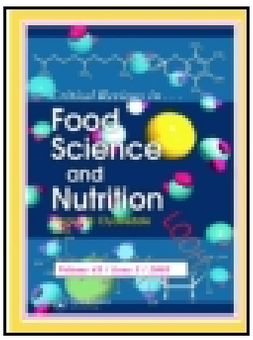

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Section II

The Role of Free Radicals and Antioxidants

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Free Radicals and Antioxidants in Food and *In Vivo*: What They Do and How They Work

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ABSTRACT: A wide variety of oxygen free radicals and other reactive oxygen species can be formed in the human body and in food systems. Transition metal ions accelerate free-radical damage. Antioxidant defenses, both enzymic and nonenzymic, protect the body against oxidative damage, but they are not 100% efficient, and so free-radical damage must be constantly repaired. Nonenzymatic antioxidants are frequently added to foods to prevent lipid peroxidation. Several lipid antioxidants can exert prooxidant effects toward other molecules under certain circumstances, and so antioxidants for food and therapeutic use must be characterized carefully. Methods of measuring oxidative damage and trapping free radicals *in vivo* are briefly discussed. Such methods are essential in checking proposals that increased intake of food-derived antioxidants (such as antioxidant vitamins) would be beneficial to humans.

KEY WORDS: oxygen radical, lipid peroxidation, oxidative stress, hydroxyl radical, hydrogen peroxide, food antioxidants, food preservation.

I. BASIC DEFINITIONS

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 atomic or molecular orbital. Perhaps the simplest free radical is an atom of the element hydrogen, but a wide range of free radicals can be generated in living systems.[1] Table 1 gives some examples. Recently, much interest has focused on the oxygen-centered radicals, (i.e., radicals in which the unpaired electron is located on oxygen), such as superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$).

When two free radicals meet, they can join their unpaired electrons to form a covalent bond. An example is the very fast reaction of $O_2^{\cdot-}$ with the nitric oxide radical ($NO^{\cdot}$) to give the nonradical peroxynitrite.[2]

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^- \qquad (1)$$

However, most molecules found in living systems are nonradicals. When radicals react with nonradicals, new radicals are generated (Table 2). Hence, the formation of reactive radicals *in vivo* is likely to set off free-radical *chain reactions*. The best-studied biologically relevant free-radical chain reaction is *lipid peroxidation* (Figure 1).

Peroxidation of lipids is also a major concern of food manufacturers,[5,6] because it can lead to the development of unpleasant "rancid" or "off" flavors as well as potentially toxic end products.[5,7]

Some oxygen-containing molecules that can be generated in biological and food systems are not themselves free radicals, but can participate in free-radical reactions. Examples are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and the $^1\Delta g$ form of singlet oxygen. H_2O_2 can react with transition metal ions to form the hydroxyl radical.[8,9]

$$M^{z+} + H_2O_2 \rightarrow M^{(z+1)} + OH^{\cdot} + HO^- \qquad (2)$$

HOCl is generated by activated phagocytes[10] and is present in many chlorine-based bleaches and disinfectants often used in food handling areas. Singlet O_2 can be generated during lipid peroxidation (Figure 1). It is also formed when the toxic gas ozone (O_3) reacts with biological molecules[11] and by photosensitization reactions.[1] Singlet oxygen reacts *directly* with many biological molecules, including lipids (to give lipid hydroperoxides). Photosensitization reactions can occur *in vivo*[1] and in illuminated food material containing photosensitizers of $^1O_2$ formation such as riboflavin[1] (e.g., brightly illuminated milk spoils quickly). Lipid hydroperoxides, formed by lipid peroxidation

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TABLE 1
Examples of Free Radicals

Name	Formula	Comments
Hydrogen atom	$H^{\cdot}$	The simplest free radical known
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
Superoxide	$O_2^{\cdot -}$	An oxygen-centered radical
Hydroxyl	$OH^{\cdot}$	An oxygen-centered radical; the most highly reactive oxygen radical known
Thiyl	$RS^{\cdot}$	General name for a group of radicals with an unpaired electron residing on sulfur
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Oxygen-centered radicals formed during the breakdown of organic peroxides
Oxides of nitrogen	$NO^{\cdot}$, $NO_2^{\cdot}$	Both are free radicals; $NO^{\cdot}$ is formed *in vivo* from the amino acid L-arginine. $NO_2^{\cdot}$ 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)

(Figure 1), attack of $^1O_2$ or even by the action of *lipoxygenase* enzymes, can decompose to generate radicals upon heating, or even at 37°C in the presence of transition metal ions.

$$LOOH + Fe^{2+}(Cu^{+}) \rightarrow LO^{\cdot} + Fe^{3+}(Cu^{2+}) + OH^{-} \quad (3)$$
$$LOOH + Fe^{3+}(Cu^{2+}) \rightarrow LOO^{\cdot} + Fe^{2+}(Cu^{+}) + OH^{+} \quad (4)$$

