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Free Radicals and Antioxidants: A Personal View

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Free radicals and other oxygen-derived species are constantly generated in vivo, both by "accidents of chemistry" and for specific metabolic purposes. The reactivity of different free radicals varies, but some can cause severe damage to biological molecules, especially to DNA, lipids, and proteins. Antioxidant defense systems scavenge and minimize the formation of oxygen-derived species, but they are not 100% effective. Hence, diet-derived antioxidants may be particularly important in diminishing cumulative oxidative damage and helping us to stay healthier for longer. Repair systems exist to deal with molecules that have been oxidatively damaged. Damage to DNA by hydroxyl radicals appears to occur in all aerobic cells, and might be a significant contributor to the age-dependent development of cancer. Lipid peroxidation probably contributes significantly to the development of atherosclerosis.

Why Do We Need Antioxidants?

When living organisms first appeared on the Earth, they did so under an atmosphere containing very little O_2, i.e., they were essentially anaerobes. Anaerobic microorganisms still survive to this day, but their growth is inhibited and they can often be killed by exposure to 21% O_2, the current atmospheric level. As the O_2 content of the atmosphere rose (due to the evolution of organisms with photosynthetic water-splitting capacity) many primitive organisms may have died out. Present day anaerobes are presumably the descendants of those primitive organisms that followed the evolutionary path of "adapting" to rising atmospheric O_2 levels by restricting themselves to environments that the O_2 did not penetrate. However, other organisms began the evolutionary process of evolving antioxidant defense systems to protect against O_2 toxicity. In retrospect, this was a fruitful path to follow. Organisms that tolerated the presence of O_2 could also evolve to use it for metabolic transformations (oxidases, oxygenases, etc.) and for efficient energy production by using electron transport chains with O_2 as the terminal electron acceptor, such as those present in mitochondria. Our mitochondria make over 80% of the ATP we need, and the lethal effects of inhibiting this, e.g., by cyanide, show how important the mitochondria are.

It is interesting to note that we have developed antioxidant defenses to protect against 21% O_2, but no greater than that. This is evidenced by the fact that all aerobes suffer demonstrable injurious effects if exposed to O_2 at concentrations greater than 21%.[1] For example, exposure of adult humans to pure O_2 at 1 atm pressure for as little as 6 hours causes chest soreness, cough, and sore throat in some subjects, and longer periods of exposure lead to alveolar damage. The incidence of ocular damage in babies known as retrolental fibroplasia (formation of fibrous tissue behind the lens) increased abruptly in the early 1940s among infants born prematurely and often led to blindness. Not until 1954 was it realized that retrolental fibroplasia is associated with the use of high O_2 concentrations in incubators for premature babies. More careful control of O_2 concentrations (continuous transcutaneous O_2 monitoring, with supplementary O_2 given only where necessary) and administration of the lipid-soluble antioxidant α-tocopherol have decreased its incidence. However, the problem has not disappeared, since many premature infants need increased levels of O_2 in order to survive at all.[2]

The damaging effects of elevated O_2 on aerobes vary considerably with the organism studied, age, physiological state, and diet. Different tissues are affected in different ways.[1] Thus, cold-blooded animals, such as turtles and crocodiles, are relatively resistant to O_2 at low environmental temperatures but become more sensitive at higher temperatures. Young rats resist lung damage in an atmosphere of 100% O_2 far more effectively than do adult rats.[1]

The earliest suggestion made to explain O_2 tox-

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Table 1. Examples of Free Radicals

Name	Formula	Comments
Hydrogen atom	$H^{\cdot}$	The simplest free radical.
Trichloromethyl	$CCl_3^{\cdot}$	A carbon-centered radical (i.e., the unpaired electron resides on carbon). $CCl_3^{\cdot}$ is formed during metabolism of CCl_4 in the liver and contributes to the toxic effects of this solvent.[5] Carbon-centered radicals usually react fast with O_2 to make peroxyl radicals, e.g., $CCl_3^{\cdot} + O_2 \rightarrow CCl_3O_2^{\cdot}$.
Superoxide	$O_2^{\cdot -}$	An oxygen-centered radical. Selectively reactive.
Hydroxyl	$OH^{\cdot}$	A highly reactive oxygen-centered radical. Attacks all molecules in the human body.
Thiyl	$RS^{\cdot}$	A group of radicals with an unpaired electron residing on sulfur.
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Oxygen-centered radicals formed (among other routes) during the breakdown of organic peroxides.
Oxides of nitrogen	$NO^{\cdot}$, $NO_2^{\cdot}$	Nitric oxide is formed in vivo from the amino acid L-arginine.[6] Nitrogen dioxide is made when $NO^{\cdot}$ reacts with O_2 and is found in polluted air and smoke from burning organic materials, e.g., cigarette smoke.[7]

icity was that O_2 is a direct inhibitor of enzymes, thereby interfering with metabolism.[1] However, very few targets of direct damage by O_2 have been identified in aerobes. In 1954, Gerschman et al.[3] proposed that the damaging effects of O_2 could be attributed to the formation of oxygen radicals. This hypothesis was popularized and converted into the "superoxide theory of O_2 toxicity" following the discovery of superoxide dismutase (SOD) enzymes by McCord and Fridovich.[4] In its simplest form, this theory states that O_2 toxicity is due to excess formation of superoxide radical ($O_2^{\cdot -}$) and that the SOD enzymes are important antioxidant defenses.

What Is a Radical?

We need antioxidants to scavenge and prevent the formation of free radicals such as superoxide. But what exactly is a radical?

