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INVITED REVIEW

Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification

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ABSTRACT

Reactive oxygen species (ROS) and other radicals are involved in a variety of biological phenomena, such as mutation, carcinogenesis, degenerative and other diseases, inflammation, aging, and development. ROS are well recognized for playing a dual role as deleterious and beneficial species. The objectives of this review are to describe oxidative stress phenomena, terminology, definitions, and basic chemical characteristics of the species involved; examine the biological targets susceptible to oxidation and the defense mechanisms of the organism against these reactive metabolites; and analyze methodologies, including immunohistochemical markers, used in toxicological pathology in the visualization of oxidative stress phenomena. Direct detection of ROS and other free radicals is difficult, because these molecules are short-lived and highly reactive in a nonspecific manner. Ongoing oxidative damage is, thus, generally analyzed by measurement of secondary products including derivatives of amino acids, nuclei acids, and lipid peroxidation. Attention has been focused on electrochemical methods based on voltammetry measurements for evaluating the total reducing power of biological fluids and tissues. This approach can function as a tool to assess the antioxidant-reducing profile of a biological site and follow changes in pathological situations. This review thus includes different topics essential for understanding oxidative stress phenomena and provides tools for those intending to conduct study and research in this field.

Keywords. Antioxidants; pro-oxidant; reducing power; cyclic voltammetry; oxidative damage markers.

INTRODUCTION AND DEFINITIONS

The related terms oxidative stress, oxidative damage, free radical, and antioxidant have become an integrated part of the scientific vocabulary and are often used in a variety of scientific discussions and issues by chemists, physicists, biologists, and researchers. The scientific literature is replete with articles concerning oxidative stress phenomena. The objectives of this review are to provide and elaborate on the terminology and definitions used in this field and describe the essence, distribution, causes, and importance of oxidative stress phenomena, as well as some of the methodology used, in particular, in toxicological pathology.

Free radicals,known in chemistry since the beginning of the 20th century, were initially used to describe intermediate compounds in organic and inorganic chemistry, and several chemical definitions for them were suggested. Only in 1954 when the pioneering work of Daniel Gilbert and Rebecca Gersham was published (77) were these radicals suggested as important players in biological environments and responsible for deleterious processes in the cell. Soon after, in 1956, Herman Denham (104) suggested that these species might play a role in physiological events and, particularly, in the aging process (105). His hypothesis, the free-radical theory of aging, inspired numerous studies and research efforts and

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This review is dedicated to the memory of Walter Yaakov Kohn, the father of Ron Kohen.

Abbreviations: AC: anodic current; ARE: antioxidant responsive element; AP: apyramidenic; ATP: adenosine triphosphate; CHAOS: Cambridge Heart Antioxidant Study; COX-2: cyclooxygenase-2; CV: cyclic voltammetry; EC: endothelial cell; EC-SOD: extracellular SOD; eNOS: endothelial NOS; FeSOD: iron SOD; Fe^{+2}: ferrous ion of iron; Fe^{+3}: ferric ion; Fe^{+4}: ferryl ion; FRAP: ferric reducing-antioxidant power; GC-MS: gas chromatograph mass spectroscopy; GSH: reduced glutathione/glutamic acid-cysteine-glycine; GSSG: oxidized glutathione; GST: glutathione S-transferase; GST-pi: glutathione S transferase-pi; HClO: hypochlorous acid; HO-1: haemoxygenase-1; $HO_2^{\cdot}$: hydroperoxyl radical; H_2O_2: hydrogen peroxide; HPLC: high pressure liquid chromatography; HPLC-ECD: high pressure liquid chromatography electrochemical detection; 2,4-Hx: 2,4 hexadienal; IL-1: interleukin-1; iNOS: inducible NOS; IP: indium phosphide; LDL: low-density lipoprotein; LMWA: low-molecular-weight antioxidants; MDA: malondialdehyde; MS: multiple sclerosis; N_2O_3, NO_2^-, NO_3^-: derivatives of NO + H_2O_2 and hypochlorous acid; NADH: reduced nicotinamide dinucleotide; NADPH: reduced nicotinamide phosphate dinucleotide; $NO^{\cdot}$: nitric oxide radical; NO^+: nitrosonium cation; NO^-: nitroxyl anion; NOS: nitric oxide synthase; Nrf2: nuclear factor 2; NSAID: non-steroidal anti-inflammatory drug; $^1O_2$: singlet oxygen; O_2: molecular oxygen; $O_2^{-\cdot}$: superoxide radical; O_3: ozone; ODS: oxygen-derived species; $OH^{\cdot}$: hydroxyl radical; OH^-: hydroxide ion; 8-OHdG: 8-hydroxydeoxyguanosine, oxidized form of guanine; 8-OHG: 8-hydroxyguanosine; $ONOO^-$: peroxynitrite; ONOOH: protonated peroxynitrite; ORAC: oxygen radical absorbance capacity; oxLDL: oxidized low-density lipoprotein; PIN: prostatic intraepithelial neoplasia; $R^{\cdot}$: radical; RNS: reactive nitrogen species; $RO^{\cdot}$: alkoxyl radical; $ROO^{\cdot}$: peroxyl radical; ROP: retinopathy of prematurity; ROS: reactive oxygen species; –SH: sulfhydryl; SOD: superoxide dismutase; STZ: streptozotocin; TEAC: Trolox equivalent-antioxidant capacity; TBARS: thiobarbituric reactive species; TRAP: total radical-trapping potential; UV-A, UV-B, UV-C: ultraviolet radiations, solar, non-ionizing; VP: ventral lobe of prostate.

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contributed significantly to our knowledge of radicals and, more specifically, oxygen-derived radicals and other oxygen-derived, nonradical reactive species. These metabolites are now considered major players in biochemical reactions, cellular response, and clinical outcome (4, 24, 51, 56, 100, 105, 229).

Since the discovery of oxygen in the early 18th century by Antoine Laurent Lavoisier, the necessity of controlling oxygen levels has been recognized (117). An elusive molecule, oxygen plays contradictory roles, one essential for life and the other as a toxic substance. In 1775 Priestly (200) described the toxicity of the oxygen molecule to the organism and compared its effect on the body as similar to that of burning a candle. As a candle burns out much faster in oxygen than in air, the body becomes quickly exhausted in this "pure kind of air." The toxicity of the atmospheric oxygen molecule had already been used by our ancestors for therapeutic purposes, such as treatment of sites infected with the anaerobic bacterium *Clostridium* by exposure to air (100). The beneficial use of the toxic effect of oxygen has been utilized in hyperbaric and irradiation therapy (260). The development of submarines, diving as a sport, and medicine have contributed significantly to the knowledge of oxygen, its derivatives, and its toxicity (15, 46, 218). Widespread cases of blindness in young infants born prematurely in the 1940s were associated with the high oxygen concentration in the newly invented incubators (80, 223). This pathology, called retinopathy of prematurity (ROP), was easily controlled by modulating the oxygen concentration in the incubators. Nowadays, we are again facing an increase in ROP due to the vulnerability of the extremely young and small infants, weighing less than 700 grams, to the minimal incubator-oxygen concentration required to sustain their lives. Today oxygen is considered toxic to bacteria, plants, eukaryotic cells, and humans.

A crucial advancement in scientific interest in the field of oxygen toxicity and free radicals occurred when McCord and Fridovich in 1969 (167) discovered the role of the protein hemocuprein in the dismutation of superoxide radicals and described the existence of superoxide dismutase (SOD) in almost all aerobic cells (74). This discovery led to the description of the superoxide theory of oxygen toxicity (100), which became the focus of much research and debate associated with aging, development, diseases, and cell signaling (10, 12, 67, 68, 83, 100, 201).

Oxygen is required by prokaryotic and eukaryotic cells for energy production, often via the electron transport chain in the mitochondria in the latter. In most cases oxygen is consumed as dioxygen in the form of a diatomic molecule, the configuration that exists in the atmosphere (100). The source of oxygen has probably been the evolution of the photosynthetic process in blue-green algae (100). The increase in oxygen concentration in the atmosphere, today at 21%, and its derivative ozone (O_3) has been beneficial, as it has allowed the absorbance of deleterious solar ultraviolet radiation (UV-C, <280 nm) and thereby enabled organisms to survive on dry land. On the other hand, in its harmful role, oxygen itself has been toxic to anaerobic bacteria and forced them to develop a variety of mechanisms to cope with the increasing concentrations. In the atmosphere oxygen concentration is a dynamic parameter that is constantly changing. That there were periods when atmospheric oxygen reached a concentration of 35% and later stabilized at 21% was suggested (100). Today, due to the massive cutting of rain forests, its concentration is decreasing again and probably will lead to changes in the biochemical response of the living cell.

