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Role of oxygen radicals in DNA damage and cancer incidence

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Abstract

The development of cancer in humans and animals is a multistep process. The complex series of cellular and molecular changes participating in cancer development are mediated by a diversity of endogenous and exogenous stimuli. One type of endogenous damage is that arising from intermediates of oxygen (dioxygen) reduction – oxygen-free radicals (OFR), which attacks not only the bases but also the deoxyribosyl backbone of DNA. Thanks to improvements in analytical techniques, a major achievement in the understanding of carcinogenesis in the past two decades has been the identification and quantification of various adducts of OFR with DNA. OFR are also known to attack other cellular components such as lipids, leaving behind reactive species that in turn can couple to DNA bases. Endogenous DNA lesions are genotoxic and induce mutations. The most extensively studied lesion is the formation of 8-OH-dG. This lesion is important because it is relatively easily formed and is mutagenic and therefore is a potential biomarker of carcinogenesis. Mutations that may arise from formation of 8-OH-dG involve GC → TA transversions. In view of these findings, OFR are considered as an important class of carcinogens. The effect of OFR is balanced by the antioxidant action of non-enzymatic antioxidants as well as antioxidant enzymes. Non-enzymatic antioxidants involve vitamin C, vitamin E, carotenoids (CAR), selenium and others. However, under certain conditions, some antioxidants can also exhibit a pro-oxidant mechanism of action. For example, β-carotene at high concentration and with increased partial pressure of dioxygen is known to behave as a pro-oxidant. Some concerns have also been raised over the potentially deleterious transition metal ion-mediated (iron, copper) pro-oxidant effect of vitamin C. Clinical studies mapping the effect of preventive antioxidants have shown surprisingly little or no effect on cancer incidence. The epidemiological trials together with *in vitro* experiments suggest that the optimal approach is to reduce endogenous and exogenous sources of oxidative stress, rather than increase intake of anti-oxidants. In this review, we highlight some major achievements in the study of DNA damage caused by OFR and the role in carcinogenesis played by oxidatively damaged DNA. The protective effect of antioxidants against free radicals is also discussed. (Mol Cell Biochem **266**: 37–56, 2004)

Key words: radicals, DNA damage, cancer incidence, antioxidants, pro-oxidants, carotenoids, oxidative stress, review

Abbreviations: OFR – oxygen-free radicals; ROS – reactive oxygen species; XO – xanthine oxidase; EPR – Electron Paramagnetic Resonance; MDA – malondialdehyde; GSH – glutathione; NADH – nicotineamide adenine dinucleotide; AFR – ascorbate-free radical; LDL – low-density lipoprotein; 8-oxoG – 8-oxoguanine; CAR – carotenoids; FAPy-G – 2,6-diamino-5-formamido-4-hydroxypyrimidine

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Introduction

Oxygen, while indisputably essential for life, can also participate in the destruction of tissue and/or impair its ability to function normally [1, 2]. Oxygen-free radicals (OFR), or more generally, reactive oxygen species (ROS) are products of normal cellular metabolism. It has been estimated that the average person has around 10,000–20,000 free radicals attacking each body cell each day. For an over-trained athlete, this figure can be increased by roughly 50%.

In some cases, ROS are produced specifically to serve essential biological functions, whereas in other cases, they represent byproducts of metabolic processes [3]. Despite the cell's antioxidant defence system to counteract oxidative damage from ORF, radical-related damage of DNA and proteins have been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and others [4, 5]. All ROS have the potential to interact with cellular components including DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases or strand breaks [6]. Oxygen radicals can also oxidise lipids or proteins thus generating intermediates that react with DNA by forming adducts [7]. Some oxidative DNA lesions are promutagenic and oxidative damage is proposed to play a role in the development of certain cancers [8].

Electronic structure of oxygen radicals

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals [9]. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems [10].

Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. In the ground state, it is a biradical with two parallel unpaired electrons in antibonding π^* orbitals thus forming a triplet state molecule [11]. These two unpaired electrons give oxygen a spin quantum number (S) of one ($S = 1$, each unpaired electron contributing 1/2) and a spin multiplicity of three ($2S + 1 = 3$), that is, dioxygen is a triplet molecule [12]. Valence bond theory describes dioxygen as a double bond species (O=O), however molecular orbital (MO) theory predicts that dioxygen is a biradical ($^{\bullet}O{\equiv}O^{\bullet}$), a structure which better accounts for the reactivity of dioxygen with other radical molecules [12].

In addition to the triplet state of dioxygen (denoted $^3\Sigma_g^-$) it also exists in two singlet states, denoted as $^1\Delta_g$ and $^1\Sigma_g^+$, higher in energy than the ground state by 23.4 and 37.5 kcal/mol, respectively (see Fig. 1). The lifetime of $^1\Sigma_g^+$ is extremely short and it rapidly interconverts to $^1\Delta_g$, which is probably the most relevant source of singlet dioxygen in biological systems [11].

The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$) (Fig. 1). This electron fills one of the π^* orbitals. The addition of another electron to the second π^*orbital forms peroxide dianion (O_2^{2-}), which has all electrons paired and therefore is not an oxygen radical [11].

The kinetic stability of dioxygen is explained by its electronic configuration. As mentioned above, in the ground state the π^* electrons are parallel. Therefore, when oxygen reacts with an atom or a molecule, the substrate also must possess parallel spins. However, electron pairs in most atoms and molecular bonds contain antiparallel electrons, therefore, the oxidising ability of molecular oxygen is reduced. A second reason for the reduced oxidative ability of dioxygen is the thermodynamic disadvantage of the one-electron reduction of oxygen ($O_2 + e^- \rightarrow O_2^{\bullet-}$) versus the two electron reduction ($O_2 + 2e^- \rightarrow O_2^{2-}$), which fills both π^* orbitals [13, 14]. The thermodynamic parameters for the reduction of dioxygen molecule are summarized in Table 1 and the four single-electron reduction steps from molecular oxygen to water are shown in Fig. 2.

Sources and properties of oxygen radicals

Superoxide radical

With the exception of unusual circumstances such as ionising radiation, ultraviolet light, and other forms of high energy exposure, free radicals are produced in cells generally by electron transfer reactions, which can be enzymatically mediated or non-enzymatically mediated. Cellular sites of superoxide generation and its compartmentalization is shown in Fig. 3. The major source of free radicals under normal circumstances is the electron leakage that occurs from electron

Table 1. Standard reduction potentials for dioxygen and related species (the values of E^{ϕ} (V) are for aqueous solutions with O_2 at 1 atm, pH = 7)

	E^{ϕ} (V)
One electron reduction	
$O_2 + e^- \leftrightarrow {}^{\bullet}O_2^-$	−0.33
${}^{\bullet}O_2^- + e^- + 2H^+ \leftrightarrow H_2O_2$	+0.94
$H_2O_2 + H^+ + e^- \leftrightarrow {}^{\bullet}OH + H_2O$	+0.38
${}^{\bullet}OH + H^+ + e^- \leftrightarrow H_2O$	+2.33
Two electron reduction	
$O_2 + 2H^+ + 2e^- \leftrightarrow H_2O_2$	+0.30
$H_2O_2 + 2H^+ + 2e^- \leftrightarrow H_2O$	+1.35
Four electron reduction	
$O_2 + 4H^+ + 4e^- \leftrightarrow 2H_2O$	+0.82

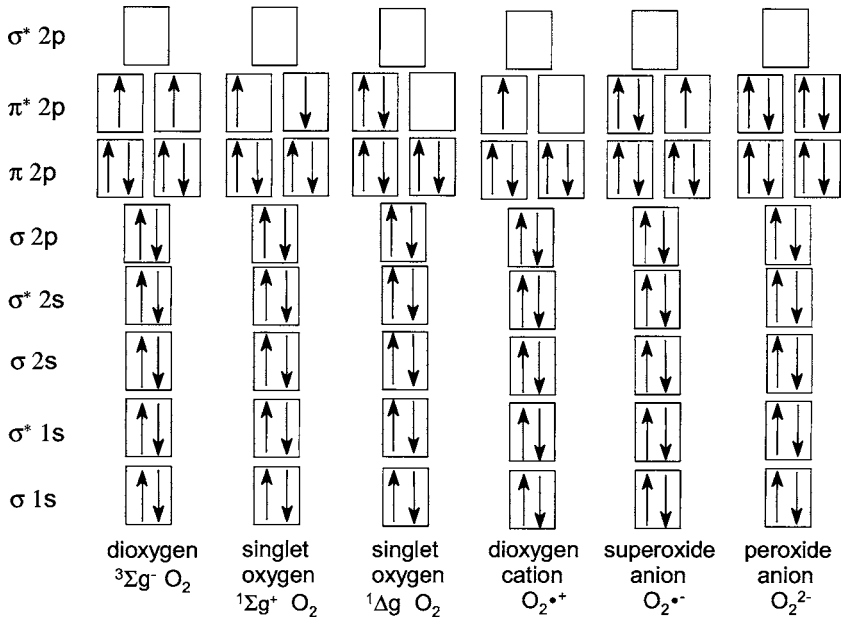

Fig. 1. Various forms of dioxygen.