The peroxyl ($LOO^{\cdot}$) and alkoxyl ($LO^{\cdot}$) radicals so generated can propagate lipid peroxidation by abstracting hydrogen atoms from adjacent fatty acid side-chains (Figure 1). Hence, transition metal ions, especially iron and copper, are powerful promoters of free-radical damage (Equations 2 to 4) in both the human body[1,8] and foods.[6]

Lipoxygenase enzymes are of importance in at least two areas. First, they can generate peroxides in food material during handling and processing.[2,6] Second, they may generate peroxides in human low-density lipoproteins *in vivo* and facilitate the development

TABLE 2
How Free Radicals Can React with Nonradicals to Give Radicals

Type of reaction	General equation	Example
Addition	$x^{\cdot} + y \rightarrow [x\text{–}y]^{\cdot}$	Addition of $OH^{\cdot}$ to guanine in DNA to give 8-hydroxyguanine radical[3]
Reduction (electron donation)	$x^{\cdot} + y \rightarrow y^{\cdot -} + x^{+}$	Reduction of O_2 to O_2^{-} by paraquat radical[1] $PQ^{\cdot +} + O_2 \rightarrow PQ^{2+} + O_2^{-}$
Oxidation (electron acceptance)	$x^{\cdot} + y \rightarrow x^{-} + y^{\cdot +}$	Oxidation of ascorbic acid[1] by $O_2^{-} + H^{+} \rightarrow$ ascorbate$^{\cdot}$ + H_2O_2
Oxidation (hydrogen atom transfer)	$x^{\cdot} + y\text{–}H \rightarrow x\text{–}H + y^{\cdot}$	Reaction of α-tocopherol with lipid peroxyl radicals[4] $\alpha TH + LOO^{\cdot} \rightarrow LOOH + \alpha T^{\cdot}$

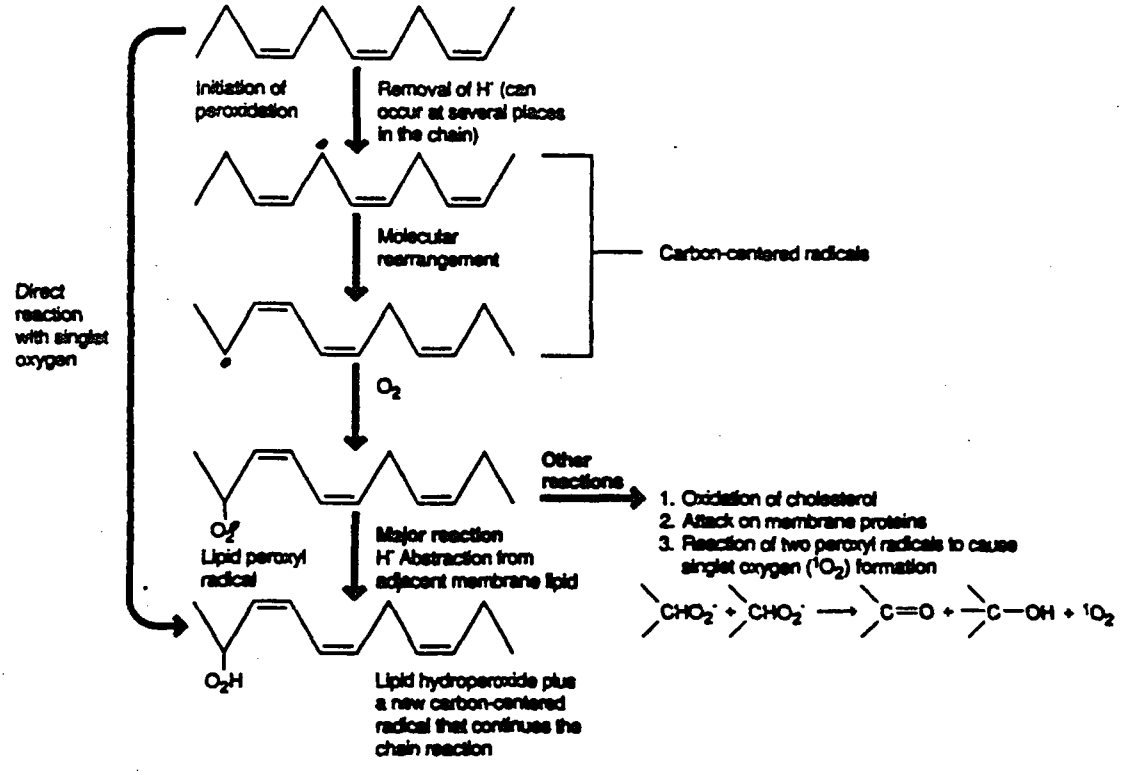

FIGURE 1. Diagram of lipid peroxidation. (Adapted from Halliwell, B., *Lipid Peroxidation, Free Radical Reactions and Human Disease*, Current Concepts Series, Upjohn, Kalamazoo, MI.)