Terminology and Chemistry

Chemically, every compound, including oxygen, that can accept electrons is an oxidant or oxidizing agent (201). In contrast, a substance that donates electrons is a reductant or reducing agent (40, 201). In general, a chemical reaction in which a substance gains electrons is defined as a reduction (225). Oxidation is a process in which a loss of electrons occurs. When a reductant donates its electrons, it causes another substance to be reduced, and, when an oxidant accepts electrons, it causes another substance to be oxidized (111). In biology, a reducing agent acts via donation of electrons, usually by donation of hydrogen or removal of oxygen. An oxidation process is always accompanied by a reduction process in which there is usually a loss of oxygen, while in an oxidation process there is a gain in oxygen (40, 111, 201, 225). Such reactions, called redox reactions, are the basis for numerous biochemical pathways and cellular chemistry, biosynthesis, and regulation (232). They are also important for understanding biological oxidation and radical/antioxidant effects. While reductant and oxidant are chemical terms, in biological environments they should be termed antioxidant and pro-oxidant, respectively (40, 201). There are many examples of the biological importance of pro-oxidants.

This review focuses on oxygen-derived pro-oxidants, which can cause damage to biological targets such as lipids, DNA, and proteins, and on the defending systems of the cell, which are composed of enzymes and reducing equivalents, or antioxidants. In general these pro-oxidants are referred to as reactive oxygen species (ROS) that can be classified into 2 groups of compounds, radicals and nonradicals. The radical group, often incorrectly called free-radical (the term is not accurate, because a radical is always free.), contains compounds such as nitric oxide radical ($NO^{\cdot}$), superoxide ion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$), and one form of singlet oxygen ($^1O_2$) as shown in Table 1 (139). These species are radicals, because they contain at least 1 unpaired electron in the shells around the atomic nucleus and are capable of independent

Table 1.—Radical and nonradical oxygen metabolites.

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	$O_2^{\cdot}$
Superoxide ion	$O_2^{\cdot -}$
Hydroxyl	$OH^{\cdot}$
Peroxyl	$ROO^{\cdot}$
Alkoxyl	$RO^{\cdot}$
Nitric oxide	$NO^{\cdot}$
Nonradical oxygen derivatives	
Hydrogen peroxide	H_2O_2
(Organic peroxide)	ROOH
Hypochlorous acid	HOCL
Ozone	O_3
Aldehydes	HCOR
Singlet oxygen	$^1O_2$
Peroxynitrite	ONOOH

existence (100, 210). The occurrence of one unpaired electron results in high reactivity of these species by their affinity to donate or obtain another electron to attain stability. By definition, the oxygen molecule itself is also a radical, because it contains 2 unpaired electrons in 2 different orbits and, thus, is biradical (100). The oxygen radical is not a reactive one, however, due to the so-called spin restriction, which does not allow the donation or acceptance of another electron before rearrangement of the spin directions around the atom. The group of nonradical compounds contains a large variety of substances, some of which are extremely reactive although not radical by definition. Among these compounds produced in high concentrations in the living cell are hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3), and O_2 as shown in Table 1. The terminologies ROS, oxygen-derived species (ODS), oxidants, reactive nitrogen species (RNS), and pro-oxidant species are often used interchangeably in the scientific literature. Radicals are described in the literature with a superscripted dot ($R^{\cdot}$), which distinguishes them from other reactive oxygen metabolites. An antioxidant (reductant or reducing agent), therefore, can be classified as a compound capable of preventing the pro-oxidation process, or biological oxidative damage (40, 201). Halliwell (92, 93) suggested a definition for antioxidant, which states that this agent, when present in low concentration, significantly prevents or delays oxidation of an oxidizable substrate. Because, however, an antioxidant may act in a variety of ways discussed here, this definition is insufficient and does not encompass the whole spectrum of antioxidants.

The organism must confront and control the presence of both pro-oxidants and antioxidants continuously. We now know that the balance between these is tightly regulated and extremely important for maintaining vital cellular and biochemical functions (111, 122, 139, 225, 232, 236). This balance, often referred to as the redox potential, is specific for each organelle and biological site, and any interference of the balance in any direction might be deleterious for the cell and organism. Changing the balance towards an increase in the pro-oxidant over the capacity of the antioxidant is defined as oxidative stress and might lead to oxidative damage. Changing the balance towards an increase in the reducing power, or the antioxidant, might also cause damage and can be defined as reductive stress (Figure 1).

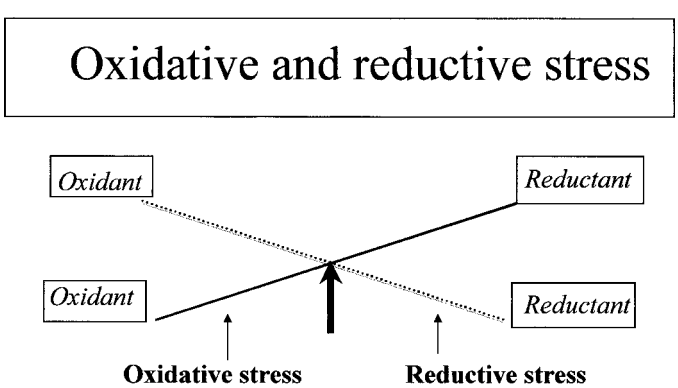


FIGURE 1.—Interference in the balance (large arrow) between oxidant and reductant defines oxidative- or reductive-stress conditions.

CHEMICAL PROPERTIES OF SOME ROS

Because most radicals are short-lived species, they react quickly with other molecules. Some of the oxygen-derived radicals are extremely reactive with a short half-life. For example, $OH^{\cdot}$ can survive for 10^{-10} sec in biological systems; its reaction rate constants ($m^{-1}s^{-1}$) for biological components are extremely high (10^7–$10^9 m^{-1}s^{-1}$) and in many cases diffusion-controlled. The life span of other radicals is also short but depends on the environmental medium. For example, the half-life of $NO^{\cdot}$ in an air-saturated solution might be a few minutes (100). $RO^{\cdot}$ can survive about 10^{-6} of a second, while the half-life of $ROO^{\cdot}$ is about 17 seconds. Nonradical metabolites also possess a relatively short half-life varying from parts of seconds to hours, as in the case of HClO. Obviously, the physiological environment, consisting of such factors as pH and the presence of other species, has a great influence on the half-life of ROS. Toxicity is not necessarily correlated with reactivity. In many cases a longer half-life of a species might imply a higher toxicity of the compound by allowing it adequate time to diffuse and reach a sensitive location where it can interact and cause damage a long distance from its site of production. For example, the relatively long half-life of superoxide radicals permits them to move to locations where they can undergo interaction with other molecules; these radicals can be produced in the mitochondrial membrane, diffuse towards the mitochondrial genome, and reduce transition metals bound to the genome. On the other hand, a highly reactive species with an extremely short life span, like $OH^{\cdot}$, is produced in locations where it can cause damage (224) by interacting with its immediate surroundings. If there is no essential biological target adjacent their production site, radicals will not cause oxidative damage. The high reactivity of radicals and their short life span illustrate the potential toxic effect and difficulties in preventing oxidative damage. To prevent the interaction between radicals and biological targets, the antioxidant should be present at the location where the radicals are being produced in order to compete with the radical for the biological substrate. This information should be used as a guideline in determining appropriate antioxidant therapy.

EXAMPLES OF CHEMICAL QUALITIES AND REACTIVITIES OF SOME ROS

Superoxide Ion Radical ($O_2^{\cdot-}$/$HO_2^{\cdot}$)

This species possesses different properties depending on the environment and pH. Due to its pKa of 4.8, superoxide can exist in the form of either $O_2^{\cdot-}$ or, at low pH, hydroperoxyl ($HO_2^{\cdot}$) (31, 100, 225). The latter can more easily penetrate biological membranes than the charged form. Hydroperoxyl can therefore be considered an important species, although under physiological pH most of the superoxide is in the charged form. In a hydrophilic environment both the $O_2^{\cdot-}$ and $HO_2^{\cdot}$ can act as reducing agents capable, for example, of reducing ferric (Fe^{+3}) ions to ferrous (Fe^{+2}) ions; however, the reducing capacity of $HO_2^{\cdot}$ is higher. In organic solvents the solubility of $O_2^{\cdot-}$ is higher, and its ability to act as a reducing agent is increased. It also acts as a powerful nucleophile, capable of attacking positively charged centers, and as an oxidizing agent that can react with compounds

capable of donating H^+ (eg, ascorbate and tocopherol—see later). The most important reaction of superoxide radicals is dismutation; in this reaction, which we have designated reaction 1, superoxide radical reacts with another superoxide radical. One is oxidized to oxygen, and the other is reduced to hydrogen peroxide (32).

$$HO_2^{\cdot}/O_2^{\cdot -} + HO_2^{\cdot}/O_2^{\cdot -} \xrightarrow{+H^+} H_2O_2 + O_2 \quad k \sim 10^6\ M^{-1}s^{-1} \tag{1}$$

Although the constant rate for this spontaneous reaction is low, it can become much higher in acidic pH where the hydroperoxyl radical is formed.