In the structure of atoms and molecules, electrons usually associate in pairs, with each pair moving within a defined region of space around the nucleus. This space is referred to as an atomic or molecular orbital. One electron in each pair has a spin quantum number of +½, the other −½. A free radical is any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons. An unpaired electron is any electron that is alone in an orbital. The simplest free radical is an atom of the element hydrogen, with one proton and a single electron. Table 1 gives examples of other free radicals[5–7] (a superscript dot is used to denote free radical species). The spectroscopic technique of electron spin resonance is specific for the detection and measurement of free radicals, recording the energy changes that occur as unpaired electrons respond to a magnetic field.[8]

What Radicals Are Made In Vivo?

Hydroxyl Radical

The chemical reactivity of free radicals varies. One of the most reactive is hydroxyl radical ($OH^{\cdot}$).[9] Exposure of living organisms to ionizing radiation causes fission of O–H bonds in water:

$$H_2O \rightarrow H^{\cdot} + OH^{\cdot}$$

to give $H^{\cdot}$ and $OH^{\cdot}$. Hydroxyl radical reacts at a diffusion-controlled rate with almost all molecules in living cells.[9] Hence, when $OH^{\cdot}$ is formed in vivo, it damages whatever it is generated next to, as $OH^{\cdot}$ cannot migrate any significant distance within the cell. Many, if not most, of the harmful effects of excess exposure to ionizing radiation upon living organisms are thought to be initiated by attack of $OH^{\cdot}$ upon proteins, carbohydrates, DNA, and lipids.

Nitric Oxide

Whereas $OH^{\cdot}$ is probably always harmful, other less reactive free radicals may often be useful in vivo. Free radicals are known to be produced metabolically in living organisms. For example, the free radical nitric oxide ($NO^{\cdot}$) is synthesized from the amino acid L-arginine by vascular endothelial cells, phagocytes, certain cells in the brain, and many other cell types.[6] Nitric oxide is a vasodilator agent and possibly an important neurotransmitter. It may also be involved in the killing of parasites by macrophages in some mammalian species.[6]

Superoxide

Superoxide radical ($O_2^{\cdot -}$) is the one-electron reduction product of oxygen. It is produced by phagocytic cells (neutrophils, monocytes, macrophages,

eosinophils) and helps them to inactivate viruses and bacteria.[10] Evidence is accumulating that $O_2^{\cdot -}$ is also produced in vivo by several cell types other than phagocytes, including lymphocytes[11] and fibroblasts.[12,13] Superoxide produced by such cells is often thought to be involved in intercellular signaling and growth regulation, and many experiments with cell cultures are consistent with this concept.[14] However, it has not yet been proven as occurring in vivo. There are many reports[15–17] that vascular endothelial cells generate $O_2^{\cdot -}$, but it is uncertain if they do this all the time in vivo or only after an insult, such as ischemia–reperfusion (or even the cell isolation process itself).

In addition to deliberate metabolic production of $O_2^{\cdot -}$, this radical can also emanate from what may be called "accidents of chemistry." Superoxide and H_2O_2 may be generated by "autoxidation" reactions, in which such compounds as catecholamines, tetrahydrofolates, and reduced flavins react directly with O_2 to form $O_2^{\cdot -}$. The $O_2^{\cdot -}$ then oxidizes more of the compound and sets up a free radical chain reaction. In fact, most so-called autoxidation reactions in vitro depend on the presence of traces of catalytic transition metal ions. Superoxide might also be made in the mitochondria. The mitochondrial electron transport chain is a gradient of redox potential, from the highly reducing NADH/NAD^+ couple to the oxidizing O_2. Thermodynamically, there is nothing to prevent constituents of the early part of the chain (nonheme-iron proteins, quinones, flavoproteins, cytochromes b) from reducing O_2 directly to make $O_2^{\cdot -}$. Fortunately, such reactions are kinetically restricted, so that most of the electrons entering the mitochondrial electron transport arrive at cytochrome oxidase, and only a small percentage (perhaps only 1–3%) may leak. These studies of the leakiness of electron transport chains are based on experiments with bacteria and mitochondria respiring in air-saturated solutions[4] and might be an overestimate. However, if we add in the deliberate $O_2^{\cdot -}$ production from phagocytes, and other sources (such as electron leakage from other electron-transport chains and autoxidation reactions), a figure of 1–3% does not seem unreasonable. Although this figure may seem trivial, remember that humans are big animals and breathe in a lot of O_2. Thus, at rest, we may produce close to 2 kg of $O_2^{\cdot -}$ per year (Figure 1).

An adult at rest utilizes 3.5 mL O_2/kg/minute
or 352.8 liters/day (assuming 70 kg body mass)
or 14.7 moles/day.
If 1% makes $O_2^{\cdot -}$
This is 0.147 moles/day
or 53.66 moles/year
or about 1.72 kg/year (of $O_2^{\cdot -}$).
During bodily exertion, this would increase
up to 10-fold
(assuming that the 1% figure still applied).

Figure 1. How much superoxide is made in the human body?

Hydrogen Peroxide, a Nonradical

Most of the $O_2^{\cdot -}$ generated in vivo probably undergoes a nonenzymatic or SOD-catalyzed dismutation reaction, represented by the overall equation:

$$2O_2^{\cdot -} + 2H^+ \dashrightarrow H_2O_2 + O^2.$$

This generates hydrogen peroxide (H_2O_2), a nonradical. H_2O_2 resembles water in its molecular structure and is very diffusible within and between cells. As well as arising from $O_2^{\cdot -}$, H_2O_2 is produced by the action of several oxidase enzymes in vivo, including amino acid oxidases and the enzyme xanthine oxidase.[18,19] Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid. Oxygen is simultaneously reduced both to $O_2^{\cdot -}$ and to H_2O_2. Low levels of xanthine oxidase are present in many mammalian tissues, especially in the gastrointestinal tract. Levels of xanthine oxidase often increase when tissues are subjected to insult, such as trauma or deprivation of oxygen.[19]