$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow{e^-,2H^+} H_2O_2 \xrightarrow{e^-,-OH^-} {}^{\bullet}OH \xrightarrow{e^-,H^+} H_2O$$

Fig. 2. The active oxygen system: four single-electron reduction steps from molecular oxygen to water.

transport chains, such as those in the mitochondria and endoplasmic reticulum, to molecular oxygen, which generates superoxide [15].

Intracellular sources of superoxide/hydrogen peroxide

Mitochondria

The production of superoxide, the most common radical in biological systems, occurs mostly within the mitochondria of a cell. The mitochondrium is a small intracelullar organelle which is responsible for energy production and cellular respiration. Mitochondria accomplish this task through a mechanism called the "electron transport chain." In this mechanism, electrons are passed between different molecules, with each pass producing useful chemical energy. Oxygen occupies the final position in the electron transport chain. Even under ideal conditions, some electrons "leak" from the electron transport chain [16, 17]. These leaking electrons interact with oxygen to produce superoxide radicals, so that under physiological conditions, about 1–3% of the oxygen molecules in the mitochondria are converted into superoxide [18–21].

The primary site of radical oxygen damage from superoxide so produced is mitochondrial DNA (mtDNA). The cell repairs much of the damage done to nuclear DNA, but mtDNA cannot be readily fixed. Therefore, extensive mtDNA damage

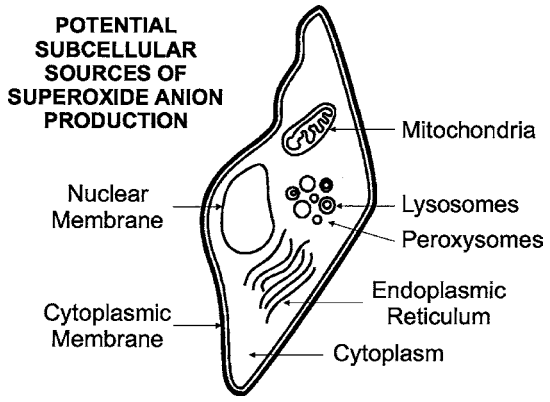

Fig. 3. Cellular sites of superoxide generation and its compartmentalization.

accumulates over time and shuts down mitochondria, causing cells to die and the organism to age.

Cytochrome P-450

The phase I cytochrome P-450 is the terminal component of the monoxygenase system found within the endoplasmatic reticulum of most mammalian cells [22]. The main role of cytochrome P-450 is that of detoxification of foreign compounds into less toxic products. In order to perform this detoxification function, this enzyme uses oxygen to oxidise the foreign compounds. This enzyme is also involved in hydroxylation reactions, which also remove/inactivate toxic compounds in the body and are heavily involved in steroidogenesis. During these oxidation and hydroxylation reactions electrons may be 'leaked' onto oxygen molecules, forming superoxide radicals – $O_2^{\bullet-}$ [22].

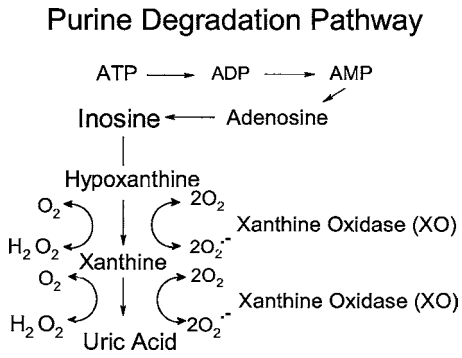

Fig. 4. Superoxide generation within enzymatic oxidations as, e.g. in the biological degradation of purines.

Cytoplasmatic oxidases

Cytochrome oxidase is found at the end of the electron transport chain in the mitochondrion [23]. The electron transport chain uses oxygen to oxidise nicotineamide adenine dinucleotide (NADH) and $FADH_2$ during aerobic respiration to generate energy. Cytochrome oxidase adds four electrons onto a molecule of dioxygen in a series of reduction reactions (see also Fig. 2). Each of these reduction reactions may potentially have superoxide radicals as a byproduct, which are potentially damaging.

Xanthine oxidase

Xanthine oxidase (XO) is a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals [24]. XO is an important source of OFR. It is a member of a group of enzymes known as molybdenum iron–sulfur flavin hydroxylases and catalyses the hydroxylation of purines [25, 26]. In particular, XO catalyses the reaction of hypoxanthine to xanthine and xanthine to uric acid (see Fig. 4). In both steps, molecular oxygen is reduced, forming the superoxide anion in the first instance and hydrogen peroxide in the second.

Microsomes and peroxisomes

Microsomes are responsible for 80% of the H_2O_2 produced *in vivo* at 100% hyperoxia sites [27]. Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiological conditions [28]. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2-generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognized as a potentially important source of H_2O_2 production with prolonged starvation.

Extracellular sources of superoxide

Membrane NADPH oxidases(s)

The NADPH oxidases are a group of plasma membrane-associated enzymes found in a variety of cells of mesodermal origin. The most thoroughly studied of these is the leukocyte NADPH oxidase (E.C.1.23.45.3), which is found in phagocytes and B-lymphocytes. If a phagocytic cell such as the neutrophil is exposed to a stimulus, it has the ability of recognizing the foreign particle and undergoing a series of reactions called the *respiratory burst* [29]. The respiratory burst enables the cell to provide oxidising agents for the destruction of the target cells. When NAD(P)H oxidase is activated, it takes NAD(P)H from the cytoplasm and passes electrons to O_2-producing superoxide within the plasma membrane or on its outer surface [30, 31]

$$2O_2 + \mathrm{NAD(P)H} \rightarrow 2O_2^{\bullet-} + \mathrm{NADP}^+ + \mathrm{H}^+ \qquad (1)$$

In chronic granulomatous disease, there is a hereditary defect of NAD(P)H oxidase enzyme resulting in decreased production of superoxide and the patient's leukocytes cannot kill the pathogens [32].

As summarized in Fig. 5, granulocytes and other phagocytic cells possess a membrane NADPH oxidase, which takes reducing equivalents from the hexose monophosphate shunt and transfers these to molecular oxygen to produce superoxide and other active oxygen species. A further myeloperoxidase converts peroxide produced in this system to microbiocidal products, probably including hypochlorite [33]. Production of activated products by this system probably plays a key role in cell-mediated immunity and microbiocidal activity. There is evidence for similar systems in T-lymphocytes, platelets, and mucus. An NADPH oxidase of non-inflammatory cells may have a role in mediating cyclic nucleotide metabolism [33].

Properties of superoxide

Despite the moderate *in vitro* chemical reactivity of superoxide in aqueous solution, it has been proven to be able to do a considerable degree of *in vivo* damage. However, superoxide can undergo a dismutation reaction [34]

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

This reaction is accelerated in biological systems by the SOD enzymes by about 4 orders of magnitude. It should be noted that SOD enzymes work in conjunction with H_2O_2–removing enzymes, such as catalases and glutathione (GSH) peroxidases [35].

