Oxygen-derived Species: Their Relation to Human Disease and Environmental Stress

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Free radicals and other reactive oxygen species (ROS) are constantly formed in the human body, often for useful metabolic purposes. Antioxidant defenses protect against them, but these defenses are not completely adequate, and systems that repair damage by ROS are also necessary. Mild oxidative stress often induces antioxidant defense enzymes, but severe stress can cause oxidative damage to lipids, proteins, and DNA within cells, leading to such events as DNA strand breakage and disruption of calcium ion metabolism. Oxidative stress can result from exposure to toxic agents, and by the process of tissue injury itself. Ozone, oxides of nitrogen, and cigarette smoke can cause oxidative damage; but the molecular targets that they damage may not be the same. — Environ Health Perspect 102(Suppl 10):5–12 (1994)

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Introduction

There is considerable current interest in the role of free radicals, oxygen radicals, and oxidative stress as mediators of tissue injury in human disease and of the effects of air pollutants, such as ozone (O_3), nitrogen dioxide (NO_2), and tobacco smoke. This article explains the meaning of these terms and summarizes current knowledge of the roles they play in these various situations.

What Is a Radical?

In the structure of atoms and molecules, electrons usually associate in pairs, each pair moving within a defined region of space (an atomic or molecular *orbital*). One electron in each pair has a spin quantum number of $+1/2$, the other $-1/2$. A free radical is any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons, an unpaired electron being one that is alone in an orbital (*1*). The simplest free radical is a hydrogen atom, with one proton and a single electron. Table 1 gives some examples of other free radicals. Note that the gases nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are free radicals, whereas ozone (O_3) is not—no unpaired electrons are present. The spectroscopic

technique of electron spin resonance is often used to measure free radicals; it records the energy changes that occur as unpaired electrons align in response to a magnetic field. A superscript dot ($\bullet$) is used to denote free radical species.

Oxygen—A Free Radical and Environmental Toxin

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 probably died. 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 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 since organisms that tolerated the presence of O_2 could also evolve to use it for metabolic transformations (involving enzymes such as oxidases and oxygenases) and for efficient energy production (by using electron transport chains with O_2 as the terminal electron acceptor, such as the mitochondrial oxidative phosphorylation system). Hence, O_2 was probably the first environ-

mental air pollutant to appear in large quantities on the planet.

However, even present-day aerobes suffer oxidative damage if they are exposed to O_2 at concentrations greater than 21% (*2*). Oxygen toxicity has been demonstrated in plants, animals and microorganisms. For example, exposure of adult humans to pure O_2 at 1 atm pressure for as little as 6 hr causes chest soreness, cough, and sore throat in some subjects; and longer periods of exposure lead to lung 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 babies born prematurely and led to many cases of 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 α-tocopherol have decreased its incidence, but the problem has not disappeared, since many premature infants need continuous high O_2 to survive at all (*3*).

The damaging effects of elevated O_2 on aerobes vary considerably with the organism studied, age, physiologic state, and diet; and different tissues are affected in different ways. 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. Neonatal rats resist lung damage in an atmosphere of 100% O_2 far more effectively than do adult rats (*2*).

Table 1. Examples of free radicals.

Name	Formula	Comments
Hydrogen atom	H•	The simplest free radical known.
Trichloromethyl	$CCl_3^•$	A carbon-centered radical (i.e., the unpaired electron resides on carbon). $CCl_3^•$ is formed during metabolism of CCl_4 in the liver and contributes to the toxic effects of this solvent [7].
Superoxide	$O_2^•$	An oxygen-centered radical.
Hydroxyl	OH•	An oxygen-centered radical. The most highly reactive oxygen radical known.
Thiyl	RS•	General name for a group of radicals with an unpaired electron residing on sulfur.
Peroxyl, alkoxyl	$RO_2^•$, RO•	Oxygen-centered radicals formed during the breakdown of organic peroxides.
Oxides of nitrogen	NO•, $NO_2^•$	Both are free radicals. NO• is formed in vivo from the amino acid L-arginine [8]. $NO_2^•$ is made when NO• reacts with O_2 and is found in polluted air and smoke from burning organic materials, e.g., cigarette smoke.

The earliest suggestion made to explain O_2 toxicity was that O_2 is a direct inhibitor of enzymes, thereby interfering with metabolism. However, very few targets of direct damage by O_2 have been identified in aerobes. In 1954, Gerschman et al. [4] 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 [5]. In its simplest form, this theory states that O_2 toxicity is due to excess formation of superoxide radical (O_2^-), the one electron reduction product of O_2, and that the SOD enzymes are important antioxidant defenses because they remove O_2^-. Ironically, with all the fuss made about oxygen radicals, it must be realized that the diatomic oxygen molecule is itself a free radical, containing two unpaired electrons [1]. Fortunately, the electronic arrangement in O_2 renders this molecule unreactive despite its free radical nature [1].

Reactive Oxygen Species in Vivo

Reactive oxygen species (ROS) is a collective term used by biologists to include not only oxygen radicals (O_2^- and hydroxyl radical, OH•) but also some derivatives of O_2 that do not contain unpaired electrons, such as hydrogen peroxide (H_2O_2), singlet $O_2(^1\Delta g)$, and hypochlorous acid (HOCl)*. Reactive is of course a relative term: O_2^- is more reactive than O_2, but neither O_2^- nor H_2O_2 in aqueous solution is anywhere near as reactive as OH•[1].

All organisms suffer some exposure to OH•, because it is generated in vivo by homolytic fission of O–H bonds in water, driven by our continuous exposure to background ionizing radiation [6]. Hydroxyl 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• with second-order rate constants of 10^9 to 10^{10} $M^{-1}sec^{-1}$ (essentially, if OH• contacts the compound, reaction occurs). Damage caused by OH•, once this radical has been formed, is probably unavoidable and is dealt with by repair processes (Table 2).