Some metabolic roles for H_2O_2 are known, and others have been proposed.[20–22] For example, H_2O_2 generated in the thyroid gland is used by a peroxidase enzyme to iodinate the thyroid hormones.[20] H_2O_2 may up-regulate the expression of certain genes by (directly or indirectly) leading to displacement of an inhibitory subunit from the cytoplasmic gene transcription factor NF-κB.[23] Displacement of the inhibitory subunit causes the active factor to migrate to the nucleus and activate many different genes by binding to specific DNA sequences in enhancer and promoter elements.[23] Thus, H_2O_2 and other peroxides can induce expression of genes controlled by NF-κB. This is of particular interest currently because in cell culture systems, H_2O_2 can activate NF-κB and induce the genetic expression of the provirus human immunodeficiency virus-1 (HIV-1). This virus is the most common cause of acquired immunodeficiency syndrome (AIDS), and its expression can be prevented by certain antioxidants.[23] In addition, peroxides in human atherosclerotic lesions may activate NF-κB.[24,25]

Some Confusing Terminology: Oxygen-Derived Species, Reactive Oxygen Species, and Oxidants

Reactive oxygen species (ROS) is a collective term often used by scientists to include not only the oxy-

INITIATION of peroxidation usually occurs by the attack of any species (R$^{\cdot}$) capable of abstracting hydrogen from a polyunsaturated fatty acid side-chain in a membrane (such fatty acid side-chains are more susceptible to free radical attack than are saturated or monounsaturated side-chains).

$$-CH + R^{\cdot} \rightarrow -C^{\cdot} + RH$$

Species able to abstract hydrogen include OH$^{\cdot}$ and peroxyl radicals (Table 1). The carbon-centered radical reacts fast with O_2

$$-C^{\cdot} + O_2 \rightarrow -CO_2$$

A fatty acid side-chain **PEROXYL RADICAL** is formed. This can attack adjacent fatty acid side-chains and **PROPAGATE** lipid peroxidation.

$$-CO_2^{\cdot} + -CH \rightarrow -CO_2H + -C^{\cdot}$$

The chain reaction thus continues and **LIPID PEROXIDES** ($-CO_2H$) accumulate in the membrane. Lipid peroxides destabilize membranes and make them "leaky" to ions. Peroxyl radicals can attack not only lipids but also membrane proteins (e.g., damaging enzymes, receptors, and signal transduction systems) and oxidize cholesterol.

Figure 2. An outline of lipid peroxidation.

gen-centered radicals ($O_2^{\cdot -}$, and OH$^{\cdot}$) but also some nonradical derivatives of O_2. These include H_2O_2, hypochlorous acid (HOCl, an oxidizing and chlorinating agent produced by activated phagocytes),[10,26] and ozone (O_3). "Reactive" is, of course, a relative term; neither $O_2^{\cdot -}$ nor H_2O_2 is particularly reactive in aqueous solution. Hence, some authors use the term "oxygen-derived species" instead. Another popular collective term is "oxidants." However, since $O_2^{\cdot -}$ and H_2O_2 can act as both oxidants and reductants in aqueous solution, I prefer to avoid that term.

How Do Radicals React?

Reactivity depends on the radical and what the radical is presented with. If two free radicals meet, they can join their unpaired electrons to form a covalent bond. Thus, atomic hydrogen forms diatomic hydrogen:

$$H^{\cdot} + H^{\cdot} \rightarrow H_2$$

A more biologically relevant example is the very fast[27] reaction of NO$^{\cdot}$ and $O_2^{\cdot -}$ to form a nonradical product, peroxynitrite[28]:

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^{-} \text{ (peroxynitrite)}$$

However, when a free radical reacts with a nonradical, a new radical results, and a chain reaction is set up. Since most biological molecules are nonradicals, the generation of reactive radicals such as OH$^{\cdot}$ in vivo usually sets off chain reactions.

For example, attack of reactive radicals upon fatty acid side chains in membranes and lipoproteins can abstract hydrogen, leaving a carbon-centered radical and initiating the process of lipid peroxidation (Figure 2).

When OH$^{\cdot}$ is generated adjacent to DNA, it attacks both the deoxyribose sugar and the purine and pyrimidine bases. Figure 3 shows the structures of some of the products generated by attack of OH$^{\cdot}$ on the DNA bases. This wide range of products is characteristic of attack by OH$^{\cdot}$ and may be used to show that such attack has occurred in vivo. For example, if DNA is extracted from a tissue and most or all of the compounds shown in Figure 3 are present, this is good evidence[29] that the DNA has suffered attack by OH$^{\cdot}$. Such "OH$^{\cdot}$-fingerprint" experiments have been used to study the role of free radicals in DNA damage by radiation and toxic agents.[29] It has also been found that the amount of these OH$^{\cdot}$-derived products in DNA from human cancerous tumors is greater than in adjacent noncancerous tissue.[30,31] Whether this is due to increased OH$^{\cdot}$ formation or to decreased repair of the damage remains to be evaluated.

Toxicity of Superoxide, Hydrogen Peroxide, and Nitric Oxide

Experimental results clearly show that removal of $O_2^{\cdot -}$ and H_2O_2 by antioxidant defense systems is essential for healthy aerobic life.[4,18,32,33] Why is this? In organic media, $O_2^{\cdot -}$ can be very reactive, but in aqueous media it is not, mainly acting as a moderate reducing agent.[34] However, superoxide can react very rapidly with a few targets. These include some bacterial iron–sulfur proteins, including enzymes essential to metabolism such as aconitase.[35,36] Whether similar $O_2^{\cdot -}$-sensitive targets exist in hu-