of atherosclerosis, a process in which lipid peroxidation appears to be intimately involved.[7,12]

The terms *reactive oxygen species (ROS)* and *oxygen-derived species* are often used by biologists to include both oxygen radicals ($O_2^{\cdot-}$, $OH^{\cdot}$, $LOO^{\cdot}$, and $LO^{\cdot}$) and nonradical oxygen-containing reactive agents (HOCl, H_2O_2, $^1O_2$, and O_3). *Reactive* is a relative term: $OH^{\cdot}$ is extremely reactive, whereas $O_2^{\cdot-}$ and H_2O_2 are much more selective in their reactions: there are few molecules with which they react quickly. $LO^{\cdot}$ and $LOO^{\cdot}$ have intermediate reactivities.

II. FORMATION OF OXYGEN-DERIVED SPECIES *IN VIVO* AND ITS BIOLOGICAL CONSEQUENCES

All organisms suffer some exposure to $OH^{\cdot}$ because it is generated *in vivo* by the homolytic fission of O-H bonds in water, driven by our continuous exposure to background ionizing radiation.[13] The 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 $OH^{\cdot}$ with second-order rate constants of 10^9 to 10^{10} M/s (essentially, if $OH^{\cdot}$ contacts the compound, a reaction occurs). Damage caused by $OH^{\cdot}$, once this radical has been formed, is probably unavoidable and is dealt with by repair processes, as summarized in Table 3.

It is now well established (for reviews, see References 1 and 21 to 23) that $O_2^{\cdot-}$ 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 process:

"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.[1,23,24] It also includes so-called "autoxidation" reactions in which such compounds as catecholamines, ascorbic acid, and reduced flavins are alleged to react directly with O_2 to form $O_2^{\cdot-}$. In fact, such "autoxidations" are usually catalyzed by the presence of transition metal ions.[1]

Deliberate synthesis — The classic example of deliberate metabolic generation of ROS for useful

TABLE 3
Repair of Oxidative Damage

Substrate of damage	Repair system
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[13,14]	A wide range of enzymes exist that recognize abnormalities in DNA and remove them by excision, resynthesis, and rejoining of DNA strands[14,15]
Proteins	
Many ROS can oxidize -SH groups; $OH^{\cdot}$ attacks many amino acid residues;[16] Proteins often bind transition metal ions, making them a target of attack by site-specific $OH^{\cdot}$ generation[16]	Oxidized methionine residues may be repaired by methionine sulfoxide reductase;[17] other damaged proteins may be recognized and preferentially destroyed by cellular proteases[16,18]
Lipids	
Some ROS ($OH^{\cdot}$, $LO^{\cdot}$, and $LOO^{\cdot}$, but not $O_2^{\cdot-}$ or H_2O_2) can initiate lipid peroxidation	Chain-breaking antioxidants (especially α-tocopherol) remove chain-propagating peroxyl radicals;[4] phospholipid hydroperoxide glutathione peroxidase can remove peroxides from membranes,[19] as can some phospholipases;[20] normal membrane turnover can release damaged lipids[1]

purposes is the production of $O_2^{\cdot-}$, HOCl, and H_2O_2 by activated phagocytes.[25] Hydrogen peroxide is additionally generated *in vivo* by several oxidase enzymes, such as glycolate oxidase, xanthine oxidase, and D-amino acid oxidase.[21,26] Evidence is accumulating that extracellular $O_2^{\cdot-}$ is also produced *in vivo* by several cell types other than phagocytes, including lymphocytes,[27] fibroblasts,[28,29] and vascular endothelial cells (reviewed in Reference 30). Such $O_2^{\cdot-}$ may serve important biological functions such as intercellular signalling and cell growth regulation.[27-31] Generation of $O_2^{\cdot-}$, HOCl, and H_2O_2 by phagocytes is known to play an important part in the killing of several bacterial and fungal strains.[25]

Similarly, some metabolic roles for H_2O_2 have been proposed.[31-34] For example, H_2O_2 is used by the enzyme *thyroid peroxidase* to help make thyroid hormones.[32] H_2O_2 (or products derived from it) can displace the inhibitory subunit from the cytoplasmic gene transcription factor NF-κB. The active factor migrates to the nucleus and activates expression of a wide range of genes by binding to specific DNA sequences in enhancer and promoter elements. Thus, H_2O_2 can induce expression of genes controlled by NF-κB. For example, NF-κB can induce the expression of genes of the provirus HIV-1, the major cause of acquired immunodeficiency syndrome.[34] H_2O_2, a nonradical, resembles water in its molecular structure and is very diffusible within and between cells.