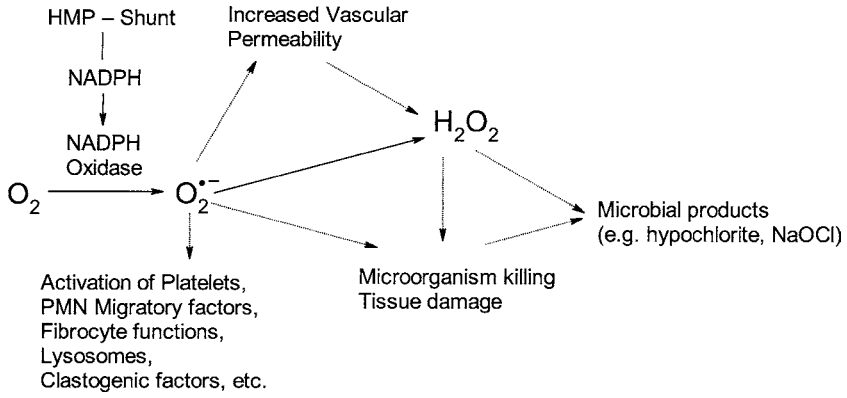

Fig. 5. Role of active oxygen species in inflammation.

Superoxide as an reducing agent

One route to generate hydroxyl radicals in biological systems is mediated by $O_2^{\bullet-}$ through a metal-catalyzed Haber–Weiss reaction [36–39];

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{2+}} O_2 + OH^{\bullet} + OH^- \quad (3)$$

which is an overall reaction and consists of two steps:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (5)$$

It is proposed that the above mechanism *in vivo* proceeds through catalytic activity of metal ions bound to biomolecules (Biol) in the close proximity of the target molecule (DNA)

$$\text{Biol-}M^{n+1} + O_2^{\bullet-} \rightarrow \text{Biol-}M^{n+} + O_2 \quad (6)$$

$$\text{Biol-}M^{n+} + H_2O_2 \rightarrow \text{Biol-}M^{n+1} + {}^{\bullet}OH + OH^- \quad (7)$$

$$DNA + {}^{\bullet}OH \rightarrow \text{damaged-DNA} \quad (8)$$

Superoxide as an oxidant

In 1976, Klug-Roth and Rabani [40] published the kinetics of copper(II)-catalyzed decomposition of superoxide to dioxygen, with the following proposed mechanism:

$$Cu^{2+} + O_2^{\bullet-} \rightarrow Cu^+ + O_2 \quad (9)$$

$$Cu^+ + O_2^{\bullet-} + 2H^+ \rightarrow Cu^{2+} + H_2O_2 \quad (10)$$

$$Cu^{2+} + O_2^{\bullet-} + 2H^+ \rightarrow Cu^{3+} + H_2O_2 \quad (11)$$

$$Cu^{3+} + O_2^{\bullet-} \rightarrow Cu^{2+} + O_2 \quad (12)$$

A similar mechanism applies to iron(III) involving Fe^{3+}/Fe^{2+} and Fe^{3+}/Fe^{4+} couples [41]. The above reactions imply that the superoxide anion is a precursor of Cu^{3+} or Fe^{4+} formation. These cations are strong oxidants and therefore may be involved in oxidative damage to biologically important molecules, as follows:

$$\text{Biol-}M^{n+} + O_2^{\bullet-} + 2H^+ \rightarrow \text{Biol-}M^{n+1} + H_2O_2 \quad (13)$$

$$\text{Biol-}M^{n+1} \rightarrow \text{(damaged-Biol)-}M^{n+} \quad (14)$$

Hydroxyl radical

Hydroxyl radical is highly reactive with a half-life in aqueous solution less than 1 ns. Thus when produced *in vivo* it reacts close to its site of formation. It can be generated through a variety of mechanisms. Ionizing radiation causes decomposition of H_2O, resulting in formation of $^{\bullet}OH$ and hydrogen atoms. $^{\bullet}OH$ is also generated by photolytic decomposition of alkylhydroperoxides. Production of OH close to DNA could lead to this radical reacting with DNA bases or deoxyribosyl backbone of DNA to produce damaged bases or strand breaks. It has been proposed that the extent of DNA strand breaking by $^{\bullet}OH$ is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone [42].

The majority of the hydroxyl radicals generated *in vivo* comes from the metal catalyzed breakdown of hydrogen peroxide, according to the reaction [43–46]

$$M^{n+}(= Cu^+, Fe^{2+}, Ti^{3+}, Co^{2+}) + H_2O_2 \rightarrow M^{(n+1)+} \times (= Cu^{2+}, Fe^{3+}, Ti^{4+}, Co^{3+}) + {}^{\bullet}OH + OH^- \quad (15)$$

where M^{n+} is a transition metal ion. The most realistic *in vivo* production of hydroxyl radical according to reaction (15) occurs when M^{n+} is iron or copper [47–49]. The Fe^{2+}-dependent decomposition of hydrogen peroxide is called the

Fenton reaction [46, 50–52]. In addition to reaction (15), the following reactions may occur:

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + H^+ + {}^{\bullet}O_2^- \quad (16)$$

$$^{\bullet}O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (17)$$

$$^{\bullet}OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad (18)$$

Generally, the hydroxyl radical may react by (i) hydrogen abstraction, (ii) electron transfer and (iii) addition reactions. The reaction of 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 reactions that can severely damage the membranes [52, 53]. Hydroxyl radical also causes addition 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) (Fig. 6) [54, 55]. Adenine reacts with $^{\bullet}$OH in a similar manner to guanine, although oxidative adenine lesions are less prevalent in DNA damage [56]. It has been demonstrated that in the presence of Fe(III) or Fe(III)–EDTA complex, endogenous reductants such as ascorbate, GSH, and the reduced form of NADH, caused DNA damage at every type of nucleotide with a slight dominance by guanine [57]. Specifically, NADH in the presence of Fe(III)–EDTA and H_2O_2 generated $^{\bullet}$OH, lead to formation of 8-oxo-dG [58]. The DNA damage was inhibited by typical $^{\bullet}$OH scavengers and by catalase [59–61], suggesting that these reductants cause DNA damage *via* the Fenton reaction.

Peroxyl radicals

The reactions of peroxyl radicals (ROO$^{\bullet}$) are prevalent in all aspects of life, ranging from interactions with DNA [62] to "knocking" in the internal combustion engines of automobiles [63]. They are high-energy species, with a reduction potential ranging from +0.77 to +1.44 V, depending on the R group. Several methods, ranging from chemical and physical to enzymatic techniques, may be used to generate peroxyl radicals [64].

The simplest peroxyl radical is dioxyl radical HOO$^{\bullet}$, the conjugate acid of superoxide, $O_2^{\bullet -}$. There are many, more complex peroxyl radicals, including cholesterol derivatives, fatty acids, etc. [65]. The chemistry of this type of molecule is variable due to the identity of the R group, the local environment, and the concentration of oxygen and other reactants.

Perhaps the most interesting feature of peroxyl radicals is the diversity of biological reactions in which they participate. The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free radical reactions in human disease and toxicology. Peroxyl radicals are involved in DNA cleavage [66] and protein backbone modification [67]. Peroxyl radicals synergistically enhance the induction of DNA damage by superoxide [68].

The pathway of reactions of peroxyl radicals is as follows [69]. Chain initiation refers to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group. Hydroxyl radical is sufficiently reactive to do this, although superoxide is not.

$$-CH_2- + {}^{\bullet}OH \rightarrow -{}^{\bullet}CH- + H_2O$$

$$(R_i = \text{Rate of initiation}) \quad (19)$$

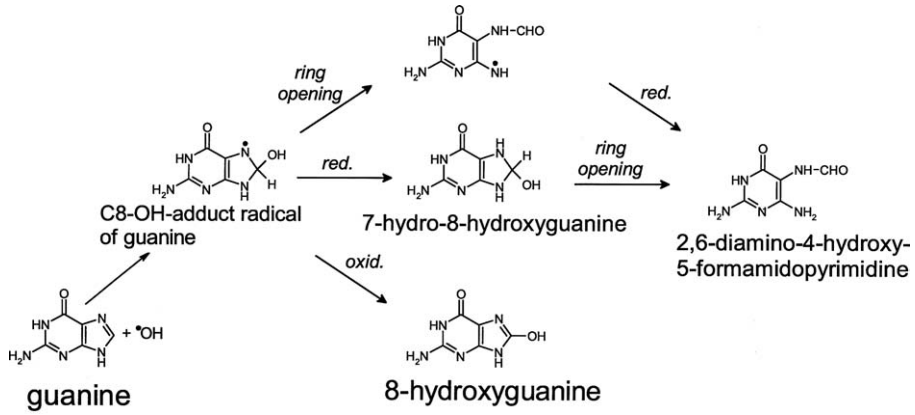

Fig. 6. Structures of various products formed from the C8-OH–adduct radical of guanine, which itself is formed by attack of $^{\bullet}$OH on the C8-position of guanine.