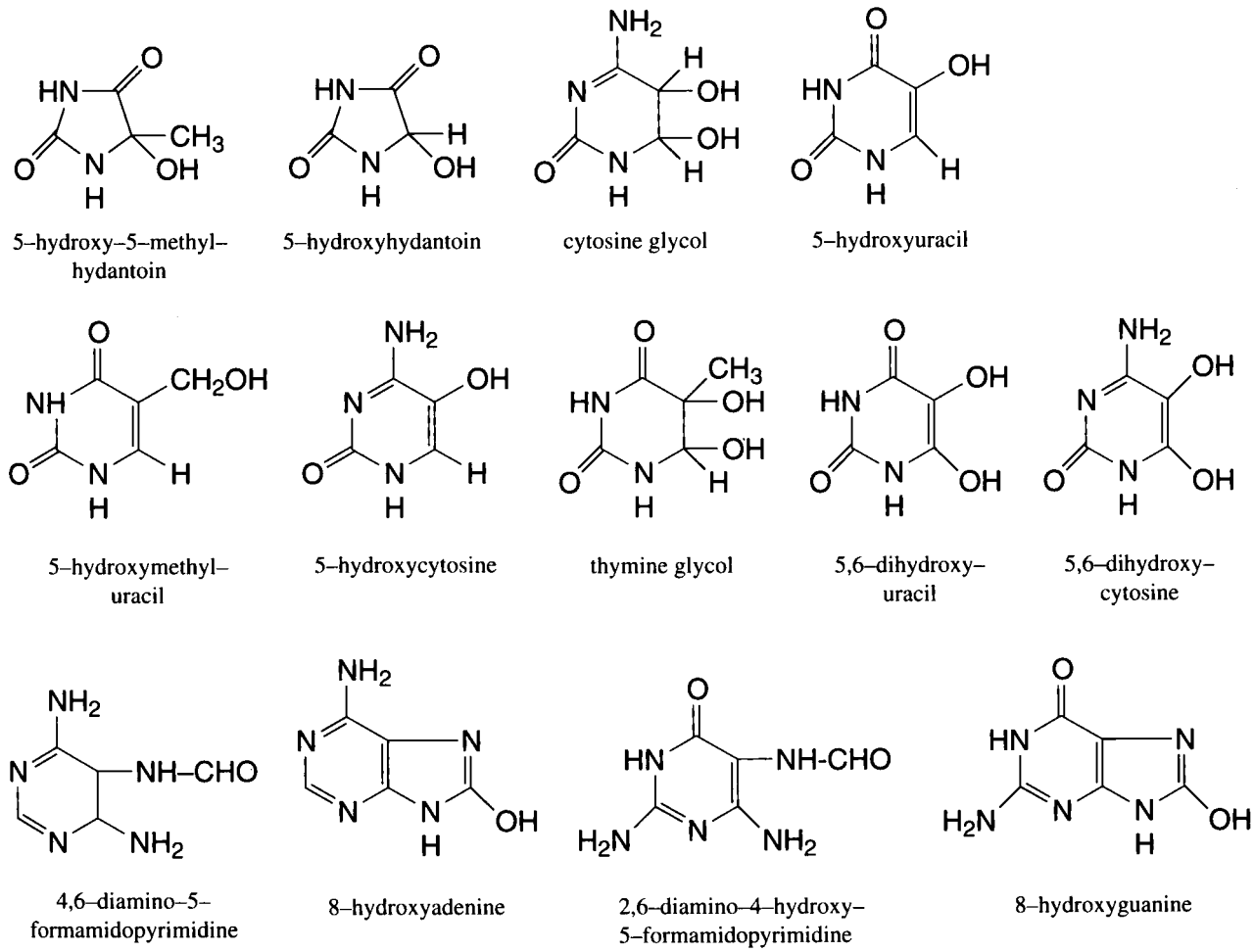

Figure 3. Products of attack of hydroxyl radicals upon purine and pyrimidine bases of DNA.

man cells remains to be established. However in isolated submitochondrial particles, $O_2^{\cdot -}$ has been claimed to inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain.[37]

One important molecule in humans that reacts with $O_2^{\cdot -}$ is $NO^{\cdot}$. $NO^{\cdot}$ is useful in human metabolism, but an excess can be cytotoxic, and this mechanism of tissue injury has been implicated in several human diseases.[6] Excess $NO^{\cdot}$ can be directly toxic, e.g., by damaging iron–sulfur proteins,[6] but evidence is increasing to show that $NO^{\cdot}/O_2^{\cdot -}$ interactions are also involved.[38,39] Since $NO^{\cdot}$ acts upon smooth muscle cells in vessel walls to produce relaxation,[6] then $O_2^{\cdot -}$, by removing $NO^{\cdot}$, can act as a vasoconstrictor, which might have deleterious effects in some clinical situations.[40,41] For example, excess vascular $O_2^{\cdot -}$ production could lead to hypertension[41] and is implicated in the development of atherosclerosis.[39] Peroxynitrite, the product of $NO^{\cdot}$ and $O_2^{\cdot -}$, can cause direct biological damage by oxidizing –SH groups.[38,42] In addition, at physiological pH, peroxynitrite can protonate and decompose to a range of noxious products, including nitrogen dioxide ($NO_2^{\cdot}$), a powerful initiator of lipid peroxidation in biological fluids,[43] $OH^{\cdot}$, and nitronium ion (NO_2^+), an agent active in nitrating aromatic rings, such as those of phenylalanine and tyrosine.[38,44,45]

The protonated form of $O_2^{\cdot -}$, perhydroxyl radical ($HO_2^{\cdot}$) is much more reactive than $O_2^{\cdot -}$ in vitro,[34] but very little $O_2^{\cdot -}$ is protonated at pH 7.4. For example, $HO_2^{\cdot}$ can initiate peroxidation of polyunsaturated fatty acids, which $O_2^{\cdot -}$ cannot[46,47]:

$$L\text{–}H + HO_2^{\cdot} \rightarrow L^{\cdot} + H_2O_2.$$

However, $HO_2^{\cdot}$ is not a very efficient initiator and there is no direct evidence that $HO_2^{\cdot}$ exerts damaging effects in vivo.