It is now well established [1,5,18–20] that O_2^- and H_2O_2 are produced in aerobes,

although the precise amounts generated and the steady-state concentrations achieved are still uncertain. Generation of these species occurs by two types of processes described below.

"Accidental" Generation. This encompasses such mechanisms as "leakage" of electrons onto O_2 from mitochondrial electron transport chains, microsomal cytochromes P450 and their electron donating enzymes, and other systems [1,5,20]. It also includes so-called autoxidation reactions in which compounds such as catecholamines, ascorbic acid, and reduced flavins are alleged to react directly with O_2 to form O_2^- [5]. In fact, such autoxidations are usually catalyzed by transition metal ions [1].

Deliberate Synthesis. The classic example of deliberate metabolic generation of ROS for useful purposes is the production of O_2^-, HOCl, and H_2O_2 by activated phagocytes [21]. Hydrogen peroxide is additionally generated in vivo by several oxidase enzymes, such as glycolate oxidase, xanthine oxidase, and D-amino acid oxidase [18,22]. Evidence is accumulating that O_2^- is also produced by several cell types other than phagocytes, including lymphocytes [23], fibroblasts [24,25], and vascular endothelial cells [26–28]. Such O_2^- might be involved in intercellular signalling and could serve important biologic functions, although more information is needed.

Table 2. Repair of oxidative damage.

Substrate of damage	Repair system	Representative recent references
DNA All components of DNA can be attacked by OH•, whereas singlet O_2 attacks guanine preferentially. H_2O_2 and O_2^- do not attack DNA [9].	A wide range of enzymes exist that recognize abnormalities in DNA and remove them by excision, resynthesis, and rejoining of DNA strand [10].	[9,10]
Proteins Many ROS can oxidize –SH groups. OH• attacks many amino acid residues [11]. Proteins often bind transition metal ions, making them a target of attack by site-specific OH• generation [1,11].	Oxidized methionine residues may be repaired by methionine sulfoxide reductase. Other damaged proteins may be recognized and preferentially destroyed by cellular proteases.	[12,13]
Lipids Some ROS (not including O_2^- or H_2O_2) can initiate lipid peroxidation [14].	Chain-breaking antioxidants (especially α-toxopherol) 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.	[15–17]

*HOCl could equally well be regarded as a "reactive chlorine species."

Generation of O_2^-, HOCl, and H_2O_2 by phagocytes is known to play an important part in the killing of several bacterial and fungal strains (21). Some other metabolic roles for H_2O_2 have been proposed (29–33). For example, H_2O_2 is used by the enzyme thyroid peroxidase to help make thyroid hormones (30). H_2O_2 or products derived from it can displace the inhibitory subunit from the cytoplasmic gene transcription factor NF-KB. The active factor migrates to the nucleus and activates genes by binding to specific DNA sequences in enhancer and promoter elements. Thus, H_2O_2 can induce expression of genes controlled by NF-KB. This is of particular interest because NF-KB can induce the expression of genes of the provirus HIV-1, the major cause of acquired immunodeficiency syndrome (33). H_2O_2, a nonradical, resembles water in its molecular structure and is very diffusible within and between cells.

Much O_2^- generated *in vivo* probably undergoes a dismutation reaction to give H_2O_2, as represented by the overall equation

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad [1]$$
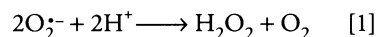
Toxicity of Superoxide and Hydrogen Peroxide

Experimental data show clearly that removal of O_2^- and H_2O_2 by antioxidant defense systems is essential for healthy aerobic life (1,5,18,34). Why is this? In organic media, O_2^- can be very reactive but in aqueous media it is not, mainly acting as a moderate reducing agent, e.g., the reduction of cytochrome *c*

$$cyt\ c\ (Fe^{3+}) + O_2^- \rightarrow cyt\ c\ (Fe^{2+}) + O_2 \quad [2]$$
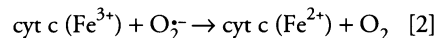
However, O_2^-, can react with some targets. In particular, O_2^- reacts fast with nitric oxide in a radical addition reaction (35).

$$O_2^- + NO^\bullet \rightarrow ONOO^-$$

$$\text{peroxynitrite} \quad [3]$$
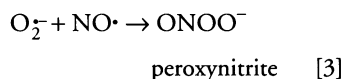
NO• is known to be produced *in vivo* by vascular endothelial cells, by some cells in the brain, and by phagocytes (8). NO• performs useful physiologic functions, such as regulation of vascular smooth muscle tone (hence controlling blood pressure) and neurotransmitter action (8). Since NO• acts upon smooth muscle cells in blood vessel walls to produce relaxation,

then O_2^-, by removing NO• (Equation 3), can act as a vasoconstrictor, and this might have deleterious effects in some clinical situations (36,37).

Considerable debate continues in the literature as to whether or not the interaction of O_2^- and NO (Equation 3) is damaging to cells (38). Peroxynitrite might be directly toxic to cells (38,39). It might also decompose to form a range of toxic products, including some OH• (38,40).

$$ONOO^- + H^+ \rightarrow OH^\bullet, NO_2^\bullet, NO_2^+ \quad [4]$$
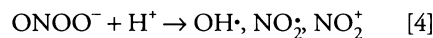
Note that Equation 4 also produces the toxic free radical gas nitrogen dioxide. However, the physiologic significance of these reactions is still uncertain, since some experiments suggest that NO• may protect against oxidative damage even when O_2^- is being generated (41,42).