Under aerobic conditions the radical $-^{\bullet}CH^{-}$ produced in (19) reacts with dioxygen to yield a peroxyl radical:

$$-^{\bullet}CH- + O_2 \rightarrow -CHOO^{\bullet} \quad (Rp1 = \text{rate of propagation}) \tag{20}$$

It should be noted that a very low oxygen pressure might favor the self-reaction of carbon-centered radicals, terminating the process.

$$2CHOO^{\bullet} \rightarrow \{\text{Non-radical products, i.e. termination}\} \tag{21}$$

$$\text{or} \quad 2CH^{\bullet} \rightarrow \{\text{Non-radical products, i.e. termination}\}$$

Peroxyl radicals produced in (20) are capable of abstracting hydrogen from another adjacent lipid molecule to propagate the process.

$$- CHOO^{\bullet} + -CH_2 \rightarrow -CHOOH + -CH^{\bullet} \times (Rp2 = \text{rate of propagation}) \tag{22}$$

Electron paramagnetic resonance spectroscopy

Electron Paramagnetic Resonance (EPR; also known as Electron Spin Resonance, ESR) is a spectroscopic technique which allows us to detect free radicals (molecular fragments or atoms possessing a single unpaired electron) and identify and quantify these species even in complex biological systems where multiple radicals and other non-radical components are present [70]. The technique is routinely used to measure free radicals such as superoxide, hydroxyl and nitric oxide. EPR also enables measurement and imaging of physiologically pertinent tissue parameters (functional imaging) such as tissue perfusion, oxygenation, metabolism, redox state, viability, pH, etc., using appropriate spin probes. Short-lived free radicals of reaction oxygen species can be determined using stabilizer molecules called spin traps. The detection of spin-trapped radical adducts by EPR is a particularly powerful technique for the sensitive and specific detection, identification, and relative quantitation of short-lived free radicals [70].

Mechanisms of free radical-induced mutagenesis and DNA base modification

It has been estimated that one human cell is exposed to approximately 10^5 oxidative hits a day from hydroxyl radical and other such species [71–76]. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step of carcinogenesis involved in mutagenesis and aging [1]. DNA alterations caused by radicals are removed by specific and non-specific repair mechanisms. Repair of DNA base damage is thought to occur mainly by base-excision [77]. However, misrepair of DNA damage could result in mutations such as base substitution and deletion, also leading to carcinogenesis [75, 76]. Mutagenic potential is directly proportional to the number of oxidative DNA lesions that escape repair. It is known that repair mechanisms decay with age and thus DNA lesions accumulate with age. The sequence specificity of DNA damage sites affects the mutation frequency [73]. Therefore, investigation of the sequence specificity of DNA damage would be beneficial for cancer prevention.

The specific mechanism by which oxidative stress contributes to the development of carcinogenesis is largely unknown. However, at least two different mechanisms are thought to play a role in oxidative damage and in the development of carcinogenesis.

The first mechanism by which oxidative damage can affect carcinogenesis is through the modulation of gene expression. Epigenetic effects on gene expression can lead to the stimulation of growth signals and proliferation [78]. Chromosomal rearrangements are thought to result from strand breakage misrepair, contributing to genetic amplifications, alterations in gene expression and loss of heterozygosity, which in turn may promote neoplastic progression [79]. Active oxygen species have been demonstrated to stimulate protein kinase and poly(ADP ribosylation) pathways, thus affecting signal transduction pathways. This further can lead to modulation of the expression of essential genes for proliferation and tumour promotion [80]. Recently, Lander *et al.* [81] suggested that free radical signalling may be mediated through *ras* signal transduction pathways.

In the second mechanism, radicals induce genetic alterations, such as mutations and chromosomal rearrangements, which can play a role in the initiation of carcinogenesis [82, 83]. Oxidative DNA damage results in a wide range of chromosomal abnormalities, causing a blockage of DNA replication and wide cytotoxicity [84]. Mutations can occur through misrepair or due to incorrect replication, while chromosomal rearrangements can result from strand breakage misrepair [71]. The initiation potential of oxidants may be contributing to carcinogenesis due to their ability to induce DNA base changes in certain oncogenes and tumour suppressor genes [85]. It has been demonstrated that the hydroxyl radical is able to activate certain oncogenes, such as K-*ras* and *C-Raf*-1. The activation proceeds through the induction of DNA point mutations in GC base pairs and N-terminal deletions in these genes [85]. Base point mutations in CpG dinucleotides are also frequently found in certain tumour suppressor genes, such as p53 and retinoblastoma, leading to their inactivation [86, 87]. Furthermore, hydroxyl radical attacks or cells containing mutant or absent p53, resulted in

a failure to arrest in G_1, reducing their capacity to repair damaged DNA [85]. This increase in replication errors can initiate additional oncogene activation and tumour suppressor gene inactivation, ultimately contributing to malignancy. Free radical-induced cytotoxicity may also contribute to the initiation of carcinogenesis by depleting the normal cell population, promoting the clonal expansion of more resistant-initiated cells, thus increasing the probability of mutation [78].

Oxygen radicals and genotoxicity of DNA damage

ROS-induced DNA damage can be described both chemically and structurally and shows a characteristic pattern of modifications. It is well established that in various cancer tissues free radical-mediated DNA damage was found [88]. The majority of these changes can be reproduced *in vitro*. The forms of DNA damage produced by ROS experimentally include the following: modification of all bases, production of base-free sites, deletions, frame shifts, strand breaks, DNA–protein cross-links, and chromosomal rearrangements. An important reaction involved in DNA damage involves generation of hydroxyl radical, e.g. through Fenton chemistry [89]. Hydroxyl radical is known to react with all components of the DNA molecule: the purine and pyrimidine bases as well as the deoxyribose backbone [90, 91]. In terms of oxidative DNA damage, major interest has focused on modifications to DNA bases, with over 20 products identified, however only a few have been investigated in detail.

Hydroxyl radical is able to add to double bonds of DNA bases at second-order rate constants of $3–10 \times 10^9$ M^{-1} s^{-1} and abstracts an H-atom from the methyl group of thymine and each of the five carbon atoms of 2′ deoxyribose at rate constants of $\sim 2 \times 10^9$ M^{-1} s^{-1} [92, 93]. While OH–adduct radicals of DNA bases are generated *via* addition reactions, the allyl radical of thymine and carbon-centered sugar radicals are formed from abstraction reactions. Peroxyl radicals are generated in oxygenated environments *via* oxygen addition to OH–adduct radicals and also to carbon-centered radicals at diffusion-controlled rates [92, 93]. Further reactions of base and sugar radicals generate a variety of modified bases and sugars, base-free sites, strand breaks, and DNA–protein cross-links.

Hydroxyl radical adds to pyrimidines: to the C5 and C6 positions of thymine and cytosine, generating C5-OH– and C6-OH–adduct radicals, respectively. Oxidation reactions of the C5-OH–adduct radicals of thymine and cytosine followed by addition of water or OH^- and deprotonation lead to the formation of glycols of cytosine and thymine, respectively [94, 95]. Oxygen adds to C5-OH–adduct radicals to give 5-hydroxy-6-peroxyl radicals that may eliminate superoxide followed by reaction with water, giving rise to cytosine glycol and thymine glycol. Oxidation of the allyl radical of thymine generates 5-(hydroxymethyl)uracil (5-OHMeUra) and 5-formyluracil. In the absence of oxygen, 5-hydroxy-6-hydro- and 6-hydroxy-5-hydropyrimidines are formed by reduction of 5-OH– and 6-OH–adduct radicals of pyrimidines, respectively, followed by protonation.

Hydroxyl radical is also able to add to purines giving rise to C4-OH–, C5-OH–, and C8-OH–adducts [96, 97]. One-electron oxidation and one-electron reduction of C8-OH–adduct radicals yields 8-hydroxypurines (7,8-dihydro-8-oxopurines) and formamidopyrimidines, respectively [94, 96, 98, 99]. The most extensively studied of these oxidised DNA products is 8-oxo-deoxyguanosine (8-oxo-dG), mainly because it is the most easily detectable. The presence of 8-oxo-dG in human urine was first reported by Shigenaga *et al.* [100]. This base modification occurs in approximately one in 10^5 guanidine residues in a normal human cell [101].