Hydroxyl Radical ($OH^{\cdot}$)

The reactivity of hydroxyl radicals is extremely high (31, 100, 260). In contrast to superoxide radicals that are considered relatively stable and have constant, relatively low reaction rates with biological components, hydroxyl radicals are short-lived species possessing high affinity toward other molecules. $OH^{\cdot}$ is a powerful oxidizing agent that can react at a high rate with most organic and inorganic molecules in the cell, including DNA, proteins, lipids, amino acids, sugars, and metals. The 3 main chemical reactions of hydroxyl radicals include hydrogen abstraction, addition, and electron transfer (100). $OH^{\cdot}$ is considered the most reactive radical in biological systems; due to its high reactivity, it interacts at the site of its production with the molecules closely surrounding it.

Hydrogen Peroxide (H_2O_2) (100, 102)

The result of the dismutation of superoxide radicals is the production of H_2O_2. There are some enzymes that can produce H_2O_2 directly or indirectly. Although H_2O_2 molecules are considered reactive oxygen metabolites, they are not radical by definition; they can, however, cause damage to the cell at a relatively low concentration (10 μM). They are freely dissolved in aqueous solution and can easily penetrate biological membranes. Their deleterious chemical effects can be divided into the categories of direct activity, originating from their oxidizing properties, and indirect activity in which they serve as a source for more deleterious species, such as $OH^{\cdot}$ or HClO, which is discussed later. Direct activities of H_2O_2 include degradation of haem proteins; release of iron; inactivation of enzymes; and oxidation of DNA, lipids, —SH groups, and keto acids.

Nitric Oxide ($NO^{\cdot}$), Peroxynitrite ($ONOO^-$), and Other Members of the Family (22, 50, 100, 102, 176, 195)

The nitric oxide, or nitrogen monoxide, radical ($NO^{\cdot}$) is produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine. In this reaction, catalyzed by the group of enzymes called nitric oxide synthase(NOS)s, L-arginine is converted to nitric oxide and L-citrulline. Three types of the enzyme exist: neuronal NOS, endothelial NOS (eNOS), and inducible NOS (iNOS). One-electron oxidation results in the production of nitrosonium cation (NO^+), while one-electron reduction leads to nitroxyl anion (NO^-), which can undergo further reactions, such as interacting with $NO^{\cdot}$ to yield N_2O and $OH^{\cdot}$. The half-life of the nitric oxide radicals depends on the square of the radical concentration. $NO^{\cdot}$ can react with a variety of radicals and substances. For example, it can react with H_2O_2 and HClO to yield a line of derivatives such as N_2O_3, NO_2^-, and NO_3^-. One of the most important reactions under physiological conditions is that of superoxide and nitric oxide radicals resulting in peroxynitrite, designated here as reaction 2. This reaction helps to maintain the balance of superoxide radicals and other ROS and is also important in redox regulation.

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^- \quad k > 10^9\ M^{-1}s^{-1} \tag{2}$$

The protonated form of peroxynitrite (ONOOH) is a powerful oxidizing agent that might cause depletion of sulfhydryl (—SH) groups and oxidation of many molecules causing damage similar to that observed when $OH^{\cdot}$ is involved. It can also cause DNA damage such as breaks, protein oxidation, and nitration of aromatic amino acid residues in proteins (eg, 3-nitrosotyrosine), (See method section later). Under physiological conditions, ONOOH can react with other components present in high concentrations, such as H_2O_2 or CO_2, to form an adduct that might be responsible for many of the deleterious effects seen in biological sites.

The Role of Transition Metals

In 1894 Fenton (66) described the interaction between Fe^{+2} and H_2O_2. Forty years later, this Fenton reaction, which we call reaction 3 (below), was found to produce hydroxyl radicals, which explain its oxidizing power (91). Even later, this reaction was recognized as one of the most important in the explanation of oxidative damage that occurs in biological environments (224). Most of the transition metals—those in the first row of the D block in the periodic table—contain unpaired electrons and can, therefore, with the exception of zinc, be considered radicals by definition (100). They can participate in the chemistry of radicals and convert relatively stable oxidants into powerful radicals. Among the various transition metals, copper and especially iron are most abundant, present in relatively high concentrations, and are major players in the Fenton reaction (66) and the metal-mediated Haber-Weiss reaction (91). The metal ions participating in this reaction are those bound to the surface of proteins, DNA, and other macromolecules or chelates. These particular ions can still undergo the reduction-oxidation process, interact with oxygen derivatives, and are often called "loosely bound metals" or "removable metals" (99, 246). Metals that are hidden in proteins, as in catalytic sites and cytochromes, or storage complexes; are not exposed to oxygen radicals; or are kept under 1 oxidation state cannot participate in this chemistry.

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^{\cdot} + OH^- \tag{3}$$

At physiological pH, most of the iron is oxidized and attached to biological chelate in the form of Fe^{+3}. To take part in the Fenton reaction, the iron must be converted to its reduced form, Fe^{+2}; in some cases metals in a higher oxidation state can participate (246). Reducing equivalents with a suitable oxidation potential can easily be responsible for this reaction. For example, ascorbic acid can reduce ferric ions to ferrous ions, enabling it to undergo the Fenton reaction and produce hydroxyl radicals for hydroxylating aromatic compounds (100). This transformation is the Udenfriend reaction. Similarly, in the transformation designated as reaction 4 here,

superoxide radicals can reduce Fe^{+3} to Fe^{+2} ions, which are more soluble and enable the Fenton reaction. The nature of the chelates, to which the iron is bound, can strongly affect the occurrence of the Fenton reaction in biological surroundings (261). Iron can also exist in a hypervallency state, as, for example, ferryl ion (Fe^{+4}) (210, 246), which is highly reactive and can account for many deleterious processes in the cell. Today, many of the deleterious effects of oxygen radicals are attributed to the continuous production of highly toxic hydroxyl radicals via the metal-mediated Haber-Weiss reaction in which ferric ions are reduced by superoxide radicals to ferrous ions (reaction 4). The latter interact with H_2O_2, produced in vivo from the spontaneous or enzymatic dismutation of superoxide radicals, to yield $OH^{\cdot}$ via the Fenton-type reaction. The sum of these two reactions (3 and 4) can explain the existence of the Haber-Weiss reaction in vivo, reaction 5, which itself is thermodynamically possible, although extremely slow. Thus, the interaction between superoxide radicals and H_2O_2 leads to the production of hydroxyl radicals in vivo.

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^{\cdot} + OH^{-} \quad (3)$$

$$O_2^{\cdot -} + Fe^{+3} \longrightarrow O_2 + Fe^{+2} \quad (4)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-} \quad (5)$$

Site-Specific Mechanism (128, 222)

The involvement of transition metal helps to clarify and explain oxidative damage to biological targets. As mentioned before, to cause biological damage the radical must be produced at the site of its target, in close proximity to DNA or protein, as examples. When hydroxyl radicals are produced in the cytoplasm following exposure to ionizing irradiation, for example, they cannot diffuse into the cellular environment and reach vulnerable targets due to their high reactivity and therefore extremely short life span. Metals such as iron or copper cannot be free under physiological conditions (pH, biological components with high affinity toward transition metals) and are always bound to biological sites such as proteins and membranes. The bound metals can undergo a Fenton-type reaction or mediate the Haber-Weiss reaction and produce $OH^{\cdot}$ at the site where they are bound. The chances that $OH^{\cdot}$ will interact with a biological molecule are high. The bound metal can catalyze a continuous production of $OH^{\cdot}$ at a single site and therefore increase the chances of causing damage. This process, called multiple-hit effect, causes double-strand breaks in DNA. The bound metals can, therefore, direct nonreactive radicals to their binding sites, convert them to highly reactive radicals, and thereby increase the chances of biological damage.

In recent years much focus has been placed upon the oxidative damage induced by the interaction between nitric oxide radicals and superoxide radicals, which yields peroxynitrite (reaction 2). In turn, $ONOO^{-}$ can cause damage similar to that caused by $OH^{\cdot}$ (22, 50, 176, 195); however, the relevance of this reaction in a biological environment where carbonate ions are present is questionable (81). In any case the production of reactive species—$OH^{\cdot}$ and carbonate radicals—from $ONOO^{\cdot}$ might be responsible for oxidative damage occurring whenever $O_2^{\cdot -}$ and $NO^{\cdot}$ are involved.