Superoxide has also been shown to be capable of inactivating several bacterial enzymes, such as *Escherichia coli* dihydroxyacid dehydratase, aconitase, and 6-phosphogluconate dehydratase (5,20,43). It appears to attack iron-sulfur clusters at the enzyme active sites. Whether such reactions of O_2^- occur in mammals is uncertain, although in isolated submitochondrial particles, O_2^- has been claimed to inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (44). The protonated form of O_2^- hydroperoxyl radical ($HO_2^\bullet$), is much more reactive than O_2^- *in vitro*. For example, HO_2^- can initiate peroxidation of polyunsaturated fatty acids and decompose lipid hydroperoxides, which O_2^- cannot (45,46). However, there is no direct evidence that HO_2^- exerts damaging effects *in vivo*. The pK_a of HO_2^- is about 4.8, so only a small fraction of O_2^- is protonated at physiologic pH values.

H_2O_2 at low micromolar levels also appears poorly reactive (1). However, higher levels of H_2O_2 can attack several cellular energy-producing systems, e.g., by inactivating the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (47).

It is usually thought that most or all of the toxicity of O_2^- and H_2O_2 involves their conversion into OH• (1,48). Several mechanisms have been proposed to explain this. The most recent is the interaction of O_2^- and NO• (Equations 3,4). An earlier proposal (5,48) was the superoxide-driven Fenton reaction

$$O_2^- + Fe(III) \rightarrow Fe(II) + O_2 \quad [5]$$

$$Fe(II) + H_2O_2 \rightarrow OH^\bullet$$

$$+ OH^- + Fe(III) \quad [6]$$
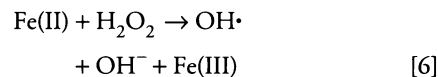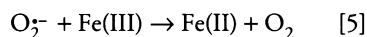
Although there has been repeated controversy in the literature as to whether OH• is formed in such reactions at physiologic pH, the evidence is now overwhelming (49–51). Copper can also catalyze OH• formation from H_2O_2 (52).

Iron and copper (and other transition metal ions) in chemical forms that can decompose H_2O_2 to OH• are in very short supply *in vivo*: organisms are very careful to ensure that as much iron and copper as possible are kept safely bound to transport or storage proteins. Indeed, the "sequestration" of metal ions into forms that will not catalyze free radical reactions is an important antioxidant defense mechanism (1,29,53). Sequestration of iron and copper ions deters the growth of many bacterial strains in human blood plasma (54): it also ensures that plasma will not convert O_2^- and H_2O_2 into OH• (29,53). Prevention of OH• formation may allow small quantities of O_2^- and H_2O_2 released into the extracellular environment (e.g., from endothelial cells, lymphocytes, and phagocytes) to perform useful metabolic roles, such as intercellular signaling, rather than causing damage (29).

In any case, any transition metal ions that do become available to catalyze free radical reactions *in vivo* will not exist in the "free" state for very long. Thus, if iron ions are liberated, they must bind to a biological molecule or else eventually precipitate out of solution as ferric hydroxides, oxyhydroxides, and phosphates. If metal ions bound to a biological molecule react with O_2^- and H_2O_2 (Equations 5,6), OH• will be formed upon the molecule. This OH•-mediated damage is said to be "site specific" (55). Binding of metal ions to a target means that any OH• generated will tend to react with that target rather than with any added scavenger, and the OH• will be very difficult to intercept by OH• scavengers.

It follows that a major determinant of the nature of the damage done by excess generation of ROS *in vivo* is the availability and location of metal ion catalysts of OH• radical formation (1,29). If, for example, "catalytic" iron or copper ions are bound to DNA in one cell type and to membrane lipids in another, then excessive formation of H_2O_2 and O_2^-, will, in the first case, damage the DNA and in the other could initiate lipid peroxidation. Evidence for OH• formation in the nucleus of cells treated with H_2O_2 has been

obtained, presumably involving metal ions bound upon, or very close to DNA (56,57).

E. coli mutants lacking SOD activity are hypersensitive to damage by H_2O_2 (34), and extra SOD can often protect cells against damage by H_2O_2, provided that it can enter the cell (58). These data are consistent with a role of O_2^- in facilitating damage by H_2O_2, and Equations 5 and 6 provide an explanation. However, many scientists are reluctant to believe that O_2^- serves only as a reducing agent for metal ions since, in general, mammalian tissues are fairly reducing environments. The arguments have been rehearsed in detail (1,49,59) but the point is not yet settled.

Antioxidant Defenses

Living organisms have evolved antioxidant defenses to remove excess O_2^- and $H_2O_2^-$ Superoxide dismutases (SODs) remove O_2^- by greatly accelerating its conversion to H_2O_2 (Equation 1). Human cells have a SOD enzyme containing manganese at its active site (Mn-SOD) in the mitochondria. A SOD with copper and zinc at the active site (Cu,Zn-SOD) is also present, but largely in the cytosol (5). Catalases in the peroxisomes convert H_2O_2 into water and O_2 and help dispose of H_2O_2 generated by peroxisomal oxidase enzymes (18).

However, the most important H_2O_2-removing enzymes in human cells are glutathione peroxidases (GSHPX), which require selenium (as an active site selenocysteine residue) for their action. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (18). Another important antioxidant defense already referred to is the sequestration of transition metal ions into forms that will not catalyze free radical reactions (1,29,53). This is particularly important in the extracellular environment, where levels of SOD, GSH, GSHPX, and catalase are often very low (53).

Antioxidant defense enzymes are essential for healthy aerobic life. For example, SOD-negative mutants of E. coli will not grow aerobically unless given a rich growth medium, due to impaired biosynthesis of certain amino acids. Even when so supplemented, SOD E. coli cells grow slowly, suffer membrane damage, are abnormally sensitive to damage by H_2O_2 (perhaps because of Equations 5 and 6) and show a high mutation rate (34).