Much of the $O_2^{\cdot-}$ generated *in vivo* probably undergoes a dismutation reaction to give H_2O_2, as represented by the overall equation

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

A. Toxicity of $O_2^{\cdot-}$ and H_2O_2

Superoxide is much less reactive than $OH^{\cdot}$, but some biological targets can react with it. Thus, $O_2^{\cdot-}$ combines with $NO^{\cdot}$ (Equation 1). The resulting peroxynitrite may be directly cytotoxic, for example, by oxidizing essential -SH groups on proteins.[35] Peroxynitrite can also decompose to generate a range of toxic species, including $OH^{\cdot}$, $NO_2^{\cdot}$ and NO_2^+ (see Reference 36).

$$ONOO^- + H^+ \rightarrow OH^{\cdot} + NO_2^{\cdot} \qquad (6)$$

Because $NO^{\cdot}$ is a vasodilator agent,[37] the ability of $O_2^{\cdot-}$ to remove it can produce vasoconstrictor effects.[37,38] For example, a role for $O_2^{\cdot-}$ in the pathogenesis of hypertension has been suggested.[39]

Superoxide also has been shown to be capable of inactivating several bacterial enzymes, such as *Escherichia coli* dihydroxyacid dehydratase, aconitase, and 6-phosphogluconate dehydratase.[24,40] It appears to attack iron-sulfur clusters at the enzyme active sites. Whether such reactions of $O_2^{\cdot-}$ occur in mammals has yet to be decided, although in isolated submitochondrial particles, $O_2^{\cdot-}$ has been claimed to inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain.[41] The protonated form of $O_2^{\cdot-}$, hydroperoxyl radical ($HO_2^{\cdot}$), is much more reactive than $O_2^{\cdot-}$ *in vitro*.[42] For example, $HO_2^{\cdot}$ can initiate peroxidation of polyunsaturated fatty acids[42] and can decompose lipid hydroperoxides to free radicals,[43] neither of which can be done by $O_2^{\cdot-}$. However, there is as yet no direct evidence that $HO_2^{\cdot}$ exerts damaging effects *in vivo*.

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; for example, it inactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase.[44] H_2O_2 also forms $OH^{\cdot}$ in the presence of transition metal ions (Equation 2) and $O_2^{\cdot-}$ can facilitate this reaction:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{\text{metals}} OH^{\cdot} + OH^- + O_2 \qquad (7)$$

The evidence for the biological importance of Equation 7 has been reviewed recently in detail.[1,8,45]

III. ANTIOXIDANT DEFENSE SYSTEMS

Living organisms have evolved antioxidant defenses to remove $O_2^{\cdot-}$ and H_2O_2. *Superoxide dismutases* (SODs) remove $O_2^{\cdot-}$ by greatly accelerating its conversion to H_2O_2 (Equation 5).[23,24] 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.[23] *Catalases* in the peroxisomes[21] convert H_2O_2 into water and O_2 and help to dispose of H_2O_2 generated by the action of oxidase enzymes located in these organelles. However, the most important H_2O_2-removing enzymes in human cells are *glutathione peroxidases* (GSHPX), enzymes that require selenium (as selenocysteine at the active site) for their action. GSHPX enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG). Glutathione reductase, an FAD-containing enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power.[21]

Another important antioxidant defense is that iron, copper, and other transition metal ions in chemical forms that can decompose H_2O_2 to $OH^{\cdot}$ are in very

short supply *in vivo*: living organisms take great care to keep as much iron and copper as possible safely bound to transport and storage proteins.[1,46] This sequestration of metal ions is particularly important in the extracellular environment, where levels of SOD, catalase, GSH, and GSHPX are usually low.[46] For example, the absence of metal ions catalytic for free-radical reactions in human blood plasma may allow O_2^- and H_2O_2 generated by cells (e.g., phagocytes, lymphocytes, and endothelial cells) to be used as intercellular signals.

A. Radical Scavenging Antioxidants: Problems and Principles *In Vivo* and in the Food Matrix

In addition to antioxidant defense enzymes, living organisms contain a variety of radical-scavenging antioxidants, including GSH, uric acid, α-tocopherol and ascorbic acid (for discussions, see References 1, 4, 22, and 46 to 49). None of these compounds is a radical. Hence, when they scavenge radicals *in vivo*, new radicals (Figure 1) can be generated (Table 2). For example, α-tocopherol delays lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains.[4] This gives a tocopherol radical, however.

$$LOO^{\cdot} + \alpha TH \rightarrow \alpha T^{\cdot} + LOOH \quad (8)$$

It is widely believed, but not yet rigorously proven,[50] that ascorbic acid (and possibly GSH) can reduce this radical back to α-tocopherol. If this is so, new radicals will form

$$\alpha T^{\cdot} + \text{ascorbic acid} \rightarrow \alpha TH + \text{semidehydroascorbate} \quad (9)$$

$$GSH + \alpha T^{\cdot} \rightarrow \alpha TH \rightarrow + GS^{\cdot} \quad (10)$$