Hydrogen peroxide at micromolar levels also appears poorly reactive, but higher (>50 μM) levels of H_2O_2 can attack certain cellular targets.[32,48] For example, it can oxidize an essential –SH group on the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, blocking glycolysis and interfering with other aspects of cell energy metabolism.[48]

In 1970, Beauchamp and Fridovich[49] proposed

that the toxicity of $O_2^{\cdot -}$ and H_2O_2 could pertain to their conversion into $OH^{\cdot}$. Three mechanisms have been proposed to explain this. One is the interaction of $O_2^{\cdot -}$ and $NO^{\cdot}$, as described above. An earlier proposal[50] was the superoxide-driven Fenton reaction:

$$O_2^{\cdot -} + Fe(III) \rightarrow Fe(II) + O_2$$

and

$$Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe(III)$$

Despite repeated controversy in the literature, this author feels that the evidence for the formation of $OH^{\cdot}$ when Fe^{2+} reacts with H_2O_2 is overwhelming.[32,50,51] This does not, of course, preclude the formation of reactive species additional to $OH^{\cdot}$, such as oxo-iron complexes (ferryl, perferryl). Copper ions also catalyze formation of $OH^{\cdot}$ from H_2O_2.[50] A third mechanism for making $OH^{\cdot}$ is the reaction of $O_2^{\cdot -}$ with hypochlorous acid:[52]

$$O_2^{\cdot -} + HOCl \rightarrow OH^{\cdot} + OH^- + O_2$$

This has a rate constant of $7.5 \times 10^6\ M^{-1}\ second^{-1}$.

Do Metal Ion "Catalysts" Exist In Vivo?

Iron and copper ions in chemical forms that can decompose H_2O_2 to $OH^{\cdot}$ are in very short supply in vivo. The human body is very careful to ensure that as much iron and copper as possible is kept safely bound to transport or storage proteins. Indeed, this "sequestration" of metal ions is an important antioxidant defense mechanism.[32,53] Sequestration of metal ions deters the growth of bacteria in human body fluids[54] and also ensures that such fluids will, in general, not convert $O_2^{\cdot -}$ and H_2O_2 into $OH^{\cdot}$. If iron or copper does become available to catalyze free radical reactions in body fluids, as happens in certain metal overload diseases, severe damage to many body tissues occurs.[55,56]

Hence, a major determinant of the nature of the damage done by excess generation of reactive oxygen species in vivo may be the availability and location of metal ion catalysts of $OH^{\cdot}$ radical formation.[32,50] If, for example, "catalytic" iron salts are bound to DNA in one cell type and to membrane lipids in another, then excessive formation of H_2O_2 and $O_2^{\cdot -}$ will, in the first case, damage the DNA and in the second case could initiate lipid peroxidation. Evidence for $OH^{\cdot}$ formation in the nucleus of cells treated with H_2O_2 has been obtained by showing that all four DNA bases are modified in the way characteristic of $OH^{\cdot}$ attack (Figure 3).[57] If this $OH^{\cdot}$ is formed by metal ion-dependent reactions, then the "catalytic" metal ions must be bound to the DNA itself.

Escherichia coli mutants lacking SOD activity are hypersensitive to damage by H_2O_2,[33] and if able to enter the cell, extra SOD can protect cells against damage by H_2O_2.[58] These and much other data are consistent with a role of $O_2^{\cdot -}$ in facilitating damage by H_2O_2, and the Fenton reactions provide a possible explanation. However, many scientists are reluctant to believe that $O_2^{\cdot -}$ serves only as a reducing agent for Fe(III), since in general, mammalian tissues are fairly good reducing environments containing thiols, NADH, NADPH, reduced folates, and ascorbate.[50] An additional, and possibly more important role for $O_2^{\cdot -}$ may be in the provision of the iron required for $OH^{\cdot}$ generation. Thus, $O_2^{\cdot -}$ can reductively mobilize iron ions from the iron storage protein ferritin.[59] Because the amount of $O_2^{\cdot -}$-releasable iron is very small, ferritin-bound iron is much safer than an equivalent amount of "free" iron.[32,60] Superoxide might also release iron if it attacks iron-sulfur proteins.[35,36]

Antioxidant Defenses

All organisms suffer some exposure to $OH^{\cdot}$, because it is generated by homolytic fission of O–H bonds in water driven by background ionizing radiation.[9] This radical is so reactive with all biological molecules that it is impossible to evolve a specific scavenger of it. Almost everything in living organisms reacts with $OH^{\cdot}$ with second-order rate constants of 10^9–$10^{10}\ M^{-1}\ second^{-1}$, so that collision of $OH^{\cdot}$ with the molecules almost always results in reaction.[9] Once $OH^{\cdot}$ has been formed, damage caused by this radical is probably unavoidable and is dealt with by repair processes (Table 2).[61–67] A large part of the body's antioxidant defenses serve to minimize any additional production of $OH^{\cdot}$.