However, antioxidant defenses exist as a balanced and coordinated system. Thus, although SOD is important, an excess of SOD in relation to peroxide-metabolizing enzymes can be deleterious (60–63). This has been shown by transfecting cells with human cDNAs encoding SOD (60). Transgenic mice overexpressing human Cu,Zn-SOD are resistant to elevated O_2 and to certain toxic agents (62,63) but they show certain neuromuscular abnormalities resembling those found in patients with Down's syndrome (62). The gene encoding Cu,Zn-SOD is located on chromosome 21 in humans, and Down's syndrome is usually caused by trisomy of this gene, raising tissue Cu,Zn-SOD levels by about 50%. The limited data available at present are consistent with the view that the excess of Cu,Zn-SOD may contribute to at least some of the abnormalities in patients with Down's syndrome (62).

In addition to antioxidant defense enzymes, some low-molecular-mass free radical scavengers exist. Reduced glutathione can scavenge various free radicals directly, as well as being a substrate for GSHPX enzymes. α-Tocopherol is the most important free radical scavenger within membranes. Attack of reactive radicals, such as OH•, upon membranes can damage them by setting off a free radical chain reaction called lipid peroxidation. α-Tocopherol (α-TH) inhibits this by scavenging peroxyl radicals (Table 1), intermediates in the chain reaction.

$$\alpha\text{-TH} + \text{LOO•} \rightarrow \alpha\text{T•} + \text{LOOH} \quad [7]$$
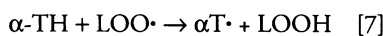
However, the tocopherol thereby becomes a radical, αT•. This illustrates a fundamental principle of free radical chemistry: when radicals react with nonradicals, new radicals are generated. Only when two radicals meet and join their unpaired electrons are the radicals lost (termination reactions). An example is the reaction of O_2^- with NO• (Equation 3).

Overall, antioxidant defenses seem to be approximately in balance with generation of oxygen-derived species in vivo. There appears to be no great reserve of antioxidant defenses in mammals, perhaps because, as pointed out previously, some oxygen-derived species perform useful metabolic roles.

Oxidative Stress: A Definition

Generation of ROS and the activity of antioxidant defenses appear more or less balanced in vivo. In fact, the balance may be slightly tipped in favor of the ROS so

that there is continuous low-level oxidative damage in the human body. This creates a need for repair systems that can deal with oxidatively damaged molecules (Table 2). However, if a greater imbalance occurs in favor of the ROS, oxidative stress is said to result (19). Most aerobes can tolerate mild oxidative stress: indeed they often respond to it by inducing synthesis of extra antioxidant defenses. For example, if rats are gradually acclimatized to elevated O_2, they can tolerate pure O_2 for much longer than naive rats, apparently due to increased synthesis of antioxidant defenses in the lung (64,65). Other examples are the complex adaptive response of E. coli treated with low concentrations of H_2O_2 (66) and the activation of NF-KB in oxidatively stressed mammalian cells (33).

However, severe oxidative stress can cause cell damage and death. In mammalian cells, oxidative stress appears to cause increases in the levels of free Ca^{2+} (67) and free iron (68) within cells, e.g., by damaging proteins that normally keep these metal ions safely bound. Iron ion release can lead to OH• generation, which has been shown to occur within the nucleus of H_2O_2-treated cells (56). An excessive rise in intracellular free Ca^{2+} can also activate endonucleases and cause DNA fragmentation (67).

Hence, oxidative stress results in damage to DNA, proteins, lipids, and carbohydrates (67,68). The relative importance of damage to these different molecular targets in mediating cell injury or death depends upon what degree of oxidative stress occurs, by what mechanism it is imposed, for how long, and the nature of the system stressed. For example, lipid peroxidation appears to be an important consequence of oxidative stress in human atherosclerotic lesions (69). Several halogenated hydrocarbons (such as CCl_4 and bromobenzene) appear to exert some, or all, of their toxic effects by stimulating lipid peroxidation in vivo (7). However, for most other toxic agents causing oxidative stress, lipid peroxidation is not the major mechanism of primary cell injury: damage to proteins and DNA is usually more important (1,47,68). For example, it has often been assumed that lipids are a major target of damage by inhaled ozone, but proteins may be equally or more important (see below).

Causes of Oxidative Stress: Toxic Agents

Oxidative stress can be imposed in several ways. Thus, severe malnutrition can deprive humans of the minerals (e.g., Cu, Mn, Zn,

Table 3. Why cigarette smoke can impose oxidative stress.

(1) Smoke contains many free radicals, especially peroxyl radicals, that might attack biological molecules and deplete antioxidants, such as vitamin C and α-tocopherol.

(2) Smoke contains oxides of nitrogen, including the unpleasant nitrogen dioxide (NO_2^-).

(3) The tar phase of smoke contains hydroquinones. These are lipid-soluble and can redox-cycle to form $O_2^{\bullet-}$ and H_2O_2. They can enter cells and may even reach the nucleus to cause oxidative DNA damage. Some hydroquinones may release iron from the iron-storage protein ferritin in lung cells and respiratory tract lining fluids.

(4) Smoking may irritate lung macrophages, activating them to make $O_2^{\bullet-}$.[a]

(5) Smokers' lungs contain more neutrophils than the lungs of nonsmokers, and smoke might activate these cells to make $O_2^{\bullet-}$.[a]

(6) Smokers often eat poorly and drink more alcohol than nonsmokers and may have a low intake of nutrient antioxidants.

[a] The effects of cigarette smoke on phagocytes are dose-related. Low levels may stimulate them, but high levels may poison them and so depress their activity.

Se) and vitamins (e.g., riboflavin—needed for the FAD cofactor of glutathione reductase, and α-tocopherol—needed for antioxidant defense) (1). More usually, however, the stress is due to production of excess ROS.