Ubiquinol (reduced coenzyme Q) might also regenerate α-tocopherol in membranes and lipoproteins:[51]

$$CoQH_2\,\alpha T^{\cdot} \rightarrow CoQH^{\cdot} + \alpha TH \quad (11)$$

The fate of all these radicals must be considered. For example, ascorbate (vitamin C) is often claimed to be an important antioxidant *in vivo*.[47] Its ability to show antioxidant properties is related to the fact that the semidehydroascorbate radical is much less reactive than are many of the radicals that can be scavenged by ascorbate.[52] Enzymic systems (reviewed in Reference 1) exist *in vivo* to reduce this radical back to ascorbate using NADH (the NADH-semidehydroascorbate reductase enzyme) or GSH (the dehydroascorbate reductase enzyme) as sources of reducing power. However, these enzymes seem to be largely intracellular, and so ascorbic acid is rapidly depleted in human extracellular fluids under conditions of oxidative stress,[47,53–56] probably by the reactions:

$$\text{ascorbate} \xrightarrow{\text{radical attack}} \text{semidehydroascorbate} \quad (12)$$

$$2\ \text{semidehydroascorbate} \xrightarrow{\text{disproportionation}} \text{ascorbate} + \text{dehydroascorbate} \quad (13)$$

$$\text{dehydroascorbate} \xrightarrow[\text{breakdown}]{\text{rapid nonenzymatic}} \text{oxalate, threonate, other breakdown products} \quad (14)$$

However, oxidizing ascorbic acid can modify proteins in a glycation-type reaction,[57] which can lead to problems in the food matrix.[58] Ascorbate can also show prooxidant effects in the presence of transition metal ions.[1,49,58] The careful sequestration of iron and copper ions in healthy humans[1,31,46,59] means that the antioxidant abilities of ascorbate normally will predominate *in vivo*. In addition, ascorbate protects isolated human low-density lipoproteins against lipid peroxidation even in the *presence* of copper ions by several mechanisms,[60] including regeneration of α-tocopherol.[61] However, copper ions can promote severe oxidative damage to DNA[62] and protein[63] in the presence of ascorbate, so that the overall effect of ascorbate on the development of atherosclerosis[64] and other human diseases is uncertain.

The basic principles of free radical chemistry must not be forgotten when designing antioxidants for therapeutic use or for addition to foods: the fate of the antioxidant radicals must be considered. Many food antioxidants (including BHA, BHT, and propyl gallate) are chain-breaking inhibitors of lipid peroxidation, acting by a mechanism of the type shown in Equation 9. There is increasing concern over their safety (for recent discussions see References 6, 65, and 66), and their replacement by "safe, natural" antioxidants such as flavonoids and other plant phenolics has been increasingly advocated. However, many plant phenolics can bind and reduce iron ions and *accelerate* free-radical damage to DNA and proteins,[67–71] both by reducing metal ions and by oxidizing in their presence to generate O_2^- and H_2O_2.[67,68,71] For example, many flavonoids have been described as antioxidants because they can inhibit lipid peroxidation. However, they

could exert prooxidant effects on other molecules *in vivo* and in the food matrix, such as proteins.[69] The biological significance of these effects remains to be fully evaluated, but it cannot be simplistically assumed that "natural" equates to "safe", or that antioxidants that protect lipids against free-radical damage will necessarily be innocuous to other biological molecules (Table 4).

IV. OXIDATIVE STRESS

Antioxidant defense enzymes are essential for healthy aerobic life,[1,23,72] as are at least some of the antioxidant nutrients (vitamins E and C). However, the optimal intakes of vitamin C, α-tocopherol, β-carotene, and other carotenoids have yet to be determined. Humans do not have a great excess of antioxidant defense systems. Indeed, although SOD is an important antioxidant, an excess of it in relation to H_2O_2-metabolizing enzymes can be deleterious.[73,74] For example, transgenic mice overexpressing human copper-zinc SOD are resistant to elevated O_2 and to certain toxins,[75] but they show certain neuromuscular abnormalities resembling those found in patients with Down's syndrome.[73] The gene encoding Cu,Zn SOD is located on chromosome 21 in humans, and Down's syndrome usually is caused by trisomy of this chromosome, raising tissue Cu,Zn SOD levels by about 50%. The data from transgenic animals 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.[73]

In fact, the balance between antioxidants and prooxidants in the human body may always be tipped slightly in favor of the latter, because there is good evidence for ongoing oxidative damage in the human body (Table 5). Hence, repair systems have evolved (for reviews see References 1, 14, and 83) to cope with such damage (Table 3). This situation may exist because a number of ROS have useful metabolic roles, so that they are not scavenged with 100% efficiency. However, if the production of ROS increases or total antioxidant defenses fall (e.g., as a result of inadequate nutrition[84]), the situation of *oxidative stress* [22] is said to result.