Enzymes

Living organisms have evolved antioxidant defenses to remove excess $O_2^{\cdot -}$ and H_2O_2. SOD enzymes remove $O_2^{\cdot -}$ by accelerating its conversion to H_2O_2 by about four orders of magnitude at pH 7.4. Mammalian cells have a SOD enzyme containing manganese (MnSOD) at its active site in the mitochondria. A SOD with copper and zinc (CuZnSOD) at the active site is also present, but largely in the cytosol.[4] It has recently been shown that the familial dominant form of amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), a fatal degenerative disorder of motor neurons in the brain and spinal cord, is somehow related to mutations in the CuZn protein. These mutations decrease activity somewhat but may also cause the protein to become toxic[68,69], perhaps by releasing copper (a powerful prooxidant) and/or by acting as a peroxidase.[69]

Because SOD enzymes generate H_2O_2, they work in collaboration with H_2O_2-removing enzymes. Catalases convert H_2O_2 to water and O_2

Table 2. Repair of Oxidative Damage

Substrate of Damage	Repair System	Representative Recent References
DNA: All components of DNA can be attacked by $OH^{\cdot}$, whereas singlet O_2 attacks guanine preferentially. H_2O_2 and $O_2^{\cdot -}$ do not attack DNA.	A wide range of enzymes exists that recognize abnormalities in DNA and remove them by excision, resynthesis, and rejoining of the DNA strand.	29,61
Proteins: Many reactive oxygen species can oxidize –SH groups. Hydroxyl radicals attack many amino acid residues. Proteins often bind transition metal ions, making them a target of attack by "site-specific" $OH^{\cdot}$ generation.	Oxidized methionine residues may be repaired by methionine sulfoxide reductase. Damaged proteins may be recognized and preferentially destroyed by cellular proteases.	62–64
Lipids: Some reactive oxygen species (not including $O_2^{\cdot -}$ or H_2O_2) can initiate lipid peroxidation.	Chain-breaking antioxidants (especially α-tocopherol) remove chain-propagating peroxyl radicals. Phospholipid hydroperoxide glutathione peroxidase can remove peroxides from membranes, as can some phospholipases. Normal membrane turnover can release damaged lipids.	65–67

$$2H_2O_2 \rightarrow 2H_2O_2 + O_2$$

Catalases are present in the peroxisomes of mammalian cells, and probably serve to destroy H_2O_2 generated by oxidase enzymes located within these subcellular organelles.[18] However, the most important H_2O_2-removing enzymes in mammalian cells are the selenoprotein glutathione peroxidase (GSHPX) enzymes. A selenocysteine residue, essential for enzyme activity, is present at the active site. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein (FAD-containing) enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power[18] (Figure 4).

Sequestration of Transition Metals

As already emphasized, an additional important antioxidant defense system is that which allows organisms to generate metal ion storage and transport proteins. These are produced to keep iron and copper safely protein-bound whenever possible.

Low-Molecular Mass Antioxidants

GSH

In addition to antioxidant defense enzymes, some low-molecular mass free radical scavengers exist. GSH can scavenge various free radicals directly, as well as being a substrate for GSHPX enzymes.[18]

GLUTATHIONE PEROXIDASE

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

$$2GSH + \text{FATTY ACID–OOH} \rightarrow GSSG + \text{FATTY ACID–OH} + H_2O$$

GLUTATHIONE REDUCTASE

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Reduced glutathione is a tripeptide, glutamic acid–cysteine–glycine. It is present at millimolar concentrations in most mammalian cells and has multiple metabolic functions.[18] In oxidized glutathione (GSSG), two tripeptides are linked by a disulfide bridge. Glutathione peroxidase can also destroy fatty acid (lipid–OOH) peroxides by converting them to alcohols (lipid–OH).[18] Mammalian cells additionally contain a phospholipid hydroperoxide glutathione peroxidase that can apparently perform the same reaction upon lipid peroxides within membranes; how exactly it works is uncertain as yet.[66]

Figure 4. The glutathione system.

Vitamin E

Alpha-tocopherol, the major constituent of the fat-soluble vitamin known as vitamin E, is the most important[65] (but by no means the only[70,71]) free radical scavenger within membranes and lipoproteins. Alpha-tocopherol inhibits lipid peroxidation by scavenging peroxyl radicals (Table 1), which are intermediates in the chain reaction:

$$\alpha TH + LOO^{\cdot} \rightarrow \alpha T^{\cdot} + LOOH$$

The α-tocopherol radical ($\alpha T^{\cdot}$), although not completely unreactive,[72] is less efficient at abstracting hydrogen than are peroxyl ($LOO^{\cdot}$) radicals, so the chain reaction of peroxidation is slowed.[65] Several biological mechanisms may exist for recycling $T^{\cdot}$ back to α-tocopherol, although none of them has yet been proven rigorously to operate in vivo in humans.[65] Likely mechanisms include the reaction of $T^{\cdot}$ with ascorbic acid at the surface of membranes and lipoproteins:[71]

$$\alpha T^{\cdot} + \text{ascorbate} \rightarrow \alpha TH + \text{ascorbate}^{\cdot}$$

and/or with ubiquinol (reduced coenzyme Q) within membranes or lipoproteins:[70,73]

$$CoQH_2 + \alpha T^{\cdot} \rightarrow \alpha TH + CoQH^{\cdot} + H^{+}.$$

Vitamin E is known to be essential in the human diet. Severe deprivation produces neurological damage.[74]

Ascorbic Acid

Ascorbic acid, the water-soluble vitamin C, plays several essential metabolic roles in vivo,[75] such as in the synthesis of collagen. There is also frequent discussion of its importance as an antioxidant. Ascorbic acid is indeed a good scavenger of several reactive oxygen species[76a] as summarized in Table 3, and it probably helps to recycle α-tocopherol in vivo. However, in the presence of transition metal ions (iron and copper), ascorbate can become pro-oxidant, acting as a reducing agent and generating $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$. Normally, since such metal ions are available in very limited amounts in vivo, the antioxidant properties of ascorbate predominate.[76] However, ascorbate can be toxic if given to iron-overloaded patients without iron ion chelators.[77] In disease and tissue injury, transition metal ions do sometimes become more available, and the possibility that pro-oxidant actions of ascorbate might occur should not be ignored.[50,53,78]