Many attempts have been made to classify ROS according to their biological significance. That $O_2^{\cdot -}$ and $NO^{\cdot}$ are the most significant ROS was recently suggested (56). Other suggestions indicate that peroxyl radicals are the most important biological species. Each of these species apparently exerts singular influence at a particular biological site and, therefore, cannot be classified according to activity alone, but also site of operation and synergistic effects with other ROS. Today's knowledge of the dual role of these metabolites, with both deleterious and beneficial effects, sheds new light on these observations. Apparently each species plays its unique and specific role as cellular mediator and signaling molecule. Similarly, each one of these reactive compounds can cause damage depending on the site of production, the biological target, and the local concentration.

SOURCES OF ROS

The cell is exposed to a large variety of ROS and RNS from both exogenous and endogenous sources (Figure 2) (136). The former include, first, exposure to di-oxygen, which, although a nonreactive biradical, can independently cause oxidation and damage to proteins and enzymes, exemplified by inhibition of aconitase and fumerase in the Krebs cycle and glutamate decarboxylase, which results in decreased γ-aminobutyric acid in the brain (100). The toxicity of the oxygen molecule itself is sometimes beneficial and used as a therapeutic aid as, for example, in hyperbaric oxygen therapy (218). Ozone (O_3) is essential in scavenging deleterious UV-C irradiation and extremely important with its presence in the upper atmosphere. On the other hand, it acts as a damaging species to biological tissues (29, 132). Ozone is not a radical like oxygen, is characterized by its sharp odor, can damage lungs, and can serve as a powerful oxidizing agent that can oxidize biological components directly (204).

Exposure of living organisms to ionizing and nonionizing irradiation constitutes another major exogenous source of ROS (196, 231). Exposure of the cell to γ-irradiation results in the production of a whole range of radical and nonradical species from ionization of intracellular water (eg, aqueous electron, $OH^{\cdot}$, H_2O_2). Even exposure to nonionizing irradiation such as UV-C (<290 nm), UV-B (290–320 nm), and UV-A (320–400 nm) can indirectly produce a variety of ROS including $^1O_2$, H_2O_2, and $O_2^{\cdot -}$ radicals; hemolytic cleavage of H_2O_2 by UV radiation yields $OH^{\cdot}$ radicals. Air pollutants such as car exhaust, cigarette smoke, and industrial contaminants encompassing many types of NO derivatives constitute major sources of ROS that attack and damage the organism either by direct interaction with skin or following inhalation into the lung (143, 258). Drugs are also a major source of ROS (178, 206). There are drugs, such as belomycinem and adreamicine, whose mechanism of activity is mediated via production of ROS, those like nitroglycerine that are $NO^{\cdot}$ donors, and those that produce ROS indirectly. Narcotic drugs and anesthetizing gases are considered major contributors to the production of ROS (43). A large variety of xenobiotics (eg, toxins, pesticides, and herbicides such as paraquat) and chemicals (eg, mustard gas, alcohol) (59, 122, 184, 265) produce ROS as a by-product of their metabolism in vivo. The invasion of pathogens, bacteria, and viruses might result in the production of many ROS species by direct release from

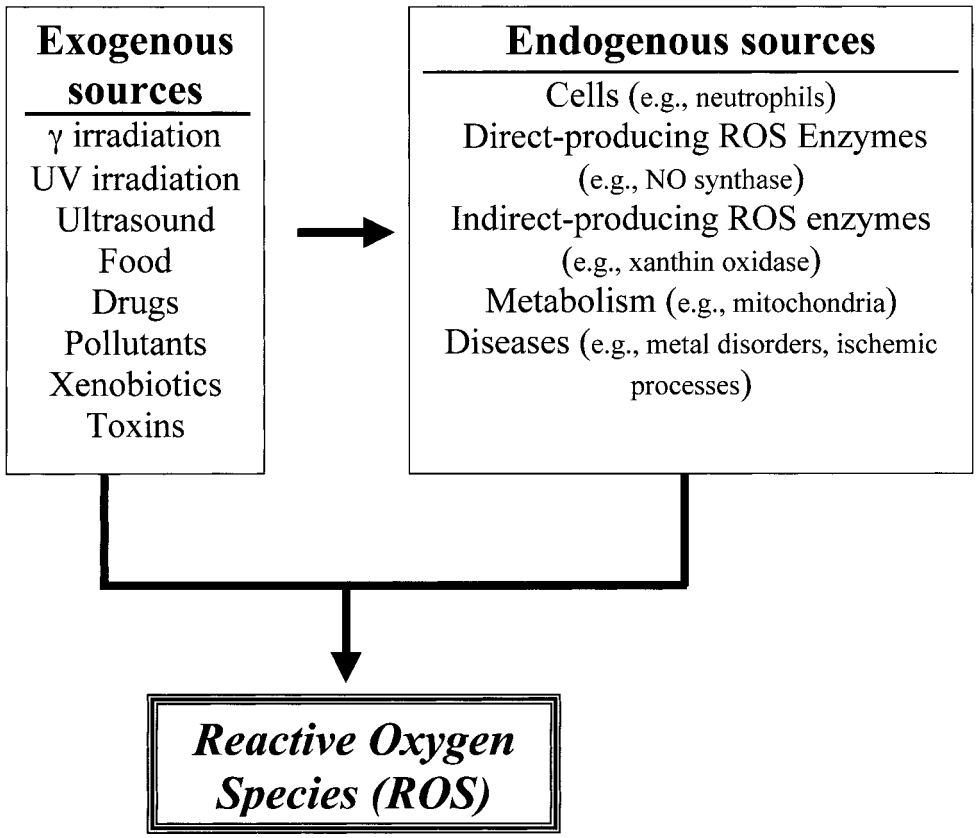


FIGURE 2.—Exogenous and endogenous sources of reactive oxygen species (ROS).

the invaders or an endogenous response induced by phagocytes and neutrophils.

One of the major sources of oxidants is food (6, 125, 158), for a large portion of the food we consume is oxidized to a large degree and contains different kinds of oxidants such as peroxides, aldehydes, oxidized fatty acids, and transition metals (6). Food debris that reaches the intestinal tract places an enormous oxidative pressure on the intestinal-tract mucosa (239).

Although the exposure of the organism to ROS is extremely high from exogenous sources, the exposure to endogenous sources is much more important and extensive, because it is a continuous process during the life span of every cell in the organism (132). The reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of 4 electrons to oxygen to produce water (69). During this process several major oxygen derivatives are formed (11). In many cases there is a leakage of ROS from the mitochondria into the intracellular environment (11). The mitochondrion serves as the major organelle responsible for ROS production and many events throughout the cell cycle (213). The massive production of mitochondrial ROS is increased further in the aging cell where the function of the mitochondrion is impaired and its membrane integrity damaged (38). Enzymes comprise another endogenous source of ROS. While most enzymes produce ROS as a by-product of their activity, exemplified by the formation of superoxide radicals by xanthine oxidase, there are some enzymes designed to produce ROS, such as nitric oxide synthase that yields $NO^{\cdot}$ radicals, those that produce H_2O_2, and those responsible for hydroxylation (39, 157, 233).

White blood cells, including neutrophils, eosinophils, basophils, and mononuclear cells (monocytes), and lymphocytes, with their mechanisms to combat bacteria and other invaders (53, 71), are major producers of endogenous ROS and other factors that act synergistically with ROS (78, 79). Following stimulation, these cells undergo a respiratory burst characterized by a 20-fold increase in oxygen consumption, which is accompanied by an increase in glucose utilization and production of reduced nicotinamide phosphate dinucleotide (NADPH) by the pentose phosphate pathway (14). NADPH serves as a donor of electrons to an activated enzymatic complex in the plasma membrane. This NADPH-oxidase complex utilizes electrons to produce superoxide radicals from the oxygen molecule. Following dismutation, the production of H_2O_2 leads to the formation of $OH^{\cdot}$ by the metal-mediated, Haber-Weiss reaction. The presence of the enzyme myaloperoxidase leads to the production of HClO by interaction between hydrogen peroxides and chlorides (106, 216), referred to as reaction 6.