Most aerobes can tolerate mild oxidative stress; indeed, they often respond to it by inducing the 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.[85] Other examples are the complex adaptive response of *E. coli* treated with low concentrations of H_2O_2 [86] and the activation of NF-κB in oxidatively stressed mammalian cells.[34]

However, severe oxidative stress can cause cell damage and death. Mechanisms of injury include excessive rises in intracellular "free" Ca^{2+}, disruption of energy metabolism, and damage to DNA, proteins, carbohydrates, and lipids.[1,16,44,87–89] The relative importance of damage to these different molecular targets in causing cell injury or death resulting from severe oxidative stress depends upon what degree of oxidative stress occurs, by what mechanism it is imposed, for

TABLE 4
Some Questions to Ask when Evaluating a Proposed Antioxidant

What biomolecule is the compound supposed to protect? For example, an inhibitor of lipid peroxidation is unlikely to be useful if the oxidative damage is mediated by an attack on proteins or DNA

Will the compound be present at or near the biomolecule in sufficient concentration?

How does the compound protect: by scavenging radicals, by preventing their formation, or by repairing damage? If the antioxidant acts by scavenging radicals, can the resulting antioxidant-derived radicals do biological damage?

Can the antioxidant cause damage in biological systems different from those in which it exerts protection? For example, several inhibitors of lipid peroxidation have the potential to accelerate free radical damage to other biomolecules[68,71]

Note: Similar questions apply whether the antioxidant is designed for therapeutic use or for addition to food.

TABLE 5
Some Evidence for Ongoing Oxidative Damage in the Human Body

Molecule	Observation
DNA	Low levels of oxidized bases are found in DNA isolated from cells and tissues;[76] damaged bases are excreted in urine after their removal from DNA by repair processes.[77]
Protein	Low levels of protein carbonyls, end products of free radical attack on proteins,[15] are present in tissues and body fluids[15,78,79]
Lipid	Accumulation of age pigments;[1] detection of end products of free radical attack on lipids in tissues and body fluids;[1,80,91] many older methods for assessing peroxidation give misleadingly-high results,[1,80] but recent methods show greater promise[81].
Uric acid	Low levels of products that can result from free radical attack on uric acid are present in human body fluids[82]

how long, and the nature of the systems stressed.[89] For example, lipid peroxidation appears to be an important consequence of oxidative stress in human atherosclerotic lesions.[12] 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*.[90] However, in only a few cases does lipid peroxidation appear to be the major mechanism of primary call injury by oxidative stress: damage to proteins and DNA is usually more important.[1,87–89] Thus, it is very important to consider molecular targets other than lipids in the design of antioxidants for therapeutic use (Table 3). However, the situation in the food matrix is different: oxidative damage to lipids causes detectable food deterioration (e.g., rancid smell, "off flavors"), whereas damage to proteins and nucleic acids may not be noticed and its occurrence has scarcely been explored.

V. OXIDATIVE STRESS AND HUMAN DISEASE

Oxidative stress can be imposed in several ways. Thus, severe malnutrition can deprive humans of the minerals (e.g., Cu, Mn, Zn, and Se) and vitamins (e.g., ascorbate, riboflavin [needed for the FAD cofactor of glutathione reductase], and α-tocopherol) needed for antioxidant defense.[84] More usually, however, the stress is due to the production of excess ROS.

Several drugs and toxins impose oxidative stress during their metabolism. Carbon tetrachloride is one example.[90] Another is paraquat, a herbicide that causes lung damage in humans. Its metabolism within the lung leads to the production of large amounts of $O_2^{\cdot-}$ and H_2O_2.[1] Cigarette smoking imposes oxidative stress, and smokers may need additional amounts of antioxidant vitamins as a result.[91]

Oxidative stress is also involved in human disease. Many of the biological consequences of excess radiation exposure are probably due to $OH^{\cdot}$-dependent damage to proteins, DNA, and lipids.[13] Oxidative damage (resulting from exposure to elevated O_2 in incubators) may contribute to retinopathy of prematurity in infants.[92] An additional complicating factor is that some premature babies (and even some normal-term babies) contain iron capable of catalyzing free-radical reactions in their plasma,[93] so that they may be exceptionally prone to oxidative stress. The presence of lipid hydroperoxides in intravenous lipid emulsions administered to preterm infants[95] is especially worrying if iron is present in their plasma.[93]

Tissue damage by disease, trauma, toxins, and other causes usually leads 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, $NO^{\cdot}$, and interleukins in adult respiratory distress syndrome and septic shock (reviewed in Reference 95). ROS can be placed in the same category, that is, *tissue damage will usually lead to increased ROS formation* and oxidative stress. Figure 2 summarizes some of the reasons for this. Indeed, in most human diseases, oxidative stress is a *secondary* phenomenon, a consequence of the disease activity.[1,95,96] That does not mean it is not important![1] For example, excess production of $O_2^{\cdot-}$, H_2O_2, and other species by phagocytes at sites of chronic inflammation can do severe damage. This seems to happen in the inflamed joints of patients with rheumatoid arthritis[95] and in the gut of patients with inflammatory bowel diseases.[97] Tissue injury can release metal ions from their storage sites within cells, leading to $OH^{\cdot}$ generation.[1,95,96,98] When generated in excess, $O_2^{\cdot-}$ can mobilize small amounts of "catalytic" iron from the iron storage protein ferritin,[99] and H_2O_2 can decompose heme proteins to release free iron.[100,101]