Several drugs and toxins impose oxidative stress during their metabolism. Carbon tetrachloride is one example (Table 1). Another is paraquat, a herbicide that causes lung damage in humans. Its metabolism within the lung leads to production of large amounts of $O_2^{\bullet-}$ and H_2O_2 (1). Gas-phase cigarette smoke also imposes some oxidative stress. Some of the reasons for this are summarized in Table 3 (70,71).

Causes of Oxidative Stress: Disease and Tissue Injury

Does oxidative damage play a role in human disease? Many of the biologic consequences of excess radiation exposure may be due to OH$\bullet$-dependent damage to proteins, DNA, and lipids (6). Oxidative damage (resulting from exposure to elevated O_2 in incubators) may account for damage to the retina of the eye (retinopathy of prematurity) in premature babies (3). However, there are many papers in the biomedical literature suggesting a role for oxidative stress in other human diseases [over 100 at the last count (1)].

Tissue damage by disease, trauma, poisons, and other causes usually lead to formation of increased amounts of putative "injury mediators," such as prostaglandins, leukotrienes, interleukins, interferons, and tumor necrosis factors (TNFs). All of these have at various times been suggested to play important roles in different human diseases. Currently, for example, there is much interest in the roles played by TNFα, NO$\bullet$, and interleukins in adult respiratory distress syndrome and septic shock (8,72). ROS can be placed in the same category, i.e., tissue damage will usually lead to increased ROS formation and oxidative stress. Figure 1 summarizes some of the reasons for this. Indeed, in most human diseases, oxidative stress is a secondary phenomenon, a consequence of the tissue injury. That does not mean it is not important (1,72). For example, excess production of $O_2^{\bullet-}$, H_2O_2, and other species by phagocytes at sites of 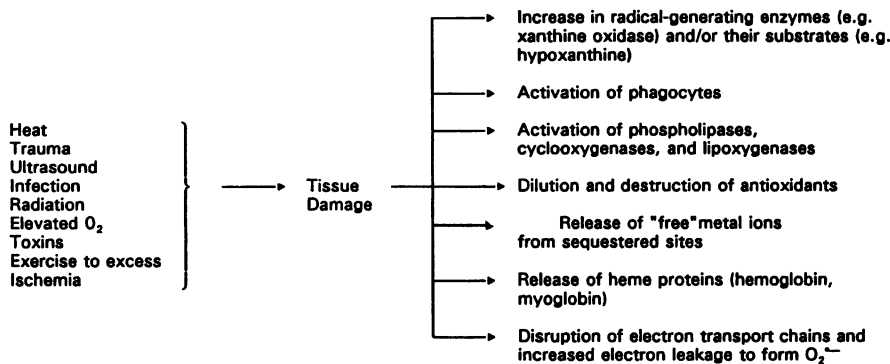chronic inflammation can cause severe dam-age. This seems to happen in the inflamed joints of patients with rheumatoid arthritis (72) and in the gut of patients with inflammatory bowel diseases (73). Tissue injury can release metal ions from their storage sites within cells, leading to OH$\bullet$ generation (72,74). Thus, the main question about ROS 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, including atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease (1,69,72–74). However, it may well be "no" in many others. Elucidating the precise role played by free radicals has not been easy because they are difficult to measure, but the development of modern assay techniques is helping to solve this problem (72).

Causes of Oxidative Stress: Environmental Air Pollutants

The role of free radicals in the toxicity of O_2 and of cigarette smoke (Table 3) has already been discussed, but oxidative damage is frequently suggested to be involved in the deleterious effects of O_3 and NO_2 (75,76). O_3 is not a free radical, but it can oxidize many biological molecules directly and, in addition, it reacts slowly with water at alkaline pH to give OH$\bullet$ (77). It has also been suggested to produce singlet O_2 when it reacts with biological molecules (78).

The first biological fluids that come into contact with inhaled O_3 are the respiratory tract lining fluids (RTLFs), that presumably serve to absorb and detoxify some of the inhaled O_3 so as to lower the amount that enters the more vulnerable peripheral gas exchange regions of the lung. Some information is available about the antioxidants of these fluids (79,80) but the problems of sampling them (by the techniques of respiratory tract lavage) have hindered elucidation of their precise comparative antioxidant capabilities, since lavage itself produces considerable and variable dilution of RTLFs and some of their constituents may be oxidized during the procedures. In addition, the antioxidants present depend upon which part of the respiratory tract is being sampled (79), i.e., nasal passages, airways, bronchioles, alveoli. Often a mixed fluid is obtained.

By contrast, the antioxidant defenses of human plasma have been well characterized (53). To approach an understanding of how O_3 might interact with a complex biological fluid, the reactions of O_3 with freshly prepared human plasma have been studied.

Heat	→ Increase in radical-generating enzymes (e.g. xanthine oxidase) and/or their substrates (e.g. hypoxanthine)
Trauma	
Ultrasound	→ Activation of phagocytes
Infection	
Radiation	→ Activation of phospholipases, cyclooxygenases, and lipoxygenases
Elevated O_2	→ Dilution and destruction of antioxidants
Toxins	
Exercise to excess	→ Release of "free"metal ions from sequestered sites
Ischemia	
	→ Release of heme proteins (hemoglobin, myoglobin)
	→ Disruption of electron transport chains and increased electron leakage to form $O_2^{\bullet-}$

Tissue Damage

Figure 1. Some of the mechanisms by which tissue damage can cause oxidative stress.

Table 4. A comparison of the effects of cigarette smoke (CS), O_3, and NO_2 on human plasma.

