for example human acrodermatitis enteropathica. Infants born with this condition developed skin lesions, serious diarrhea, hair loss, and became very sick. All of these symptoms could be resolved by giving intravenous zinc to replete zinc stores [466]. The main organ affected was the thymus, which is known as a "barometer of nutrition" because children dying from infections associated with protein calorie malnutrition were found to have little thymic tissue. The cells, called "precursor" cells, of the immune system arise in the bone marrow and, after circulating through the thymus, emerge as active "Thymus-cells", or "T" cells. Babies with low zinc levels had poor thymic development which led to reduced and weak T cells which were not able to recognize and fight off certain infections.

The field of zinc immunology is focused on the interaction of zinc with human leukocytes on a cellular and molecular basis, the influence on immunostimulants and the therapeutic use of zinc. Zinc has been successfully used to restore impaired immune functions of various syndromes [467]. A major and unresolved question is the optimal therapeutic dosage of zinc. The limiting concentration of zinc in plasma should not exceed 30 mmol/L.

Recent studies have revealed diminished plasma zinc levels in patients suffering rheumatoid arthritis, an autoreactive T-cell disease [468]. Oral application of zinc sulfate over a 12-weeks period has shown a clear clinical benefit [469]. This effect was correlated with the T-cell inhibitory influence of zinc. Zinc deficiency causes thymic atrophy, and the thymus changes are reversible by zinc supplementation, confirming that zinc interferes with the earliest steps of T-cell maturation. The presence of serum zinc also induce T cells to produce lymphokines. This effect on T cells is mediated by cytokines produced by monocytes. Low zinc serum levels were correlated with decreased production of TH1 cytokines and interferon-α by leukocytes in healthy elderly persons. The defect in interferon-α production is reconstituted by the addition physiologic amounts of zinc *in vitro*. Zinc complexes were found to have anti-convulsant activities, suggesting possible protection against radiation-induced seizures [470].

The zinc has a number of effects on leukocytes *in vivo* and *in vitro* [464]. The interaction within the immune system is complex and delicately regulated by zinc. Whereas zinc deficiency leads to dysfunction of the immune system, high doses of zinc have negative effects on leukocyte functions. Although knowledge about the molecular mechanisms of zinc has increased during the past years, it is still not know the most effective therapeutic dosage. From *in vitro* studies, zinc levels of >30 mM were found to have more inhibiting than stimulating effects on the immune system. However, these inhibiting effects might be useful as a new therapeutic tool. Because most experimental systems in immunologic research depend on the stimulation of leukocytes *in vivo* or *in vitro*, the modulation of immunostimulants by zinc is a trap. Zinc-specific alteration of the activity of stimulants might mimic effects on the immune system.

Neuroprotective or Neurodegenerative Role of Zinc in Alzheimer's Diseases

As mentioned in the section "Copper and neurodegenerative disorders" outlined above, the main feature of AD is a marked accumulation of amyloid-β peptide (Aβ), the main constituent of senile plaques in brain, as well as deposition of neurofibrillary tangles and neurophil threads [471]. The Aβ peptide toxicity depends on its conformational state and peptide length. It is known, that Aβ aggregates into two different conformational states: (i) the non-β-sheet, an amorphous, nonfibrillar, state and (ii) the β-sheet, a highly ordered, fibrillar, state. While the non-β-sheet is benign, the highly ordered, fibrillar Aβ, is cytotoxic. The aggregated state and structure of Aβ peptide are influenced by the concentration of peptide, pH, and ionic concentration of zinc, copper and iron. The neurotoxicity of Aβ depends also on peptide length, with Aβ(1-42) being more toxic than Aβ(1-40). Aβ(1-42) is the most likely candidate to generate hydrogen peroxide and other reactive species.

The role of zinc in the etiology of AD is very questionable. A growing number of reports indicate, that zinc in micromolar concentration inhibits Aβ-induced toxicity. The exact mechanisms of the protective effect of zinc against Aβ toxicity is unclear, however one of the reasons might be cytoprotection through blockage of the membrane calcium channel pore formed by Aβ(1-40).

Zinc and copper have a clear relationship in the context of AD [128]. The argument advocating protective role of zinc is its competition with copper (or iron) to bind Aβ. Zinc binding to Aβ changes its conformation to the extent that copper ions cannot reach its metal-binding sites. Preventing

copper from interacting with Aβ may preclude the Cu-Aβ induced formation of hydrogen peroxide and free radicals.