An example illustrating the mechanisms of the formation of 8-hydroxyguanine (7,8-dihydro-8-oxoguanine, 8-OH-G) is given in Fig. 6. 8-Hydroxyguanine and 8-hydroxy-29-deoxyguanosine undergo keto–enol tautomerism, which favours the 6,8-diketo form. Hence, 8-OH-G is often called 8-oxy-7-hydroguanine or 8-oxoG. The nucleoside is then called 8-oxo-7-hydro-29-deoxyguanosine or 8-oxo-dG (therefore, 8-oxo-dG and 8-OH-dG are the same compounds). Throughout this paper 8-oxoguanine (8-oxoG) or deoxynucleosides (8-oxo-dG) will be used. We note that the analogous reactions of adenine yield 8-hydroxyadenine (8-OH-Ade).

Multiple methods for measuring oxidative DNA damage exist; a popular method employs enzymatic digestion of DNA, which liberates 8-hydroxypurines for analysis by HPLC usually with electrochemical detection (HPLC-EC) [102–106]. Another method employs acidic hydrolysis of DNA, which liberates the free base, because the glycosidic bond is cleaved by acid. Detection is *via* HPLC or, after conversion to volatile derivatives, by gas chromatography mass spectrometry (GC-MS) [107].

The 8-oxoG lesion is important because it is relatively easily formed and is mutagenic, therefore is a potential biomarker of carcinogenesis [108]. The experimental proposed mutagenic potential of 8-oxo-dG is supported by a loss of base pairing specificity, misreading of adjacent pyrimidines, or insertion of adenine opposite the lesions [109]. Mutations that may arise due to formation of 8-oxo-dG involve GC $\rightarrow$ TA transversions [110]. Previous work has shown that the mispairing of 8-oxo-dG with adenine appears to be possible due to the energetically favoured *syn* glycosidic conformation, whereas pairing with dG assumes the *anti* form.

Measurements by Kasai [109] demonstrated that factors such as hard physical labour, day–night shift work, smoking, low meat intake, and low BMI (<21.8) significantly increased the 8-oxo-dG level, while moderate physical exercise, such as sports, reduced its level. These results were comparable with previous data obtained from studies on rats [111]. These

findings suggest that the lifestyle may significantly affect the level of oxidative damage.

Another important mechanism of mutations is formation of 2-oxy-dA in the nucleotide pool and its incorporation into DNA. It has been presented that the incorporation of 2-oxy-dA opposite G induced GC → TA transversions in the chromosomal lac I gene [112]. It is known that the human sanitization enzyme hMTH1 hydrolyzes 8-oxy-dGTP and prevents mutations. In a series of experiments testing sanitization enzymes for 2-oxy-dA it has been unexpectedly evidenced that 2-oxo-dATP is also hydrolyzed by hMTH1 [113].

Lipid peroxidation and DNA damage

While major attention has focused on direct DNA damage by free radicals because of the genetic consequences of such damage, reactive radical species may also cause damage to other cellular components [114]. Cell membrane phospholipids are very sensitive to oxidation and have been found to be frequent targets of radical-induced damage that enables them to participate in free radical chain reactions. Many of the fatty acids are polyunsaturated, containing a methylene group between two double bonds that makes the fatty acid more sensitive to oxidation. The high concentration of polyunsaturated fatty acids in phospholipids enables them to participate in free radical chain reactions [115]. The most common fatty acid in cells is linoleic acid. The best biomarker of lipid peroxidation is a set of arachidonic acid oxidation products termed isoprostanes [116, 117]. They can readily be detected by GC-MS.

The initial products of unsaturated fatty acid oxidation are short-lived lipid hydroperoxides. When they react with metals they produce a number of products (e.g. aldehydes and epoxides) which are themselves reactive.

Malondialdehyde (MDA) is one of the major aldehyde products of lipid peroxidation [118]. It is mutagenic in mammalian cells and carcinogenic in rats [119, 120]. MDA can react with DNA bases dG, dA, and dC to form adducts M_1G, M_1A and M_1C (Fig. 7). M_1G has been detected in human liver, white blood cells, pancreas, and breast tissue [121]. The M_1G level corresponds approximately to 6500 adducts per cell. Several studies concluded that M_1G is a reactive electrophile in the genome [122]. N^2-Oxo-propenyl-dG, the product of rapid and quantitative ring-opening of M_1G, is also electrophilic, but targets regions of DNA different from M_1G. Thus the interconversion of N^2-oxo-propenyl-dG and M_1G may reveal varying reactive groups of DNA that could participate in the formation of DNA–DNA interstrand cross-links or DNA–protein cross-links.

It has been demonstrated that hydroxypropanodeoxoguanosines (OH-PdGs) are present in human and rodent liver DNA [123]. It has been suggested that these propano adducts are mediated by the reaction of DNA with acrolein and crotonaldehyde, which in turn are products of lipid peroxidation. Acrolein and crotonaldehyde are mutagenic in bacteria and mammalian cells [124]. There is little known about the repair of OH-PdGs. PdG is a substrate for the nucleotide excision repair complex of *E. coli* and mammalian cells and is recognized and repaired by the mismatch repair system [125].

Several exocyclic etheno DNA adducts arising from lipid peroxidation have been detected by ^{32}P-post-labeling and GCMS analysis in DNA from healthy human volunteers [126]. The most important involves etheno-dA, etheno-dC and etheno-dG. Their biological activity has been widely studied by site-specific mutagenesis experiments. Etheno-dA and etheno-dC were found to be strongly genotoxic but weakly mutagenic in single-stranded *E. coli* [127, 128]. However, the same adducts were found to be highly mutagenic when introduced into monkey kidney cells, implying that marked differentials exists in mutagenic potency between bacterial and mammalian cells [129]. The kind of cell system used to evaluate the mutagenic activity of a given lesion is therefore of key importance.

Inflammation, alcohol, smoking and DNA damage

Recent data have expanded the concept that inflammation is a critical component of tumour progression [130]. It is now becoming clearer that the neoplastic process, proliferation, survival, and migration is linked with tumour microenvironement synchronized with inflammatory cells.

Several studies have demonstrated a direct link between chronic inflammation and DNA damage [131]. Liver tissue from patients suffering chronic inflammatory diseases such as hepatitis, hepatitis B, and cirrhosis exhibit increased levels of 8-oxo-dG compared to control liver tissue [132]. Similar findings were reported for patients infected with *Helicobacter pylori* [133]. Chronic atrophic gastritis shows also increased level oxidised bases, however, chronic non-atrophic gastritis does not.

An inverse correlation between alcohol consumption and lymphocyte levels of 8-oxo-dG in humans has been recently presented in an international experimental study [134]. The study was conducted in four different regions of Europe, including Potsdam (Germany), Turin (Italy), Malmö (Sweden) and Granada (Spain). Mean 8-oxo-dG levels differed significantly across study centres, with the highest levels in Granada and lowest levels in Turin [134]. Mean levels of total alcohol intake and of types of alcoholic beverages consumed (wine, fortified wines, beer and cider) also differed across the study centres, with the highest total alcohol consumption in Turin, and the lowest intake in Granada [134]. When combining all the data, but adjusting for study centre, individual 8-oxo-dG level correlated inversely with alcohol intake. The finding of a relationship between alcohol consumption and 8-oxo-dG

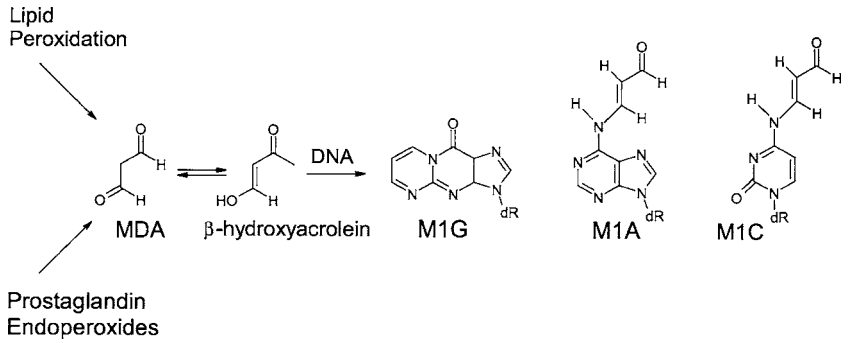

Fig. 7. Synthesis of malondialdehyde (MDA) and its reaction with DNA bases.

in lymphocytes was unexpected and not based on a prior hypothesis. This finding consequently requires confirmation from a randomised intervention study. Additional progress in understanding alcohol's enhancing effect on DNA damage will depend on a better understanding of the interactions between alcohol and other risk factors and on additional insights into the multiple biological mechanisms involved. The enhancing effect of alcohol may also be affected by other dietary factors (such as low folate intake), lifestyle habits (such as use of hormone replacement therapy), etc.