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase (MPO)}} HClO + OH^{\cdot} \qquad (6)$$

Numerous pathologies and disease states serve as sources for the continuous production of ROS (20, 90, 127, 156, 168, 257). More then 200 clinical disorders have been described in the literature in which ROS were important for the initiation stage of a disease or produced during its course. ROS may be

important initiators and mediators in many types of cancer (37, 56, 82, 182, 183), heart diseases, endothelial dysfunction (1, 162, 179), atherosclerosis and other cardiovascular disorders, inflammation and chronic inflammation (148), burns (149), intestinal tract diseases (33–35, 138), brain degenerative impairments (70, 76, 97, 118, 177), diabetes (56, 58, 170, 189), eye diseases (44), and ischemic and postischemic (eg, damage to skin, heart, brain, kidney, liver, and intestinal tract) pathologies (39, 86, 247). In several normal conditions ROS are produced and play a role in the pathogenesis of the physiological condition. These are exemplified during the aging process where ROS production significantly increases as a result of impaired mitochondrial function and in the early stages of embryonic development (151, 190, 267). Other pathological disorders, which are associated with impaired metal metabolism, such as hemochromatosis (153), Wilson disease (219, 244), and thalassemia (169), in which iron is deposited in many organs, are known to increase significantly the concentration of ROS.

OXIDATIVE-DAMAGE TARGETS AND TYPES

The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components (48) and alters many cellular functions (83). Among the biological targets most vulnerable to oxidative damage are proteinaceous enzymes (100, 154), lipidic membranes (52, 100), and DNA (23, 100) (Figure 3). Our understanding of the chemistry of radicals clarifies the interaction of these species in the locations at which they are being produced. For example, hydroxyl radicals, produced in mitochondrial compartments, are responsible for damage occurring in the mitochondrion but not the nucleus. Their high reactivity with biological molecules, which leads to their extremely short life span, does not permit their distribution within the intracellular environment and limits their ability to cause damage a long distance from their site of formation. On the other hand, oxygen metabolites that are not extremely reactive, such as $HO_2^{\cdot}$, may exist in the intracellular environment for longer periods of time and reach locations far from their production site. For example, H_2O_2 produced in mitochondria may interact elsewhere in the cytoplasm or in the nucleolus.

EXAMPLES OF SPECIFIC TARGETS OF OXIDATIVE DAMAGE

Lipids (100, 169)

All cellular membranes are especially vulnerable to oxidation due to their high concentrations of unsaturated fatty acid. The damage to lipids, usually called lipid peroxidation, occurs in 3 stages. The first stage, initiation, involves the attack of a reactive oxygen metabolite capable of abstracting a hydrogen atom from a methylene group in the lipid. The presence of a double bond adjacent the methylene group weakens the bond between the hydrogen and carbon atoms so that it can easily be removed from the molecule. Following hydrogen abstraction, the remaining fatty acid radical retains 1 electron and is stabilized by rearrangement of the molecular structure

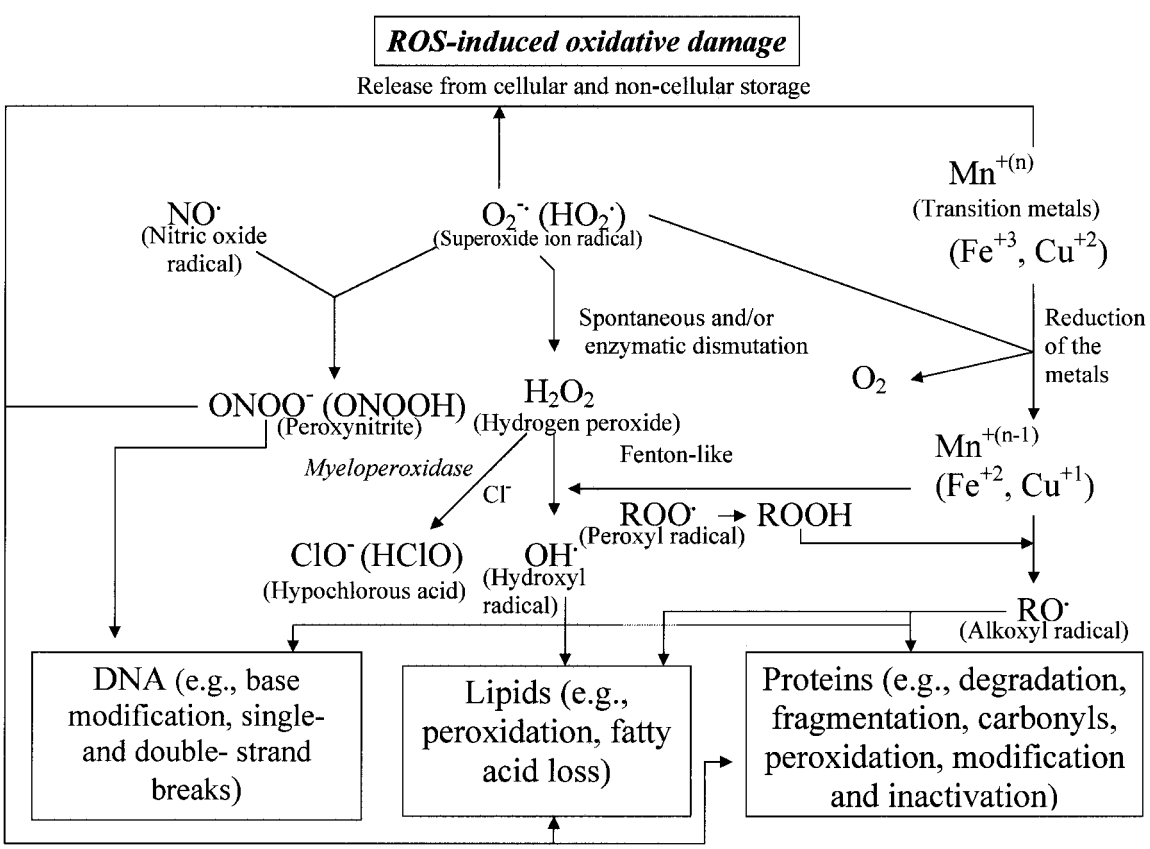


FIGURE 3.—Reactive oxygen species (ROS)-induced oxidative damage.

to form a conjugated diene. When oxygen is in sufficient concentration in the surroundings, the fatty acid radical will react with it to form $ROO^{\cdot}$ during the propagation stage. These radicals themselves are capable of abstracting another hydrogen atom from a neighboring fatty acid molecule, which leads again to the production of fatty acid radicals that undergo the same reactions—rearrangement and interaction with oxygen. The $ROO^{\cdot}$ becomes a lipid hydroperoxide that can further decompose to an aldehyde or form cyclic endoperoxide, isoprotans, and hydrocarbons. The propagation stage allows the reaction to continue. A single initiation can lead to a chain reaction resulting in peroxidation of all the unsaturated lipid in the membrane. An antioxidant that can stop this process is therefore defined as a chain-breaking antioxidant. Fatty acids with no double bonds or with 1 double bond can undergo oxidation but not a chain lipid-peroxidation process; for example, oleic acid with 18 carbon atoms and 1 double bond (18:1) cannot undergo the lipid peroxidation process. The last stage, chain termination, occurs following interaction of one $ROO^{\cdot}$ with another radical or antioxidants.

Proteins (52, 87, 100, 154, 240)

Proteins, also major constituents of membranes, can serve as possible targets for attack by ROS. Among the various ROS, the $OH^{\cdot}$, $RO^{\cdot}$, and nitrogen-reactive radicals predominantly cause protein damage. Hydrogen peroxide itself and superoxide radicals in physiological concentrations exert weak effects on proteins; those containing —SH groups, however, can undergo oxidation following interaction with H_2O_2. Proteins can undergo direct and indirect damage following interaction with ROS, including peroxidation, damage to specific amino-acid residues, changes in their tertiary structure, degradation, and fragmentation. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins. Protein oxidation products are usually aldehydes, keto compounds, and carbonyls. One of the major adducts that can easily be detected and serve therefore as a marker for protein oxidative damage is 3-nitrotyrosine. This adduct is produced following the interaction between $ONOO^{-}$ and other nitrogen reactive radicals with the amino acid tyrosine. Following $OH^{\cdot}$ attack, a series of compounds can be formed, including hydroxyproline, glutamyl semialdehyde, and others. Following protein oxidation, modified proteins are susceptible to many changes in their function. These include chemical fragmentation, inactivation, and increased proteolytic degradation (52, 87, 240).