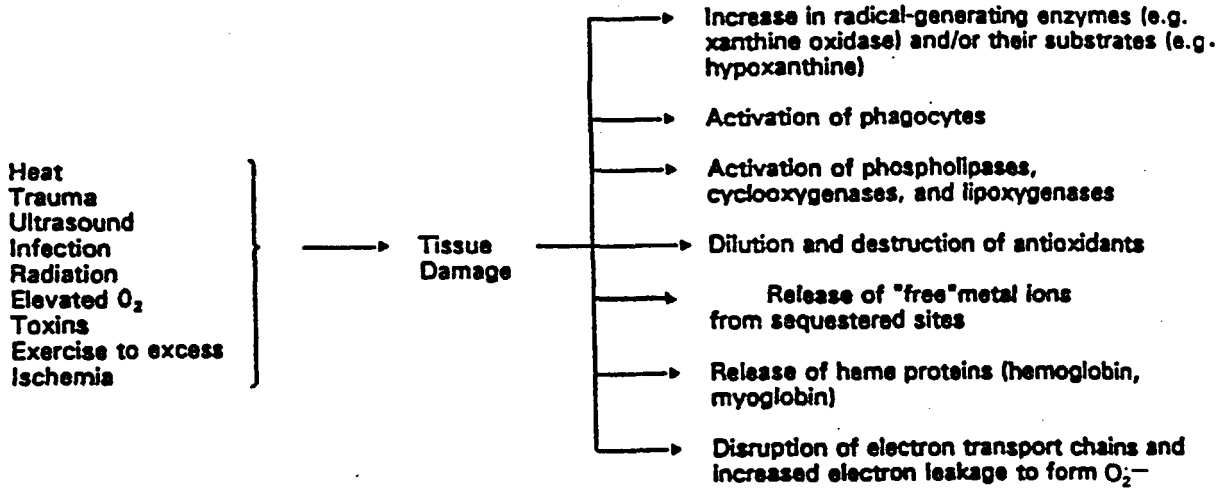

FIGURE 2. Reasons for increased oxidative damage as a consequence of injury to human tissues.

Thus, the main question 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. 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.[95]

VI. OXIDATIVE DAMAGE AND FOOD PROCESSING

The handling, processing, and cooking of food materials can also cause oxidative damage.[5-7,102,103] Indeed, the processing of meat and fish can release iron ions and heme proteins that cause oxidative deterioration[102,103] by mechanisms similar to those that occur in injured human tissues (Figure 2). Table 6 shows further illustrative data using plant material. Fresh-frozen spinach stored for several weeks contained the same levels of vitamin E and lipid peroxidation end

TABLE 6
Levels of Lipid Peroxidation and Nutritional Characteristics of Raw, Fresh-Frozen, and Canned Spinach (*Spinacia oleracea*)

	Raw	Fresh-Frozen[a]	Canned
Vitamin E[b]	35.8 ± 2.0	34.0 ± 1.7	5.0 ± 0.7
Lipid peroxidation[b] (TBARS)	2.5 ± 0.1	1.5 ± 0.3	3.4 ± 0.7

[a] Frozen immediately after harvesting and then stored at −20°C.

[b] Mean values and standard deviations corresponding to five and three different experiments, respectively. TBARS (thiobarbituric acid reactive substances) are expressed as the micromolar equivalent of MDA per milliliter of extract using an HPLC-based TBA test.[80] Vitamin E is expressed as nanomoles per milliliter of extract.

products as the freshly harvested plant, but the canning process led to decreased vitamin E and a small increase in steady-state peroxidation levels. Storage of raw spinach at 4°C (to simulate refrigerator conditions) also led to slow increases in peroxidation and falls in vitamin E.

VII. ASSAY METHODOLOGY: A PREREQUISITE FOR EVALUATING THE ROLE OF FREE RADICALS IN HUMAN DISEASE AND THE EFFECTS OF ANTIOXIDANT SUPPLEMENTATION ON OXIDATIVE STRESS *IN VIVO*

At least some of the antioxidant nutrients are essential to human health, and others (such as carotenoids) may be highly beneficial. However, we do not yet know what dietary intakes are optimal. In principle, this could be investigated by varying the dietary intake of antioxidants and measuring free- radical damage in the human body.

Several measurement techniques are available.