Event	CS	O_3	NO_2
Oxidation of ascorbate	+ + + +	+ + + +	+ + + +
Breakdown of uric acid	+ +	+ + + +	+ + +
Loss of plasma -SH groups	+ + +	+ + +	+ + +
Formation of protein carbonyls	+ + + +	+ +	O
Depletion of α-tocopherol	+ +	O	+ +
Lipid peroxidation	+	O	+ +

$$L-H + LOO\cdot \rightarrow L\cdot + LOOH \qquad [10]$$

Initiation of peroxidation by $NO_2^\bullet$ (Equation 8) presumably sets up the auto-catalytic chain reaction of lipid peroxidation (Equations 9,10) resulting in the accumulation of lipid peroxides (LOOH). Uric acid and ascorbate were found to be important antioxidants protecting plasma against oxidative damage by $NO_2^\bullet$, but the lipid-soluble antioxidants α-tocopherol and ubiquinol probably also play an important protective role in limiting lipid peroxidation (86).

Hence, as summarized in Table 4, O_3 and NO_2 damage different molecular targets, which could be one reason why they might sometimes exert synergistic damaging effects in vivo. For comparison, Table 4 also includes the effects of cigarette smoke upon plasma. Ascorbic acid is rapidly oxidized and damage to both proteins (carbonyl formation, loss of -SH groups) and lipids (lipid peroxide formation) occurs. Ascorbic acid protects the plasma against lipid damage by ozone, but does not protect against protein damage (87,88).

Indeed, oxidant injury to the lung causes increased influx of plasma constituents, so that the lung lining fluids become more like plasma in composition. Uric acid and ascorbic acid were found to be the major plasma scavengers of O_3 (81). Although it is often assumed that lipids are a major target of attack by O_3, no evidence of substantial lipid damage by O_3 was obtained (81), in keeping with other studies in the literature (82,83). Instead, oxidative protein damage was observed in O_3-exposed plasma, as -SH group loss and protein carbonyl formation (84). However, it will be necessary to study human lung lining fluids to substantiate this conclusion, especially as Cueto et al. (85) found end products of lipid damage in lung lining fluids from rats after O_3 exposure.

Since uric acid is a major constituent of upper airway lining fluid in humans (79), it could act as a "scrubber," decreasing the concentration of O_3 in inhaled air so as to protect the more vulnerable alveolar regions of the lung (81).

By contrast with the effect of O_3, $NO_2^\bullet$ did not generate protein carbonyls in plasma, although nitration of aromatic amino acids took place and protein -SH groups were lost, presumably by direct reaction with $NO_2^\bullet$ (86). Again unlike O_3, NO_2 induced lipid peroxidation in plasma, presumably by the reactions

$$L-H + NO_2^\bullet \rightarrow HNO_2 + L\cdot \qquad [8]$$

$$L\cdot + O_2 \rightarrow LOO\cdot \qquad [9]$$

REFERENCES

1. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, 2nd ed. Oxford:Clarendon Press, 1989.
2. Balentine J. Pathology of Oxygen Toxicity. New York:Academic Press, 1982.
3. Penn JS. Oxygen-induced retinopathy in the rat: a proposed role for peroxidation reactions in the pathogenesis. In: Free Radical Mechanisms of Tissue Injury (Moslen MT, Smith CV, eds). Boca Raton, FL:CRC Press, 1992;77–188.
4. Gerschman K, Gilbert DL, Nye SW, Dwyer S, Fenn WO. Oxygen poisoning and X-irradiation: a mechanism in common. Science 119:623–626 (1954).
5. Fridovich I. Superoxide dismutases. An adaptation to a paramagnetic gas. J Biol Chem 264:7761–7764 (1989).
6. von Sonntag C. The Chemical Basis of Radiation Biology. London:Taylor and Francis, 1987.
7. Comporti M. Glutathione-depleting agents and lipid peroxidation. Chem Phys Lipids 45:143–169 (1987).
8. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109–143 (1991).
9. Halliwell B, Aruoma OI, eds. DNA and Free Radicals. Chichester, UK:Ellis-Horwood, 1993.
10. Breimer L. Repair of DNA damage induced by reactive oxygen species. Free Radic Res Commun 14:159–171 (1991).
11. Stadtman ER, Oliver CN. Metal-catalyzed oxidation of proteins. Physiological consequences. J Biol Chem 266:2005–2008 (1991).
12. Brot N, Weissbach H. Biochemistry of methionine sulfoxide residues in proteins. Biofactors 3:91–96 (1991).
13. Davies KJA. Protein modification by oxidants and the role of proteolytic enzymes. Biochem Soc Trans 21:346–353 (1993).
14. Halliwell B, Chirico S. Lipid peroxidation: its mechanism, measurement and significance. Am J Clin Nutr 57:S715–S725 (1993).
15. Burton GW, Traber MG. Vitamin E: antioxidant activity bioki-