DNA (7, 23, 55, 96, 100, 108)

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage: modification of DNA bases, single- and double-DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system. Not all ROS can cause damage; most is attributable to hydroxyl radicals. For example, following exposure of DNA to hydroxyl radicals, like those induced by ionizing irradiation, a variety of adducts are formed. The $OH^{\cdot}$ can attack guanine at its C-8 position to yield an oxidation product, 8-hydroxydeoxyguanosine (8-OHdG) (117) (See method section below). Other positions could be attacked, and other possible products could be formed. Hydroxyl radicals can also attack other bases like adenine to yield 8 (or 4-, 5-)-hydroxyadenine. Other products are the result of interactions between pyrimidines and hydroxyl radicals leading to the formation of thymine peroxide, thymine glycols, 5-(hydroxymethyl) uracyl, and other such products. The direct interaction of DNA with other less reactive ROS, such as $O_2^{\cdot-}$ and H_2O_2, does not lead to damage at their physiological concentrations; however, these species serve as sources for other reactive intermediates that can easily attack and cause damage. For example, H_2O_2 and superoxide might lead to the production of the $OH^{\cdot}$ via the Haber-Weiss reaction, and NO and $O_2^{\cdot-}$ might lead to the formation of $ONOO^{-}$ that can easily cause DNA damage similar to that obtained when hydroxyl radicals are involved. Transition metals like iron that possess high-binding affinity to DNA sites can catalyze the production of $OH^{\cdot}$ in close proximity to the DNA molecule, thus ensuring repeated attack upon the DNA by an efflux of hydroxyl radicals (8, 94, 126, 198).

Defense Mechanisms of the Cell Against Oxidative Stress

Continuous exposure to various types of oxidative stress from numerous sources has led the cell and the entire organism to develop defense mechanisms for protection against reactive metabolites. These mechanisms described in Figure 4 encompass both indirect and direct activities. Indirect approaches may involve control of the endogenous production of ROS (132, 136) by, for example, altering the activity of enzymes, which indirectly produce oxygen metabolites; one such enzyme is xanthine oxidase. An efficient repair system, one of the most important methods for the organism to cope with oxidative damage, consists of enzymes and small molecules that can efficiently repair an oxidative-damage site on macromolecules. The DNA repair system, for example, can identify a DNA-oxidized adduct [eg, 8-hydroxy-2-deoxyguanosine , thiamine glycol, and apurinic and apyramidenic (AP) sites] (13), remove it, and incorporate an undamaged base (13, 55). Molecules that can donate hydrogen atoms to damaged molecules are also considered repair compounds; one such example is the donation of a hydrogen atom by ascorbate or tocopherol to a fatty acid radical that was previously attacked by a radical and lost its hydrogen. Physical defense of biological sites such as membranes is also an important mechanism allowing the cell to cope with oxidative stress. Compounds such as tocopherols can provide enhanced stability to cellular membranes, and steric interference can prevent ROS from approaching the target.

Among the various defense mechanisms, the one involving antioxidants is extremely important due to its direct removal of pro-oxidants and the variety of compounds that can act as antioxidants and ensure maximum protection for biological sites. This system apparently developed throughout the evolutionary process, perhaps in response to the changing concentration of oxygen in the atmosphere. The uniqueness of

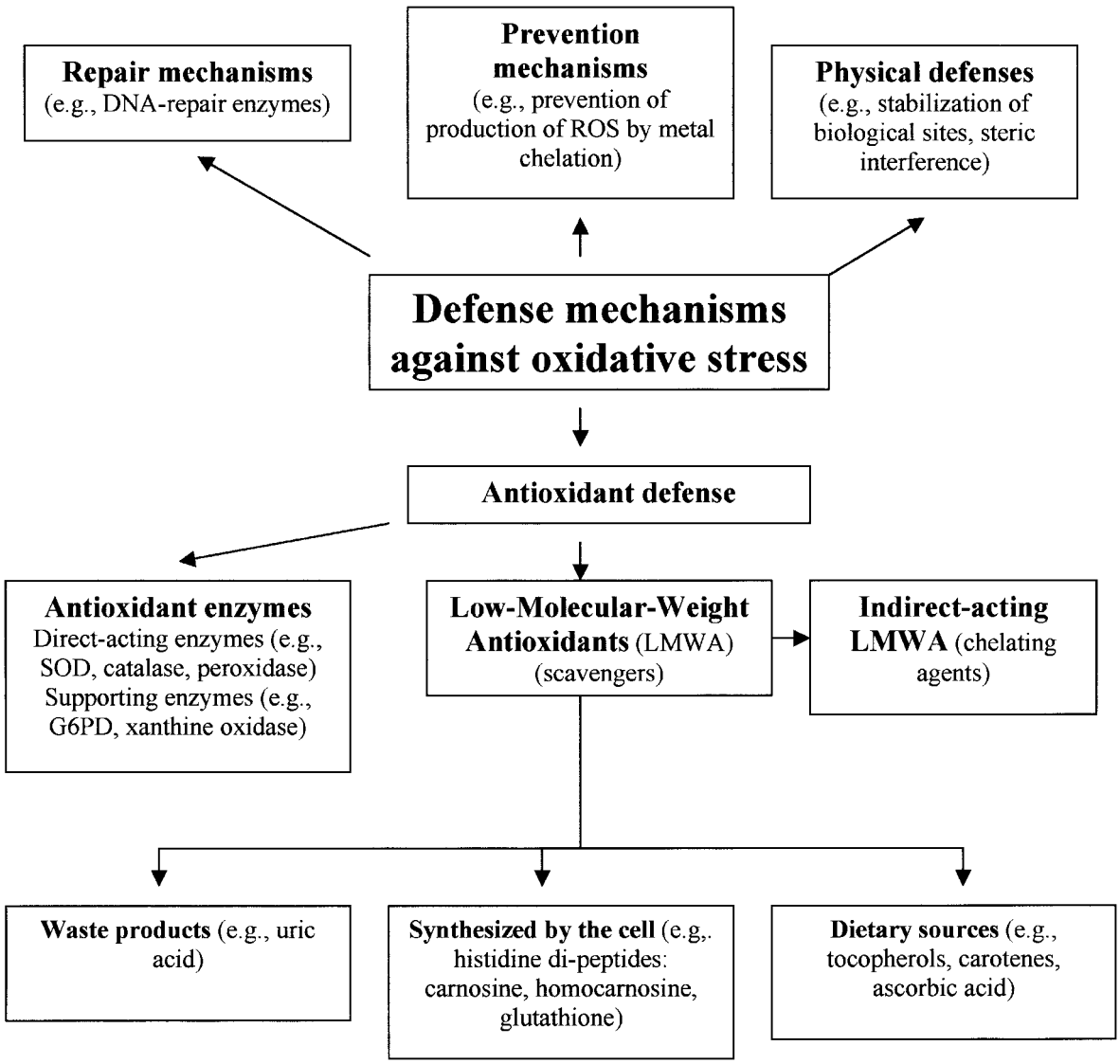


FIGURE 4.—Classification of antioxidant cellular-defense mechanisms.

this system is its direct interaction with ROS of various kinds and its provision of protection for biological targets. The system contains 2 major groups—antioxidant enzymes and low-molecular-weight antioxidants (LMWA) (Figure 4). The enzyme-containing group is composed of direct-acting proteins, such as SOD; the proteins in this family differ in their structure and cofactors. Cu-Zn SOD is an enzyme of molecular mass of approximately 32,000; contains 2 subunits, each of which possesses an active site; and is widely distributed in eukaryotic cells localized in the cytoplasm, while Mn-SOD, a protein of about 40,000, can be found in prokaryotic cells and eukaryotic mitochondria (100). Other types of SOD exist, such as extracellular SOD (EC-SOD) and Fe-SOD in plants. These enzymes possess different structures, molecular masses, and reaction rate constants. The enzyme activity itself, first discovered by McCord and Fridovich in 1969, is capable of enhancing the spontaneous dismutation of superoxide radicals to H_2O_2 (designated as reaction 7) (167). There are significant changes among the rate constants of the various SODs depending on pH and site of activity.

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2 \quad k_{CuZnSOD} = 1.6 \times 10^9\ M^{-1}s^{-1} \tag{7}$$

The end product of the dismutation reaction—H_2O_2—can be removed by the activity of the enzyme catalase and members of the peroxidase family including glutathione peroxidase (42). Catalase is a unique enzyme with a very high K_M for its substrate and can remove H_2O_2 present in high concentrations. The enzyme consists of 4 protein subunits, each of which contains ferric ions of the haem group that undergo oxidation following interaction with the first molecule of H_2O_2 to produce Fe^{+4} in a structure called compound 1 (147). A second molecule of H_2O_2 serves as an electron donor and results in the destruction of the two H_2O_2 molecules involved to produce an oxygen molecule (designated herein

as reactions 8–10).

$$H_2O_2 \xrightarrow{\text{catalase}} \{\text{Compound 1}\} \quad (8)$$

$$\{\text{Compound 1}\} + H_2O_2 \longrightarrow O_2 + 2H_2O \quad (9)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \quad (10)$$

In contrast to catalase, peroxidase possesses high affinity for and can remove H_2O_2 even when it is present in low concentration (42, 100); however, the electron donors in these reactions are small molecules, such as glutathione or ascorbate (in plants). Thus, the removal of H_2O_2 is an "expensive" reaction from the cell's point of view, as it consumes valuable molecules in the cellular environment; 2 molecules of glutathione are consumed for the removal of 1 molecule of H_2O_2 (reaction 11).

$$2GSH + H_2O_2 \xrightarrow{\text{Peroxidase}} GSSG + 2H_2O \quad (11)$$

That no oxygen is produced in the latter reaction distinguishes the activity of peroxidase from that of catalase.