Recently published trials have concluded that cigarette smoking has a low impact upon certain pathways involved in DNA damage and the antioxidative defence system [135]. Various markers of oxidative DNA damage and repair, and antioxidative defence mechanisms have been studied in smokers. Lymphocytic 8-oxo-dG levels were significantly lower in smokers as compared with non-smokers. The levels of oxidised pyrimidine bases in lymphocytes of smokers quantified by the endonuclease III-modified comet assay were non-significantly lower than those of non-smokers. Urinary excretion levels of 8-OH-dG assessed by enzyme-linked immunosorbent assay did not differ significantly between smokers and non-smokers. Plasma antioxidative capacity measured by the Trolox equivalent antioxidant capacity assay was slightly higher in smokers as compared with non-smokers, and it was significantly related to lymphocytic 8-oxo-dG levels [135].

Repair of DNA lesions

Oxygen radicals may induce a number of DNA base alterations that can lead to mutagenesis. However, there are specific and general repair mechanisms that can repair DNA base modifications [83, 136].

The first evidence of a repair mechanism for the 8-oxo-dG lesion was observed in irradiated mouse liver, where levels of this lesion were found to decrease with time [137]. A repair enzyme was partially purified from *E. coli* [138] and was later found to be identical to the cloned DNA repair enzyme, formamidopyrimidine–DNA glycosylase FPG protein, previously isolated from *E. coli*. This enzyme has both glycosylase and apurinic endonuclease activity. The repair pathway for this oxidative lesion in *E. coli* includes at least three pathways characterized by mutant strains: MutM, which lacks the Fpg protein and exhibits increased G → T transversions [139, 140]; MutY, that recognizes dA mismatches with 8-oxo-dG and removes the adenosine inserted opposite to the 8-oxo-dG [141]; and MutT, which degrades (hydrolyses) the nucleotide pool of 8-oxoGTP [142]. These three proteins cooperate in the prevention of spontaneous oxidative mutations in *E. coli* and represent the multilevel security against 8-oxo-dG-related damage [143].

Two separate glycosylase and endonuclease enzymes responsible for repair of the 8-oxo-dG lesion were isolated from mammalian cells [144]. These two enzymes are the mammalian counterparts to the MutM repair enzyme in *E. coli*.

Some endonucleases without glycosylase activity could recognise sites damaged by free radicals. The endonuclease with glycosylate activity, DNA glycosylase endonuclease III, recognizes thymine glycol and a selection of other oxidative and non-oxidative base modifications. An intriguing observation linked to DNA damage by free radicals is that nitric oxide inhibits some DNA repair enzymes including FAPY glycosylase that removes 8-oxo-dG [145].

Antioxidant defense system

Antioxidant is a classification of several organic substances, including vitamins C, E and vitamin A (which is converted from β-carotene), selenium (a mineral), and a group known as the carotenoids (CAR) [146–149]. CAR, of which β-carotene is the most popular, are pigments that add colour to many fruits and vegetables – for example, without them carrots would not be orange. These substances at the molecular and cellular level are thought to be effective in helping to deactivate free radicals and prevent cancer, heart disease and

stroke. Antioxidants play the housekeeper's role, "mopping up" free radicals before they get a chance to cause damage. Despite numerous studies carried out on the role of antioxidants in cancer and heart disease prevention, the jury is still out as to which groups of people, if any, benefit from taking antioxidant supplements [76, 150, 151]. Some studies have shown that smokers with diets high in CAR have a lower rate of lung cancer development than their smoking counterparts whose CAR intake is relatively low [152]. However, a recent trial indicated that some β-carotene takers (see below), primarily smokers, actually had higher death rates [153]. Other research efforts have suggested that diets high in CAR may also be associated with a decreased risk of breast cancer [154, 155]. Also, vitamin C has been found to prevent the formation of N-nitroso compounds, the cancer-causing substances from nitrates and nitrites found in preserved meats and in some drinking water [156].

Vitamin C

Vitamin C's major role is to make collagen, the main protein substance of the human body that holds connective tissues together in skin, bone, teeth, and other parts of the body. Vitamin C is also critical for the proper function of our immune system, for manufacturing certain nerve transmitting substances and hormones, and for the absorption and utilization of other nutrients, such as vitamin E and iron [157, 158]. Vitamin C is also a very important and powerful antioxidant that works in aqueous environments of the body, such as the lungs and lens of the eye [159]. Its primary antioxidant partners are vitamin E and the carotenes (β-carotene), as well as works along with the antioxidant enzymes [160, 161]. The recommended daily allowances (RDAs) for adults are 40 mg per day in the Europe and 60 mg per day in the US.

Ascorbic acid has two ionizable hydroxyl groups and therefore is a di-acid ($AscH_2$). Because the pK_a of the first is 4.25 and the pK_a of the second is 11.8, formation of the mono-anion is favoured at physiological (cellular) pH (see Fig. 8) [162–164]. 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 $AscH^-$ and we will use the term ascorbate throughout the paper to refer to this species. The initial product of ascorbate oxidation by many of these species is the semi-dehydroascorbate 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) [162–164].

$AscH^-$ is a donor antioxidant and donates a hydrogen atom to an oxidising radical to produce the resonance stabilized tricarbonyl ascorbate-free radical (AFR). $AscH^{\bullet}$ has a pK = –0.86 thus it is not protonated and is present in the form of $Asc^{\bullet-}$(see Fig. 8).

In practically all metabolic activities, ascorbate reduces transition metal ions (Cu and Fe). Since Fe(III) has very low solubility, the ability of ascorbate to reduce Fe(III) to Fe(II) has significance in iron sorption in gut [165]. Ascorbate also reduces Cu(II) to Cu(I).

Ascorbate converts ROS into poorly reactive ascorbate-derived products that is, ascorbate acts as one of the many antioxidants that can protect biomolecules against damage by such species *in vivo*. $Asc^{\bullet-}$ is considered to be a terminal, small-molecule antioxidant and the level of this radical is a good measure of the degree of oxidative stress in biological systems [163, 166].

It its known that vitamin C protects membranes against oxidation. Low-density lipoprotein (LDL) is well known to play an important role in atherosclerosis, the underlying cause of coronary heart disease and strokes. Oxidatively modified LDL is taken up by macrophages *via* scavenger receptors at a much greater rate than native LDL [167, 168]. This leads to the formation of cholesterol-laden foam cells, which are characteristic of many atherosclerotic lesions. Two mechanisms for a protective role of vitamin C against LDL oxidation have been suggested by Retsky *et al.* [169]. In one mechanism, ascorbic acid may scavenge free radicals in the aqueous phase. In the other, dehydroascorbic acid (the oxidation product of ascorbic acid), or its decomposition products, might modify LDL such that copper can bind less strongly to the LDL particle, thus increasing the resistance of LDL to oxidation by copper. However, very recently, Retsky *et al.* [170] have shown that when LDL has already been partially oxidised by copper, then ascorbate and dehydroascorbate no longer protect against further oxidation by copper.

The intake of high doses of vitamin C, initially suggested by Linus Pauling, has been a subject of the intense debate over years [171, 172]. The benefit of a high intake of vitamin C has never been established. However, a positive effect of vitamin C intake was reported from clinical trials of stomach cancer incidence and cardiovascular disease [173, 174]. Vitamin C cooperates with vitamin E to regenerate α-tocopherol from α-tocopheryl radicals in membranes and lipoproteins [175] (see also below).