netics and bioavailability. Annu Rev Nutr 10:357–382 (1990).
16. Schuchelt R, Brigelius-Flohé R, Maiorino M, Roveri A, Reumkens J, Strassburger W, Ursini F, Wolf B, Flohé L. Phospholipid hydroperoxide glutathione peroxidase is a selenoenzyme distinct from the classical glutathione peroxidase as evident from cDNA and amino acid sequencing. Free Radic Res Commun 14:343–361(1991).
17. Sevanian A, Wratten ML, McLeod LL, Kim E. Lipid peroxidation and phospholipase A2 activity in liposomes composed of unsaturated phospholipids: a structural basis for enzyme activation. Biochim Biophys Acta 961:316–327 (1988).
18. Chance B, Sies H, Boveris A. Hydroperoxide metabolism mammalian organs. Physiol Rev 59:527–605 (1979).
19. Sies H, ed. Oxidative Stress. London:Academic Press, 1985.
20. Imlay JA, Fridovich I. Assays of metabolic superoxide production in Escherichia coli. J Biol Chem 266:6957–6965 (1991).
21. Babior BM, Woodman RC. Chronic granulomatous disease. Semin Hematol 27:247–259 (1990).
22. McCord JM. Oxygen-derived radicals: a link between reperfusion injury and inflammation. Fed Proc 46:2402–2406 (1987).
23. Maly FE. The B-lymphocyte: a newly-recognized source of reactive oxygen species with immunoregulatory potential. Free Radic Res Commun 8:143–148 (1990).
24. Murrell GAC, Francis MJO, Bromley L. Modulation of fibroblast proliferation by oxygen free radicals. Biochem J 265:659–665 (1990).
25. Meier B, Radeke HH, Selle S, Raspe HH, Sies H, Resch K, Habermehl GG. Human fibroblasts release reactive oxygen species in response to treatment with synovial fluids from patients suffering from arthritis. Free Radic Res Commun 8:149–160 (1990).
26. Arroyo CM, Carmichael AJ, Bouscarel B, Liang JH, Weglicki WB. Endothelial cells as a source of oxygen-free radicals. An ESR study. Free Radic Res Commun 9:287–296 (1990).

27. Babbs CF, Cregor MD, Turek JJ, Badylak SF. Endothelial superoxide production in buffer perfused rat lungs, demonstrated by a new histochemical technique. Lab Invest 65:484–496 (1991).

28. Britigan BE, Roeder TL, Shasby DM. Insight into the nature and site of oxygen-centered free radical generation by endothelial cell monolayers using a novel spin trapping technique. Blood 79:699–707 (1992).

29. Halliwell B, Gutteridge JMC. Oxygen free radicals and iron in relation to biology and medicine. Some problems and concepts. Arch Biochem Biophys 246:501–508 (1986).

30. Dupuy C, Virion A, Ohayon R, Kniewski J, Deme D, Pommier J. Mechanism of hydrogen peroxide formation catalyzed by NADPH oxidase in thyroid plasma membrane. J Biol Chem 266:3739–3743 (1991).

31. Shapiro BM. The control of oxidative stress at fertilization. Science 252:533–536 (1991).

32. Riley JCM, Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 5:781–791 (1991).

33. Schreck PA, Albermann KAJ, Bauerle P. Nuclear factor KB: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun 17:221–237 (1992).

34. Touati D. The molecular genetics of superoxide dismutase in *E. coli*. An approach to understanding the biological role and regulation of SODs in relation to other elements of the defence system against oxygen toxicity. Free Radic Res Commun 8:1–9 (1989).

35. Saran M, Michel C, Bors W. Reactions of NO with O_2^-. Implications for the action of endothelium-relaxing factor. Free Radic Res Commun 10:221–226 (1990).

36. Laurindo FRM, da Luz PL, Uint L, Rocha TF, Jaeger RG. Evidence for superoxide radical-dependent coronary vasospasm after angioplasty in intact dogs. Circulation 83:1705–1715 (1991).

37. Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sako T, Inoul M. Does superoxide underly the pathogenesis of hypertension? Proc Natl Acad Sci USA 88:10045–10048 (1991).

38. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624 (1990).

39. Radi R, Beckman JS, Bush KKM Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 266:4244–4250 (1990).

40. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. Biochem J 281:419–424 (1992).

41. Jessup W, Mohr D, Gieseg SP, Dean RT, Stocker R. The participation of nitric oxide in cell free—and its restriction of macrophage-mediated oxidation of low density lipoprotein. Biochim Biophys Acta 1880:73–82 (1992).

42. Yates MT, Lambert LE, Whitten JP, McDonald I, Mano M, Ku G, Mao SJT. A protective role for nitric oxide in the oxidative modification of low density lipoproteins by mouse macrophages. FEBS Lett 309:135–138 (1992).

43. Gardner PR, Fridovich I. Inactivation-reactivation of aconitase in *Escherichia coli*. A sensitive measure of superoxide radical. J Biol Chem 267:8757–8763 (1992).

44. Zhang Y, Marcillat O, Giulivi C, Eynster L, Davis KJ. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J Biol Chem 265:16330–16336 (1990).

45. Bielski BHJ, Arudi RL, Sutherland MW. A study of the reactivity of HO_2/O_2^- with unsaturated fatty acids. J Biol Chem 258:4759–4761(1983).

46. Aikens J, Dix TA. Perhydroxyl radical (HOO•)initiated lipid peroxidation. The role of fatty acid hydroperoxides. J Biol Chem 266:15091–15098 (1991).

47. Cochrane CG. Mechanisms of oxidant injury of cells. Mol Aspects Med 12:137–147 (1991).

48. Halliwell B. Superoxide-dependent formation of hydroxyl radi-

cals in the presence of iron chelates. FEBS Lett 92:321-326 (1978).

49. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease. Methods Enzymol 186:1–85 (1990).

50. Halliwell B, Gutteridge JMC. Biologically-relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 300:108–112 (1992).

51. Burkitt MJ. ESR spin trapping studies into the nature of the oxidizing species formed in the Fenton reaction: pitfalls associated with the use of 5,5-dimethyl-1-pyrroline-*N*-oxide in the detection of the hydroxyl radical. Free Radic Res Commun 18:43–57 (1993).

52. Aruoma OI, Halliwell B, Gajewski E, Dizdaroglu M. Copper ion dependent damage to the bases in DNA in the presence of hydrogen peroxide. Biochem J 273:601–604 (1991).

53. Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. Arch Biochem Biophys 200:1–8 (1990).