Other enzymes exist in the cellular environment that support the activity of antioxidant enzymes. For example glucose-6-phosphate dehydrogenase supplies reducing equivalents (NADPH) necessary for cellular function and important for the regeneration of oxidized antioxidants; the regeneration of oxidized glutathione, GSSG, to the reduced form, GSH, by reduced nicotinamide dinucleotide (NADH) is but one example (42). That some of the supporting enzymes can provide scavengers is exemplified by xanthine dehydrogenase that produces uric acid, an effective endogenous antioxidant (9, 88).

The low-molecular-weight antioxidant (LMWA) group contains numerous compounds capable of preventing oxidative damage by direct and indirect interaction with ROS (132, 136). The indirect mechanism involves the chelation of transition metals that prevents them from participating in the metal-mediated Haber-Weiss reaction (91, 222). The direct-acting molecules share a similar chemical trait that allows them to donate electrons to the oxygen radical so that they can scavenge the radical and prevent it from attacking the biological target. Scavengers possess many advantages over the group of enzymatic antioxidants. Because scavengers are small molecules, they can penetrate cellular membranes and be localized in close proximity to the biological target. The cell can regulate their concentrations, and they can be regenerated within the cell. They possess a wide spectrum of activities toward a large variety of ROS. The scavenging mechanism can proceed only if the concentration of the scavenger is sufficiently high to compete with the biological target on the deleterious species (136). The action of the LMWA can be considered synergistic, and the interrelationships between LMWA are crucial for the development of guidelines for antioxidant therapy (see below). Scavengers originate from endogenous sources, such as biosynthetic processes and waste-product generation by the cell, and exogenously from diet. That the number of LMWA synthesized by the living cell or generated as waste products is so limited is surprising [eg, histidine dipeptides (36), glutathione (100), uric acid (10), lipoic acid (193), and bilirubin (243)]; most LMWA are derived from dietary sources. Scavengers

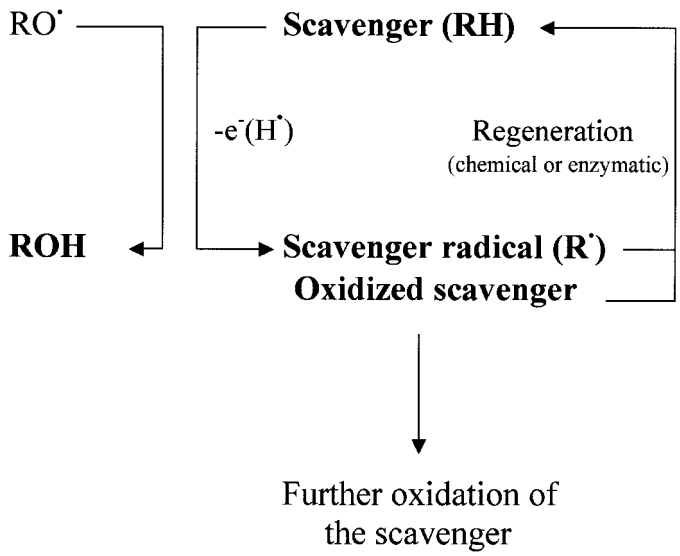


FIGURE 5.—Schematic showing mechanism of scavenger activity.

are characterized by their common mechanism of activity, reacting directly with the radical and removing it by donating an electron(s) to the reactive species. This reaction results in the conversion of the scavenger by itself to a radical, although not a reactive one (Figure 5). The scavenger radical can undergo further oxidation or be regenerated to its reduced form, a reducing antioxidant, by another scavenger possessing a suitable oxidation potential; the ascorbyl radical, for instance, can be recycled to its reduced form, ascorbic acid, by glutathione. The latter itself becomes a radical, which can also receive electrons from another donor, such as NADH. The regeneration process can be purely chemical, or an enzyme can be involved in the electron transfer (Figure 6). This cooperative activity may explain the synergism obtained when several scavengers are involved and the beneficial use of large combinations of LMWA in antioxidant therapy.

EXAMPLES OF LMWA

Although antioxidants are crucial for the maintenance of cellular functions, the cell itself produces, surprisingly, only a limited number of LMWA. None of the compounds synthesized endogenously in the human cell can be considered purely antioxidant and designed to act only as antioxidant molecules. A few examples of these compounds, which function as scavengers and in many other biological roles, are discussed subsequently.

Glutathione is a low-molecular-mass, thiol-containing tripeptide, glutamic acid-cysteine-glycine (GSH) in its reduced form and GSSG in its oxidized form, in which 2 GSH molecules join via the oxidation of the —SH groups of the cysteine residue to form a disulphide bridge (100). GSH is present in humans, animals, plants, and aerobic bacteria at high concentrations reaching the millimolar range. It acts as a cofactor for the enzyme peroxidase, thus serving as an indirect antioxidant donating the electrons necessary for its decomposition of H_2O_2. This compound is also involved in many other biochemical pathways and cellular

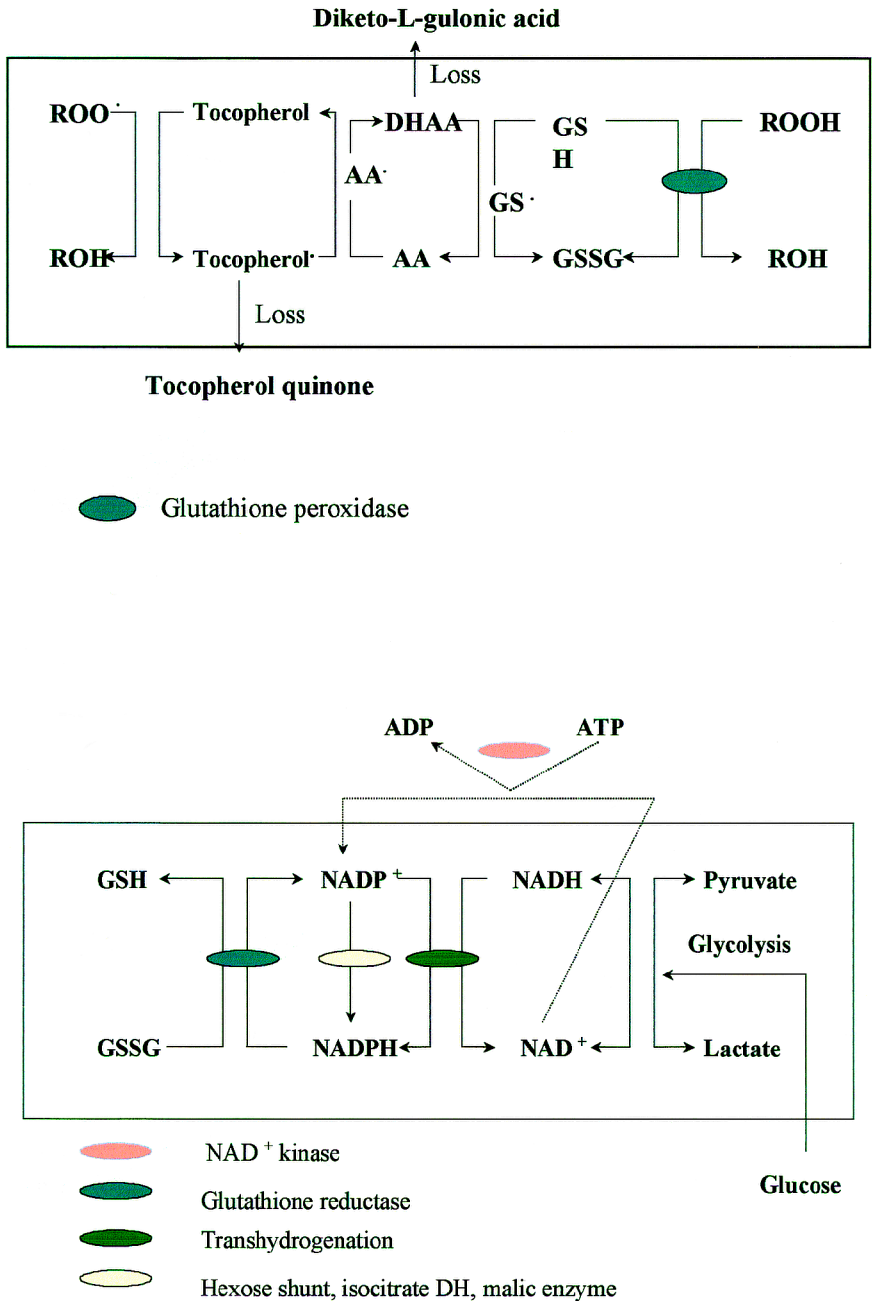


FIGURE 6.—Interrelationships among low-molecular-weight antioxidants (LMWA).

functions including metabolism, such as that of ascorbic acid; maintenance of communication between cells (18); prevention of oxidation in —SH protein groups; and copper transport (42, 88). Glutathione can act as a chelating agent for copper ions and prevent them from participating in the Haber-Weiss reaction; serve as a cofactor for several enzymes, such as glyoxylase and those involved in leukotriene biosynthesis; and play a role in protein folding, degradation, and cross-linking. In addition to its biochemical functions, it can scavenge ROS directly. GSH can interact with $OH^{\cdot}$, $ROO^{\cdot}$, and $RO^{\cdot}$ radicals as well as with HCLO and $^{\prime}O_2$ Upon reaction with ROS, it becomes a glutathione radical, which can be regenerated to its reduced form (88, 100).