Besides the positive role of ascorbate it should be noted that there are studies exploring pro-oxidant properties of ascorbate [176, 177]. Concerns have been raised over potentially deleterious transition metal ion-mediated pro-oxidant effects. It has been determined that vitamin C induces the decomposition of lipid hydroperoxides into the DNA-reactive bifunctional electrophiles 4-oxo-2-non-enal, 4,5-epoxy-2(E)-decenal, and 4-hydroxy-2-non-enal [176]. The compound 4,5-epoxy-2(E)-decenal is a precursor of etheno-2′-deoxyadenosine, a highly mutagenic lesion found in human DNA [176]. Vitamin C-mediated formation of genotoxins from lipid hydroperoxides in the absence of transition

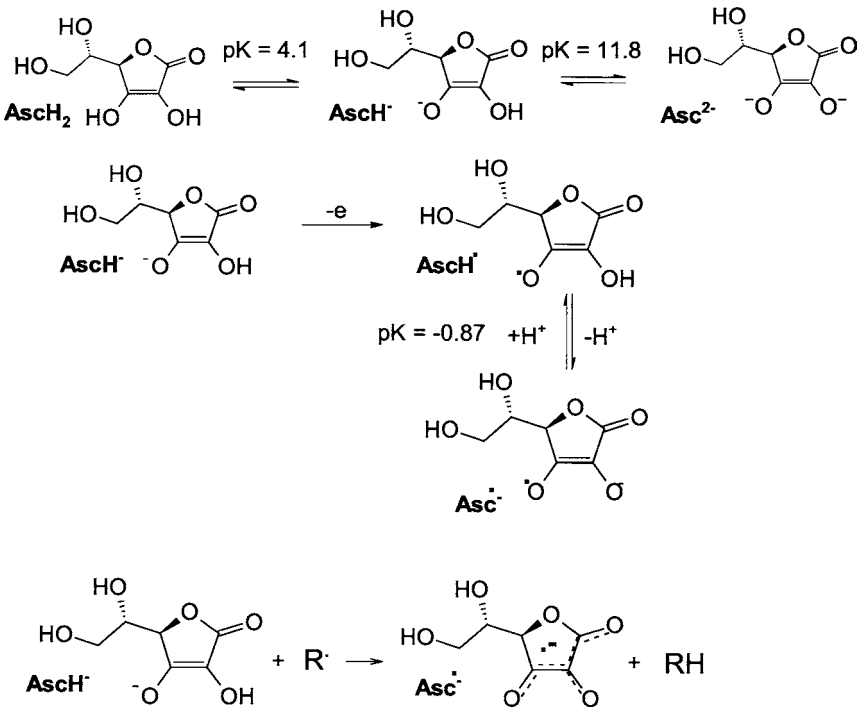

Fig. 8. Forms of ascorbate at various pH levels and its reaction with radicals.

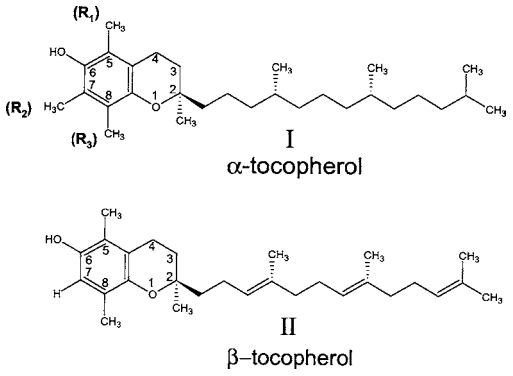

Fig. 9. Vitamin E and its derivatives: compound I, where $R^1 = R^2 = R^3 =$ Me, is α-tocopherol; compound II, where $R^1 = R^3 =$ Me; $R^2 =$ H, is β-tocopherol.

metal ions could help explain its lack of efficacy as a cancer chemoprevention agent.

Vitamin E

Vitamin E is a fat-soluble vitamin that exists in eight different forms. Each form has its own biological activity, the measure of potency or functional use in the body. α-Tocopherol (Fig. 9) is the most active form of vitamin E in humans, and is a powerful biological antioxidant [178]. Antioxidants such as vitamin E protect cells against the effects of reactive radicals, which are potentially damaging byproducts of the body's metabolism [179].

The term vitamin E should be used as the generic descriptor for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol. This term should be used in derived terms such as vitamin E deficiency, vitamin E activity, vitamin E antagonist.

The term tocol is the trivial designation for 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol ($R^1 = R^2 = R^3 =$ H). Compound I ($R^1 = R^2 = R^3 =$ Me) is designated α-tocopherol (Fig. 9) or 5,7,8-trimethyltocol. Compound II ($R^1 = R^3 =$ Me; $R^2 =$ H), is designated β-tocopherol or 5,8-dimethyltocol [180].

α-Tocopherol is considered to be the major membrane-bound antioxidant employed by the cell [181] and its main antioxidant activity is protection against lipid peroxidation [182, 183]. As mentioned above, ascorbic acid is regarded as the major aqueous-phase antioxidant [184]. Recent evidence suggests that α-tocopherol and ascorbic acid function together in a cyclic-type reaction [178]. During this process, α-tocopherol is converted to a radical by donating a labile hydrogen to a lipid or lipid peroxyl radical [181, 182]. The oxidised α-tocopherol radical is energetically stable and has low reactivity with other molecules within the membrane (Fig. 10). Oxidised α-tocopherol can then be re-reduced to its original form by ascorbic acid (see also above). This regeneration of reduced α-tocopherol presumably occurs at the surface

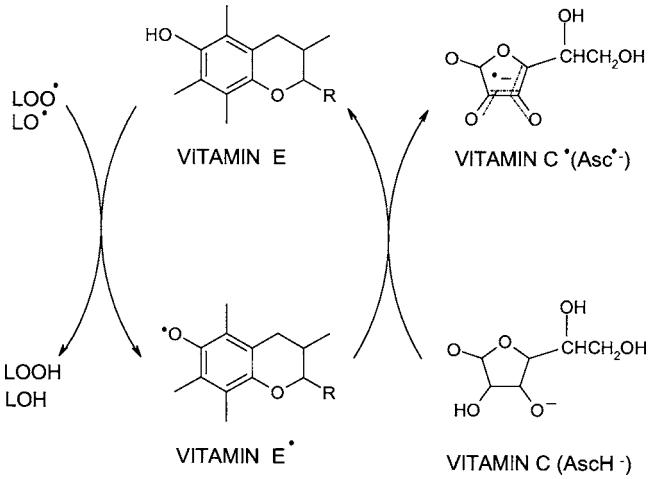

Fig. 10. Reaction of vitamin E with peroxyl free radicals and regeneration of vitamin E radical (tocopheroxyl radical) through one-electron oxidation of vitamin C.

of the membrane where ascorbic acid and α-tocopherol can meet [181]. Along with acting as a reducing agent for α-tocopherol, ascorbic acid is also considered a preventative antioxidant because of its ability to scavenge for reactive radicals.

Some studies advocate vitamin E to inhibit the incidence of stomach cancer, however a recent trial on North American populations found that there is no association between stomach cancer mortality and regular use of vitamin E [185]. It should be noted that in North American populations, stomach cancer rates are relatively low, so the results do not rule out a beneficial effect of vitamin supplementation in areas in which stomach cancer rates are high and stomach cancer etiology may differ.

A long-term epidemiological study of cancer incidence was carried out by the US National Institute of Health in Finland (**A**lpha-**T**ocopherol/**B**eta-**C**arotene, ATBC Trial) [186]. The results show no reduction in lung cancer and 40% decreased incidence of clinically significant prostate cancer in volunteers taking vitamin E. Prostate cancer mortality also decreased 41%.

In conclusion, vitamin E, in particular together with selenium have been shown to decrease cancer incidence and mortality in large-scale, well-controlled prospective randomised clinical trials.

Carotenoids

CAR are pigments that are found in plants and microorganisms but are not synthesized by animals [187]. They are responsible for the red, yellow, and orange colour of fruits and vegetables. There are over 600 CAR occurring in nature, which can be grouped into carotenes, xanthophylls (CAR containing oxygen), and lycopene. In the last decade several epidemiological studies have indicated that CAR may prevent or inhibit certain types of cancer, artherosclerosis, age-related muscular degeneration, and other diseases [188].