On the other hand a trigger caused by endogenous (genetic) and exogenous (e.g. environmental) factors results in oxidative and nitrosative stress which in turn leads to abnormal metabolism of Aβ accompanied by uncontrolled flooding of the vesicular zinc pool [472]. Thus, while low levels of zinc protect against Aβ toxicity, the excess of zinc released by oxidants could trigger neuronal death that is independent or even synergistic with the toxic effect of Aβ. This conclusion is in agreement with other studies documenting that at higher concentrations of zinc its binding to Aβ force the Aβ to precipitate over a wide range of pH (6-8) [471]. Zinc binding has been found to preserve the α-helical conformation of Aβ(1-40) and highly ordered conformational state of Aβ(1-40) upon binding of zinc has been interpreted as producing toxic, fibrillar, Aβ aggregates. Consequently, immunological/inflammatory responses to nonsoluble Aβ plagues is disruption of zinc homeostasis followed by uncontrolled cerebral zinc release, typical for oxidative stress.

It can be hypothesized that under normal physiological conditions a sensitive balance exists between zinc, copper and Aβ metabolism. However, oxidative and nitrosative stress may perturb this balance which leads to uncontrolled zinc elevation and amyloid deposition. Uncontrolled accumulation of zinc or Aβ may lead to zinc-induced and Aβ-mediated oxidative stress and cytotoxicity.

CONCLUSION

The above discussion provides an insight into the role of metal-induced toxicity and carcinogenesis. The results provide evidence that toxic and carcinogenic metals are capable of interacting with nuclear proteins and DNA causing site-specific damage. The "direct" damage may involve conformational changes to biomolecules due to the coordinated metal while "indirect" damage is a consequence of metal-driven formation of reactive oxygen species and reactive nitrogen species involving the superoxide and hydroxyl radicals, nitric oxide, hydrogen peroxide and/or other endogenous oxidants.

Metals are known to activate signalling pathways. Cadmium related carcinogenicity studies explored induction of immediate early response genes (IEGs), stress response genes and activation of transcription factors. The carcinogenic effect of certain other metals has been related to activation of redox-sensitive transcription factors. These involve NF-κB, AP-1 and p53. Chromium(VI) was found to induce activation of NF-κB *via* Cr(V)/Cr(VI)-mediated generation of hydroxyl radicals. Chromium(VI) species also stimulated AP-1. For example nickel-induced allergic responses and skin hypersensitivity were related to activation of NF-κB. Nickel was also found to induce hypoxia-inducible factor I (HIF-1) and mutations in p53. Formation of free radicals as a result of metal toxicity can cause DNA damage, lipid peroxidation, and alter sulfhydryl homeostasis. Also alterations in metal-mediated calcium homeostasis have been reported as a result of membrane damage, leading to activation of various calcium-dependent systems including endonucleases. The exact role of calcium and its relation to intracellular transition metal ions, in particular iron, however, needs to be further investigated.

Metal-induced formation of free radicals has most significantly been evidenced for iron and copper (both essential elements) then for nickel, chromium, and cadmium, all three well-known carcinogenic metals.

Common mechanisms involving Fenton generation of the superoxide and hydroxyl radical appear to be involved for iron, copper, chromium, vanadium and cobalt (Me = metal)

$$Me^{(n+1)+} + O_2^{\bullet -} \rightarrow Me^{n+} + O_2 \quad (27)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (28)$$

$$Me^{n+} + H_2O_2 \rightarrow Me^{(n+1)+} + {}^{\bullet}OH + OH^- \text{ (Fenton reaction)} \quad (29)$$

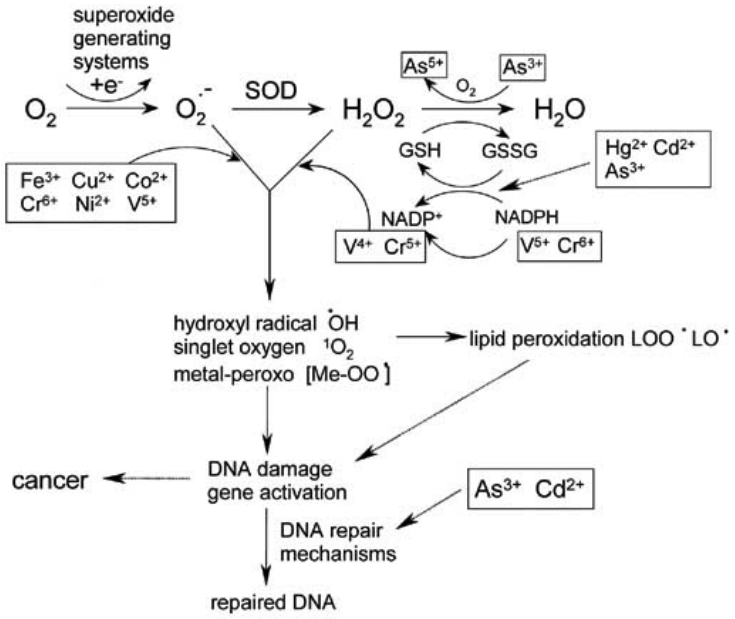


Fig. (5). Pathways of metal-induced oxidative stress.