Table 3. Ascorbic Acid as an Antioxidant

Scavenges O_2^{-} and $HO_2^{\cdot}$ (overall rate constant about 2.7×10^5 M^{-1} $second^{-1}$ at pH 7.4).
Scavenges water-soluble peroxyl ($RO_2^{\cdot}$) radicals.
Scavenges thiyl and sulfenyl radicals.
"Repairs," and so prevents damage by, radicals arising by attack of $OH^{\cdot}$ upon uric acid.
Can reduce carcinogenic nitrosamines to inactive products.
Powerful scavenger of hypochlorous acid and an alternative substrate for the enzyme myeloperoxidase (possibly slowing HOCl formation).
Inhibits lipid peroxidation by hemoglobin– or myoglobin–H_2O_2 mixtures and prevents heme breakdown to release iron ions, by being preferentially oxidized.
Powerful scavenger and quencher of singlet O_2 in aqueous solution.
Regenerates α-tocopheryl radicals in membranes and lipoproteins.
Scavenges nitroxide radicals.
Scavenges $OH^{\cdot}$ radicals (rate constant $>10^9$ M^{-1} $second^{-1}$).
Protects plasma lipids against peroxidation induced by activated neutrophils.
May protect against oxygen-derived species present in cigarette smoke.
Powerful scavenger of O_3 and $NO_2^{\cdot}$ in human body fluids, especially lung-lining fluids.

Repair Systems

Normally, the production of ROS is approximately balanced by the antioxidant defense systems, i.e., the antioxidants are not present in great excess (hence the toxicity of extra O_2). One reason for this may be that production of some $O_2^{\cdot -}$ and H_2O_2 is useful in vivo, so that the body does not scavenge them with 100% efficiency. Indeed, there is good evidence for ongoing oxidative damage in the human body (Table 4),[79–84] making clear the importance of repair systems.

Repair of oxidative DNA damage is particularly important,[29,61] as the constant assault on our DNA by free radicals may contribute to the development of "spontaneous" human cancers.[80,85] Indeed, it has been suggested that the aging process involves cumulative free radical damage over a lifetime.[86]

Oxidative Stress

Because production of ROS and antioxidant defense are approximately balanced, it is easy to tip this balance in favor of the ROS and create the situation of oxidative stress.[87] Oxidative stress can result from:

1) depletions of antioxidants due to malnutrition[87a] (e.g., through inadequate dietary intakes of α-tocopherol, ascorbic acid, sulfur-containing amino acids needed for GSH manufacture, or riboflavin) (needed to make the FAD cofactor in glutathione reductase);
2) excess production of reactive oxygen species,

Table 4. Evidence that Oxidative Damage Occurs In Vivo

Target of Damage	Evidence
DNA	Urinary excretion of DNA base damage products.[79,80] Low baseline levels of DNA damage products in DNA isolated from human cells (reviewed in Halliwell and Dizdaroglon[81]).
Protein	Attack of free radicals upon proteins produces protein carbonyls. Low levels of these can be detected in human tissues and body fluids.[62]
Lipid	Accumulation of "age pigments" in tissues (reviewed in Halliwell and Gutteridge[32]). Lipid peroxidation in atherosclerotic lesions.[82] Presence of end products of peroxidation in animal body fluids (for a recent review that examines the methodological problems in measuring such products see Halliwell and Chirico[83]).
Uric acid	Attacked by several reactive oxygen species to generate allantoin, cyanuric acid, parabanic acid, oxonic acid, etc. These products are found in human body fluids and levels increase during oxidative stress.[84]

e.g., by exposure to elevated O_2 concentrations, the presence of toxins that are metabolized to produce free radicals, or excessive activation of "natural" radical-producing systems (e.g., inappropriate activation of phagocytic cells in chronic inflammatory diseases, such as rheumatoid arthritis and ulcerative colitis).[32,88,89]

Cells can tolerate mild oxidative stress, which often results in up-regulation of the synthesis of antioxidant defense systems in an attempt to restore the balance. For example, if rats are gradually acclimatized to elevated O_2, they can tolerate pure O_2 for much longer than control rats, apparently due to increased synthesis of antioxidant defense enzymes and of GSH in the lung.[90,91] However, severe oxidative stress can produce major interdependent derangements of cell metabolism, including DNA strand breakage (often an early event), rises in intracellular "free" Ca^{2+}, damage to membrane ion transporters and/or other specific proteins, and peroxidation of lipids. Cell injury and death may result.[48,62,92,93] Oxidative stress can also lead to the release of transition metal ions able to promote such deleterious events as $OH^{\cdot}$ formation.[32,93] It may, for example, cause Fe^{2+} release from mitochondria.[94] Superoxide can reductively mobilize small amounts of ferrous iron from ferritin.[59,60] H_2O_2 can degrade hemoglobin, myoglobin, and probably other heme proteins to release iron ions from the heme ring.[95,96]

Oxidative Stress and Human Disease

Does oxidative damage play a role in human disease? We have already seen that many of the biological consequences of excess radiation exposure may be due to $OH^{\cdot}$-dependent damage, and oxidative damage may account for damage to the retina of the eye (retinopathy of prematurity) in premature babies. Indeed, there are many papers in the biomedical literature suggesting a role for oxidative stress in other human diseases.[97]

Tissue damage by disease, trauma, toxic agents, and other causes usually leads to formation of increased amounts of putative "injury mediators," such as prostaglandins, excess $NO^{\cdot}$, leukotrienes, interleukins, interferons, and tumor necrosis factors (TNFs). All of these at various times have been suggested as playing important roles in different human diseases. Currently, for example, there is much interest in the roles played by TNFα, $NO^{\cdot}$, and interleukins in adult respiratory distress syndrome and septic shock. ROS can be placed in the same category, in that tissue damage will usually lead to increased ROS formation and oxidative stress. Figure 5 summarizes some of the reasons for this. Indeed, in most human diseases, oxidative stress is a secondary phenomenon, a consequence of the disease activity. That does not mean it is not important! Its importance varies in different disease states.[88] For example, oxidative damage to lipids in blood vessel walls seems to be a significant contributor to the development of atherosclerosis.[24,25,82] Oxidative DNA damage may contribute to cancer and aging.[80,85,86] Excess production of radicals probably contributes significantly to tissue damage in rheumatoid arthritis[88] and in inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis.[89] There is growing evidence[98] that oxidative damage occurs in the neurodegenerative diseases (including the familial dominant form of ALS)[68,69] and after traumatic brain injury.[99]