54. Weinberg ED. Cellular iron metabolism in health and disease. Drug Metab Rev 22:531–579 (1990).

55. Borg DC, Schaich KM. Cytotoxicity from coupled redox cycling of autoxidizing xenobiotics and metals; a selective critical review and commentary on work in progress. Israel J Chem 24:38–53 (1984).

56. Dizdaroglu M, Nackerdien Z, Chao BC, Gajewski E, Rao G. Chemical nature of *in vivo* base damage in hydrogen peroxide-treated mammalian cells. Arch Biochem Biophys 285:388–391 (1991)

57. Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett 281:9–19 (1991).

58. Kyle ME, Nakae D, Sakaida I, Miccadei S, Farber JL. Endocytosis of superoxide dismutase is required in order for the enzyme to protect hepatocytes from the cytotoxicity of hydrogen peroxide. J Biol Chem 263:3784–3789 (1988).

59. Sutton HC, Winterbourn CC. On the participation of higher oxidation states of iron and copper in Fenton reactions. Free Radic Biol Med 6:53–60 (1989).

60. Amstad P, Peskin A, Shah G, Mirault ME, Movet R, Zbinden I, Cerutti P. The balance between Cu, Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. Biochemistry 30:9305–9313 (1991).

61. Scott MD, Eaton JW, Kuypers FA, Chiu DT, Lubin BH. Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense. Blood 74:2542–2549 (1989).

62. Groner Y, Elroy-Stein O, Avraham KB, Yarom R, Schickler M, Knobler M, Rotman G. Down syndrome clinical symptoms are manifested in transfected cells and transgenic mice overexpressing the human Cu/Zn-superoxide dismutase gene. J Physiol (Paris) 84:53–77 (1990).

63. White CW, Avraham KB, Shanley PF, Groner Y. Transgenic mice with expression of elevated levels of copper-zinc superoxide dismutase in the lungs are resistant to pulmonary oxygen toxicity. J Clin Invest 87:2162–2168 (1991).

64. Frank L, Iqbal J, Hass M, Massaro D. New "rest period" protocol for inducing tolerance to high O_2 exposure in adult rats. Amer J Physiol 257:L226–L231 (1989).

65. Iqbal J, Clerch LB, Hass MA, Frank L, Massaro D. Endotoxin increases lung Cu, Zn superoxide dismutase mRNA: O_2 raises enzyme synthesis. Amer J Physiol 257:L61–L64 (1989).

66. Storz G, Tartaglia L. OxyR: a regulator of antioxidant genes. J Nutr 122:627–630 (1992).

67. Orrenius S, McConkey DJ, Bellomo G, Nicotera P. Role of Ca^{2+} in toxic cell killing. Trends Pharmacol Sci 10:281–285 (1989).

68. Halliwell B. Oxidants and human disease: some new concepts. FASEB J 1:358–364 (1987).

69. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase atherogenicity. N Engl J Med 320:915–924 (1989).

70. Halliwell B. Cigarette smoking and health: a radical view. J Roy Soc Health 113:91–96 (1993).

71. Pryor WA, Stone K. Oxidants in cigarette smoke: radicals, hydrogen peroxide, peroxynitrate and peroxynitrite. Ann NY

Acad Sci 686:12–27 (1993).

72. Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clinic Med 119:598–620 (1992).

73. Harris ML, Schiller HJ, Reilly PM, Donowitz M, Grisham MB, Bulkley GB. Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon? Pharmacol Ther 53:375–408 (1992).

74. Halliwell B. Reactive oxygen species and the central nervous system. J Neurochem 59:1609–1623 (1992).

75. Pryor WA. Can vitamin E protect humans against the pathological effects of ozone in smog? Am J Clin Nutr 53:702–722 (1991).

76. Mustafa MG. Biochemical basis of ozone toxicity. Free Radic Biol Med 9:245–265 (1990).

77. Glaze WH. Reaction products of ozone: a review. Environ Health Perspect 69:151–157 (1986).

78. Kanofsky JR, Sima P. Singlet oxygen production from the reactions of ozone with biological molecules. J Biol Chem 266:9039–9042 (1991).

79. Hatch GE. Comparative biochemistry of airway lining fluid. In: Comprehensive Treatise on Pulmonary Toxicology, Vol 1 (Parent R, ed). Boca Raton, FL:CRC Press, 1991;17–632.

80. Pacht ER, Davis WB. Role of transferrin and ceruloplasmin in antioxidant activity of lung epithelial lining fluid. J Appl Physiol 64:2092–2099 (1988).

81. Cross CE, Motchnik PA, Bruener BA, Jones DA, Kaur H, Ames BN, Halliwell B. Oxidative damage to plasma constituents by ozone. FEBS Lett 298:269–272 (1992).

82. Freeman BA, Mudd JB. Reaction of ozone with sulfhydryls of human erythrocytes. Arch Biochem Biophys 208:212–220 (1981).

83. Mudd JB, Leavitt R, Ongun A, McManus TT. Reaction of ozone with amino acids and proteins. Atmospher Environ 3:669–682 (1969).

84. Cross CE, Reznick AZ, Packer L, Davis PA, Suzuki YJ, Halliwell B. Oxidative damage to human plasma proteins by ozone. Free Radic Res Commun 15:347–352 (1992).

85. Cueto R, Squadrito GL, Bermudez E, Pryor WA. Identification of heptanal and nonanal in bronchoalveolar lavage from rats exposed to low levels of ozone. Biochem Biophys Res Commun 188:129–134 (1992).

86. Halliwell B, Hu ML, Louie S, Duvall TR, Tarkington BK, Motchnik P, Cross CE. Interaction of nitrogen dioxide with human plasma. Antioxidant depletion and oxidative damage. FEBS Lett 313:62–66 (1992).

87. Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Biochem J 277:133–138 (1991).

88. Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L, Halliwell B. Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. Biochem J 286:607–611 (1992).