Fenton reactions are predominantly associated with mitochondria, microsomes and peroxisomes. Considering copper as a metal participating in the Fenton chemistry *in vivo*, one should bear in mind a recent discovery that upper limit of "free" pools of copper is far less than a single atom per cell. This suggests a significant overcapacity for chelation of copper in the cell.

Studies with other groups of metals, involving mercury, cadmium, nickel, revealed that the primary route for their toxicity is depletion of glutathione and bonding to sulfhydryl groups of proteins. Arsenic is also known to bind directly to critical thiols, however, another mechanism involving the formation of hydrogen peroxide by oxidation of As(III) to As(V) under physiological conditions has been proposed. Nitric oxide seems to be involved in arsenite-induced DNA damage and pyrimidine excision inhibition.

The most convincing link between oxidative damage and carcinogenesis comes from mutagenecity of DNA base modifications resulting from the attack of metal-mediatedfree radicals. Metal-mediated formation of free radicals causes a variety of modifications to DNA bases. Since many of these DNA base modifications are pro-mutagenic, there is a strong link between oxidative damage and the carcinogenesis of metals. Certain carcinogenic metals, including Cd, As, Ni are known to inhibit DNA repair mechanisms. The major oxidative effects in DNA linked with exposure of cells and experimental animals to toxic metals include base modification (Cr, Ni), cross-linking (Ni, Fe and oxidant, Cu and oxidant), strand scission (Cd, Ni, Cr and oxidant), and depurination (Cr, Cu, Ni). Various pathways for metal-induced oxidative stress and metal-carcinogenesis are outlined in Fig. (**5**).

The ability of zinc as an antioxidant to reduce oxidative stress may be due to (i) protection of sulfhydryl groups of proteins and enzymes against oxidation or (ii) reduction of hydroxyl radical formation from hydrogen peroxide through the prevention of free radical formation (antagonism of redox-active transition metals). Zinc also interacts with components of immune system. The role of zinc in the etiology of Alzheimer's diseases is very questionable; it has been proposed, that uncontrolled accumulation of zinc and amyloid-β peptide (Aβ) may lead to zinc-induced and Aβ-mediated oxidative stress and cytotoxicity.

Various antioxidants (both enzymatic and non-enzymatic) provide protection against deleterious metal-mediated free radical attacks. Generally, antioxidants can protect against iron toxicity by (i) chelating ferrous ion and preventing the reaction with molecular oxygen or peroxides, (ii) chelating iron and maintaining it in a redox state that makes iron unable to reduce molecular oxygen and (iii) trapping any radicals formed. One of the most effective classes of antioxidants are thiol compounds, especially glutathione, which provide significant protection by trapping radicals, reduce peroxides and maintain the redox state of the cell. The non-enzymatic antioxidant vitamin E can prevent the majority of metal (iron, copper, cadmium)-mediated damage both *in vitro* systems and in metal (iron, copper, cadmium)-loaded animals. However, a very recent epidemiological study has shown that daily intake of vitamin E in doses of more than 400 IU increase the risk of death and should be avoided. Toxicity studies involving chromium have shown that the protective effect of vitamin E against lipid peroxidation may be associated rather with the level of non-enzymatic antioxidants than the activity of enzymatic antioxidants.

While several *in vitro* studies proposed deleterious pro-oxidant effect of ascorbate in the presence of iron or copper, several other studies confirmed that, both in animal models and in humans, no evidence of increased oxidative damage to DNA due to the combined effect of iron (or copper) and ascorbate is observed. The results have shown that even in the presence of redox-active iron (or copper) and hydrogen peroxide ascorbate acts as an antioxidant that prevents lipid peroxidation and does not promote protein oxidation in humans *in vitro*.

In conclusion, the present stage of knowledge of the role of metals in biological systems indicate that enhanced formation of free radicals and other reactive species can be regarded as a common factor in determining metal-induced toxicity and carcinogenicity.

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ABBREVIATIONS

OFR	=	Oxygen free radicals
ROS	=	Reactive oxygen species
NF-κB	=	Nuclear factor kappa B
AP-1,	=	Activator protein-1
MDA	=	Malondialdehyd
HNE	=	4-hydroxynonenal
LDL	=	Low-density lipoprotein
HDL	=	High-density lipoprotein
Aβ	=	Amyloid-β peptide
MTF-1	=	Metal response element-binding transcription factor
8-OH-dG	=	8-hydroxyl-2'deoxyguanosine
HIF-1	=	Hypoxia-inducible factor 1 alpha
TNF- α	=	Tumour necrosis factor- α
PKC	=	Protein kinase C
Hsp	=	Heat shock protein
Asc	=	Ascorbate
SOD	=	Superoxide dismutase
LIP	=	Labile iron pool
EDTA	=	Ethylenediaminetetraacetic acid
NTA	=	Nitrilotriacetic acid

PUFA = Polyunsaturated fatty acids

LPO = Lipoperoxidation

TBARS = Thiobarbituric acid reactive species

EPR = Electron Paramagnetic Resonance

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