Since oxidative stress occurs to some degree after every tissue injury, the main question to be asked in evaluating its role in human disease is not "can we demonstrate oxidative stress?" but rather "does the oxidative stress that occurs make a significant contribution to disease activity?" The answer to the latter question appears to be "yes" in at least some cases. These include rheumatoid arthritis, the familial dominant form of ALS, and perhaps some other neurodegenerative diseases,[98] neurotrauma,[99] atherosclerosis,[82,100] and ulcerative colitis.[89] However, it may well be "no" in many others. Elucidating the precise role played by free

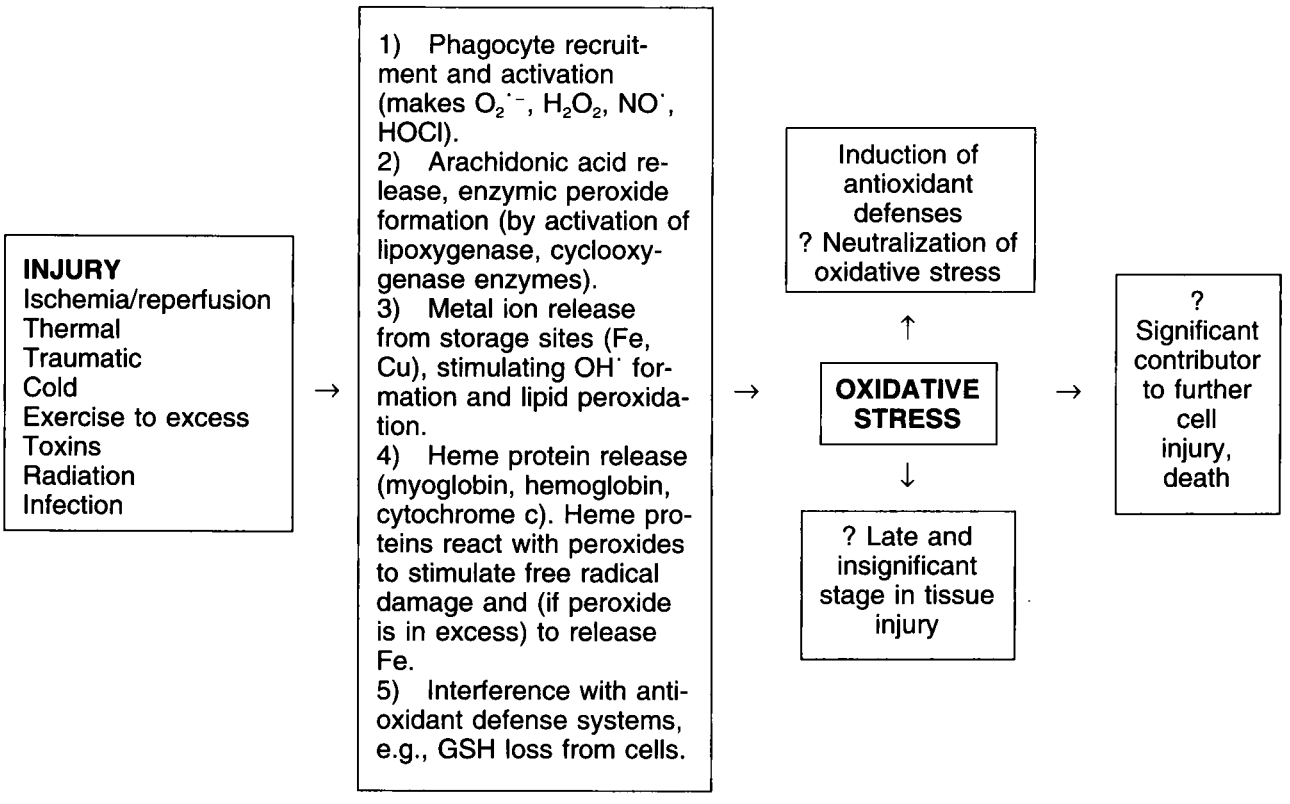

Figure 5. How tissue damage can cause oxidative stress.

radicals has not been easy because they are difficult to measure, but the development of modern assay techniques is helping to solve this problem.[88]

Conclusion: Nutritional Implications

We obtain certain essential antioxidants from the diet, primarily the tocopherols. Ascorbic acid may also have antioxidant functions. Many plant constituents have antioxidant activities in vitro, e.g., the flavonoids and other plant phenolics, but their bioavailability to humans and the significance of any role they might play are not clearly established, although research in this area is growing.[101] Like ascorbate, many plant phenolics exert pro-oxidant effects in the presence of transition metal ions in vitro,[102] but, again, the significance of this in vivo is uncertain. Epidemiologic evidence is accumulating to support the view that tocopherols decrease atherosclerosis and delay death from myocardial infarction, presumably by inhibiting lipid peroxidation.[103] Carotenoids, such as β-carotene and other plant pigments, may also have preventive effects against cancer and cardiovascular disease.[104] Several carotenoids can be made to exert antioxidant effects in vitro, but there is no evidence as yet that this mechanism produces any beneficial action in humans.[105,106]

Our endogenous antioxidant defenses are inadequate to prevent oxidative damage completely. Hence, sources of dietary antioxidants may be especially important to us. Determining their optimal intake is one of the greatest challenges in the nutrition and free radicals field today.

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