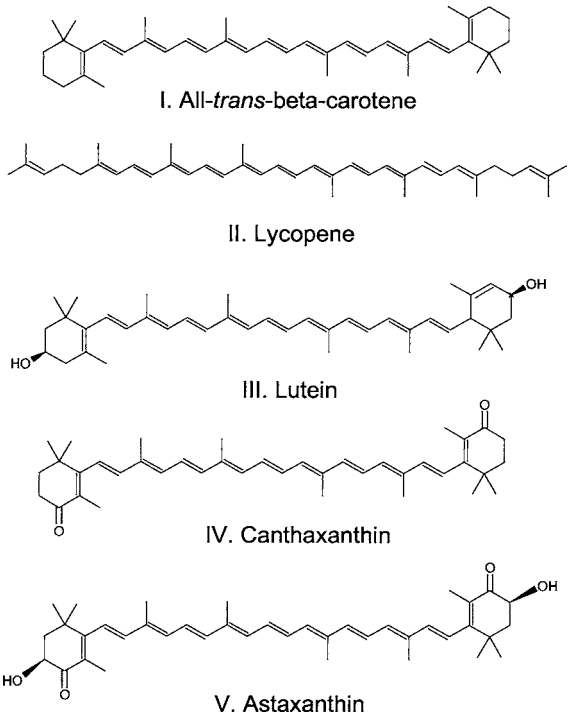

Fig. 11. Structures of carotenoids.

The antioxidant activity of CAR arises as a result of the ability of the conjugated double bond structure (Fig. 11) to delocalise any unpaired electrons. This is primarily responsible for the excellent ability of β-carotene to physically quench singlet oxygen without degradation and for the chemical

reactivity of β-carotene with free radicals such as peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), and superoxide radical ($O_2^{\bullet -}$) [189, 190]. In general, the longer the polyene chain, the greater the peroxyl radical stabilizing ability. It has been shown that the peroxyl radical ($ROO^{\bullet}$) was about 100–1000-fold more reactive with CAR than with the allylic hydrogen sites on polyunsaturated fatty acids, hence at sufficient concentrations, CAR could protect lipids from peroxidative damage [191].

In the ATBC Trial [186] supplemental β-carotene was administered to 29,133, 50–69-year-old male smokers in Finland for 5–8 years. The dosage of 20 mg per day was substantially higher than what is typically contained in the Finnish diet. In the men taking β-carotene, there was a significant, 18%, increase in the incidence of lung cancer, which contributed to an 8% excess in total mortality! The ATBC trial results imply that β-carotene or, more specifically, the all-*trans* isomer of β-carotene in a water-soluble beadlet is not correlated with the reduced cancer risk associated with vegetable and fruit intake [192]. However, these results do not totally rule out a protective role for β-carotene; for example, if it is given in higher dosages or earlier in the process of lung carcinogenesis.

Another multi-centre lung cancer prevention trial Beta-**Ca**rotene and **Ret**inol Efficiacy (CARET) [193] was started. The trial was randomised, double blind, and placebo controlled. In this trial 18,314 smokers, former smokers, and workers exposed to asbestos were examined. The treatment group has a relative risk of lung cancer of 1.28 compared with the placebo group. However, the trial was stopped early.

Antioxidant mechanisms of carotenoids

Generally three mechanisms are discussed for the reaction of free radicals ($ROO^{\bullet}$, $R^{\bullet}$) with CAR: (i) radical addition, (ii) hydrogen abstraction from the CAR, and (iii) electron transfer reaction.

It has been proposed that peroxyl radicals add to CAR polyene chain to form a resonance-stabilized, carbon-centered CAR radical adduct [194]:

$$ROO^{\bullet} + Car \rightarrow ROO\text{-}Car^{\bullet} \quad (23)$$

which then interacts with another $ROO^{\bullet}$:

$$ROO\text{-}Car^{\bullet} + ROO^{\bullet} \rightarrow ROO\text{-}Car\text{-}ROO \quad (\text{non-radical products}) \quad (24)$$

yielding a non-radical product terminating the chain reaction. A second type of reaction is hydrogen abstraction from the CAR forming the neutral CAR radical, $Car^{\bullet}$:

$$R^{\bullet} + Car(H) \rightarrow RH + Car^{\bullet} \quad (25)$$

and finally an electron transfer reaction [195]:

$$R^{\bullet} + Car \rightarrow R^{-} + (Car)^{+\bullet} \quad (26)$$

Recently, there has accumulated a growing body of evidence which suggests that scavenging of lipid $ROO^{\bullet}$, where R is an aliphatic group, by β-carotene may not proceed *via* an electron transfer mechanism, reaction (26), but rather by adduct formation (reactions (23) and (24)) and/or hydrogen abstraction (reaction (25)).

Pro-oxidant mechanisms of carotenoids

Burton and Ingold [190] were among the first to propose that β-carotene might participate in lipid peroxidation as a pro-oxidant. The term 'pro-oxidant activity' involves the 'ability' of β-carotene to increase the total radical yield in the system [196–200].

The key factors in converting carotenoids from antioxidants to pro-oxidants are the partial pressure of dioxygen (pO_2) and carotenoid concentration. At higher pO_2, a carotenoid radical, $Car^{\bullet}$ (generated through the hydrogen abstraction reaction (25)) could react with dioxygen to generate a carotenoid-peroxyl radical, $Car\text{-}OO^{\bullet}$ [201]:

$$Car^{\bullet} + O_2 \rightarrow Car\text{-}OO^{\bullet} \quad (27)$$

This is an autoxidation process and $Car\text{-}OO^{\bullet}$ could act as a pro-oxidant by promoting oxidation of unsaturated lipid (RH):

$$Car\text{-}OO^{\bullet} + RH \rightarrow Car\text{-}OOH + R^{\bullet} \quad (28)$$

The pro-oxidant mechanism of β-carotene has also been reported for the radical adduct reactions (reactions (27) and (28)). Liebler and his group reported [202, 203] that at higher pO_2 the radical adduct $ROO\text{-}Car^{\bullet}$ can react directly with O_2 forming ROO-Car-$OO^{\bullet}$ radical (reaction (27)). Alternatively, it has been reported that decomposition of non-radical product ROO-Car–ROO yields carotenoid-epoxide (5,6-epoxy-β,β-carotene) and reactive $RO^{\bullet}$, reaction (29) [202]:

$$ROO\text{-}Car^{\bullet} + O_2 \rightarrow ROO\text{-}Car\text{-}OO^{\bullet} \quad (29)$$

$$ROO\text{-}Car\text{-}ROO \rightarrow Car\text{-epoxide} + RO^{\bullet} \quad (30)$$

The experimental conditions, such as pO_2, may determine which of these simultaneous reaction pathways would be the primary one.

Although there were some discernible trends in carotenoid reactivity for individual radicals, rate constants varied by no greater than a factor of 2.5. The mechanism and rate of scavenging is strongly dependent on the nature of the oxidising radical species but much less dependent on the carotenoid structure [204].

Conclusions and perspectives

The generation of OFR is a consequence of aerobic life. OFR represent a constant source of assaults to our genetic material that can be either enhanced or reduced by nutritional, hormonal, and environmental influences. At present, we do not know the exact role that damage by oxygen radical plays in carcinogenesis and its synergetic role with other forms of genetic events accelerating cell transformation and malignant progression. However, it is known that oxidative stress can participate in the initiation of proliferation of tumour cells. The effect of oxidative stress at a certain stage of carcinogenesis is directly related to the type and the reactivity of the radicals involved. Antioxidant enzymes together with non-enzymatic antioxidants are involved in OFR conversion. However, antioxidant protection against free radicals should be taken with caution since the antioxidant action might actually stimulate cancer progression through the enhanced survival of tumour cells.

In preventing OFR-related cancer, the key role seems to be reduction of endogenous and exogenous sources of oxidative stress and the elimination of environmental carcinogens. There is also the possibility that cancer treatment could make use of the results of OFR studies. Two classes of genes, referred to as oncogenes and tumour suppressor genes, have been demonstrated to participate in the sequence of events that results in pathological cell growth. Therefore, the involvement of oncogenes in all stages of radical-induced carcinogenesis seems to open the possibility of gene therapy for OFR-related cancer.

Acknowledgements

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