A. Radical Trapping

The only technique that can "see" free radicals directly is electron paramagnetic resonance (EPR) spectroscopy, which measures the energy changes that occur as unpaired electrons align in response to an external magnetic field. However, biologically important oxygen radicals do not accumulate to high enough concentrations to be directly observable by ESR. Direct ESR of biological material detects fairly unreactive radicals, such as semidehydroascorbate.[52] Identifying highly reactive radicals formed in biological systems can be achieved by two general approaches, the first of which is trapping. The radical is allowed to react with a trap molecule to give one or more stable products, which are then measured. Mention of trapping usually brings to mind the technique of spin trapping, in which the radical reacts with a spin trap to form a more stable radical, which is detectable by EPR (for reviews see References 104 and 105). Spin traps such as phenyl-*t*-butyl nitrone have proved very useful in detecting certain free radicals in whole animals.[105]

However, there are many trapping methods other than spin trapping. For example, Babbs and Griffin[106] used dimethylsulfoxide as a trap for $OH^{\cdot}$ in animals, measuring the end products produced. The technique of aromatic hydroxylation has been introduced as an assay for $OH^{\cdot}$, based on the fact that $OH^{\cdot}$ generated under physiological conditions reacts with aromatic compounds at a diffusion-controlled rate, giving rise predominantly to hydroxylated end products.[107] An aromatic hydroxylation assay was first applied to humans[108] using salicylate (2-hydroxybenzoate) as a "trap" for $OH^{\cdot}$. The high doses of aspirin (acetylsalicylate) sometimes given to rheumatoid patients result in concentrations of salicylate in synovial fluid that could conceivably trap some $OH^{\cdot}$. The attack of $OH^{\cdot}$ upon salicylate produces two major hydroxylated products: 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate. The latter product can be produced by the action of cytochromes P-450 on salicylate, whereas 2,3-dihydroxybenzoate apparently cannot.[109] Hence, the formation of the latter product may be an index of $OH^{\cdot}$ production *in vivo*.[108]

Another aromatic trap for $OH^{\cdot}$ is the amino acid, phenylalanine. *In vivo*, the L-isomer of this amino acid is hydroxylated by phenylalanine hydroxylase at position 4 on the benzene ring to give L-*p*-tyrosine. D-Phenylalanine is not recognized by this enzyme. By contrast, $OH^{\cdot}$ cannot distinguish between the two isomers: it acts upon both L- and D-phenylalanine to produce a mixture of *o*-, *m*-, and *p*-tyrosines. Formation of these tyrosines has been used to measure $OH^{\cdot}$ production by activated neutrophils[110] and reperfused heart,[111] and to detect the generation of $OH^{\cdot}$ in irradiated foods.[112]

B. Radical "Fingerprinting"

Trapping methods have been very useful in the laboratory, but are probably of very limited use for extensive studies on humans, for example, to determine if nutritional antioxidant supplementation actually does decrease oxidative damage *in vivo*. The principle of fingerprinting methods is the measurement of end products of free-radical damage. Table 5 list some of the products that could be measured. Thus, end products of free-radical damage to lipids (e.g., hydroperoxides, isoprostanes), proteins (e.g., protein carbonyls), and DNA (e.g., 8-hydroxyguanine) may be measured.

The attack of $OH^{\cdot}$ on DNA produces a pattern of chemical changes to all four of the purine and pyrimidine bases that seems characteristic of $OH^{\cdot}$: other ROS either do not attack DNA at all ($O_2^{\cdot-}$, H_2O_2) or they preferentially modify guanine. 8-Hydroxyguanine is usually a major product of ROS attack on DNA.[3,113–115] Both HPLC-based[113,114] and GC/MS-based[3,76,115] technologies for measuring DNA damage by ROS have been described. Ames et al.[77,116,117] and Stilwell et al.[118] have measured DNA base damage products in urine as a putative measure of oxidative DNA damage *in vivo*. However, the 8-hydroxyguanine content of human urine

may be influenced by diet (just as the lipids in food can become oxidized, so can the DNA and proteins). 8-Hydroxydeoxyguanosine may originate by degradation of oxidatively damaged dGTP rather than from DNA itself.[119] Thus, several questions need to be answered before the routine use of urinary measurement of guanine-derived products as an index of DNA damage *in vivo* can be validated.

Uric acid is degraded on exposure to $OH^{\cdot}$, HOCl, O_3 and mixtures of heme proteins with H_2O_2.[55,82] The major product is allantoin, but others include oxonic, oxaluric, parabanic, and cyanuric acids. Hence, measurement of these products is a putative index of damage by ROS in humans.[82]

It must be remembered that because of the activity of repair systems (Table 3), the levels of oxidative damage products measured in biological systems (Table 5) are *steady-state* levels, that is, products are generated and then removed. Thus, a rise in, say, the level of 8-hydroxyguanine in DNA or protein carbonyls could be due not only to increased oxidative stress but also to diminished repair.

C. Conclusions

ROS are formed *in vivo* both usefully and "accidentally". Their formation increases in all human disease, and sometimes makes a significant contribution to disease severity. ROS-induced damage occurs constantly in the human body and has to be repaired. The food matrix cannot repair itself, and so excessive oxidative damage, especially to lipids, must be prevented by the addition of antioxidants.

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