Melatonin, a hormone synthesized by the pineal gland, helps to regulate circadian rhythms, possesses a powerful antioxidant capacity in vitro, and may scavenge a variety of ROS, probably through donation of the hydrogen atom by the (—NH) group. In vivo, the concentrations of melatonin are relatively low, however, and explaining its antioxidant activity in terms of its scavenging ability is difficult. It possibly indirectly alters the antioxidant activity of the cell by, for example, induction of synthesis of antioxidant enzymes or modulating other cellular responses leading to secretion and accumulation of other antioxidants. High local concentrations of melatonin in the brain may explain its great protective effect against head injury in rats (207, 208, 249).

Histidine dipeptides comprise a family of compounds synthesized in the brain and skeletal muscles, 2 tissues extremely susceptible to oxidants in high, millimolar concentrations. These compounds—carnosine, homocarnosine, and anserine—possess strong antioxidant capabilities in vitro. They are considered multifunctional antioxidants, because they can act in many ways to destroy and remove ROS. They can scavenge directly $OH^{\cdot}$, $ROO^{\cdot}$, and $RO^{\cdot}$ radicals; bind H_2O_2; quench efficiently $^{\prime}O_2$; and bind transition metals and prevent them from participating in the metal-mediated Haber-Weiss reaction. In vivo they diminish oxidative damage in many systems, including the ischemic process. These compounds do not exert pro-oxidant effects as other reducing antioxidants sometimes do. That these compounds can act as endogenous buffers and prevent protein glycosylation has been suggested (36, 142, 247).

Uric acid provides an excellent example of the adaptation of the organism to oxidative stress. It is a cellular waste product originating from the oxidation of hypoxanthine and xanthine by xanthine oxidase and dehydrogenase. Humans and other primates lack the enzyme urate oxidase, which converts uric acid to allantoin; therefore, ureate accumulates in the human blood plasma to a concentration of approximately 300 μM. An excess of ureate might lead to its precipitation and crystallization in the joint leading to joint inflammation known as gout. High uric acid levels may, however, also provide efficient antioxidant activity for the organism. Urate, the physiological state of uric acid, reacts with hydroxyl radicals producing a stable urate radical that can be regenerated by ascorbate to its prior state, urate. This compound can act with peroxyl radicals, $^1O_2$, O_3, $NO^{\cdot}$, and other nitrogen-oxygen radicals. Urate also protects protein from nitration; it can chelate metal ions, such as copper and iron, and prevent them from participating in redox cycling. The degradation product of urate, allantoin, was found in vivo, supporting claims that urate indeed is an efficient in vivo antioxidant (9, 21, 262).

Most of the reducing antioxidants are derived from dietary sources. Although many dietary habits exist, and populations around the world consume a variety of food ingredients that vary from each other and contain different arsenals of antioxidant compounds, the processes governing cellular antioxidant activity are the same, and, thus, total antioxidant activity is similar among different food consumers, although changes in the levels of specific reducing antioxidants may be detected. Large numbers of antioxidant molecules exist in green vegetables, fruit, and fish, including small molecules such as ascorbic acid, tocopherols, polyphenols, and carotenoids. Both lipophilic and hydrophilic compounds can be found that possess different oxidation potentials reflecting their ability to donate electrons and act as antioxidants.

Ascorbic acid (ascorbate, vitamin C) (41, 72, 73, 98, 100, 165, 214) is an example of a water-soluble antioxidant that can be synthesized by plants and some animals. Humans, primates, guinea pigs, and fruit bats have lost the enzyme needed for its synthesis and are completely dependent on dietary sources to obtain it. Similar to other antioxidants, ascorbate, which at physiological pH exists as a mono anion, possesses many biochemical functions in addition to its activity as a scavenger. It is required as a cofactor for many enzymes, such as proline hydroxylase and dopamine-β-hydroxylase. Ascorbate is a vitamin; its deficiency in the human diet causes scurvy, although daily consumption of fruits and vegetables readily overcomes the symptoms associated with the syndrome.

As an antioxidant, ascorbate is an efficient scavenger, or reducing antioxidant, capable of donating its electrons to ROS and eliminating them. Ascorbate can donate 2 electrons; following donation of 1 electron, it produces the ascorbyl (semidehydroascorbate or ascorbate) radical, which can be further oxidized to produce dehydroascorbate. Because the ascorbyl radical is relatively stable, it makes ascorbate a powerful, important antioxidant. This radical can lose its electron and be transformed to dehydroascorbic acid or regenerated to the reduced form by obtaining an electron from another reducing agent, such as GSH or NADH, via the mediation of an enzyme like NADH-semidehydroascorbate reductase. The oxidation product, dehydroascorbic acid, can also be regenerated by the enzyme dehydroascorbate reductase at the expense of 2 molecules of GSH. The compound dehydroascorbate is not stable and is decomposed to di-keto-L-gulonic acid and then to oxalic and L-threonic acids, which can be further decomposed to oxalic acid. In vitro, ascorbate can act as an efficient antioxidant and scavenge a variety of ROS including hydroxyl, peroxyl, thyil, and oxosulphuric radicals. Ascorbate is also a powerful scavenger of HClO and peroxynitrous acid and can inhibit the peroxidation process. It can react with $^1O_2$ and act synergistically with other antioxidants to regenerate, for example, the tocopherol radical to its reduced form. Indirect evidence has shown that, in vivo, ascorbate acts directly as an antioxidant. Data have been accumulating to demonstrate its necessity for normal cellular functioning, and indirect evidence indicates a decreased peroxidation process and beneficial involvement in disease states associated with ROS; however, direct and convincing data do not yet exist indicating that, in vivo, ascorbate acts as a direct antioxidant. The chemical nature of enabling it to perform as a reducing agent might imply a deleterious consequence that may occur when ascorbate is present in the environs of available transition metals. As described before, cooperation between ascorbate and ferric ions may lead to the production of $OH^{\cdot}$ via the Udenfriend reaction (100). Thus, ascorbate under specific conditions may act as a pro-oxidant and produce radicals that might contribute to oxidative damage (194, 197), especially in iron-overload situations, such as thalassemia, haemochromatosis, and Wilson disease (45, 60).

Exemplifying lipophilic LMWA derived from dietary sources is the family of tocopherol antioxidants, including vitamin E (110). These compounds, chain-breaking antioxidants, can scavenge $ROO^{\cdot}$ to inhibit the lipid peroxidation process in biological membranes (designated reaction 12).

$$\alpha\text{-tocopherol} + ROO^{\cdot} \longrightarrow \alpha\text{-tocopherol}^{\cdot} + ROOH \quad (12)$$

Eight naturally occurring substances are known to be members of the vitamin E family. These compounds have 3 asymmetric carbon atoms, giving 8 optical isomers. The most effective form in animals is RRR-α-tocopherol, or

d-α-tocopherol. Other members of the family consist of d-β, d-γ, and d-δ-tocopherols and d-α-, d-β-, d-γ-, and d-δ-tocotrienols. Although all possess antioxidant activity, the RRR-α-tocopherol is considered the most effective one, as the others are not retained and absorbed well in body tissues. These compounds can scavenge other ROS, such as $^1O_2$. Following interaction, tocopherol is converted to tocopherolquinone and subsequently to tocopherylquinone. As with other scavengers, the α-tocopheryl radical can be recycled to its active form. That ascorbic acid is capable of converting the radical to its reduced form without the involvement of an enzyme, leaving the ascorbic acid as an ascorbyl radical, has been suggested (Figure 6). Other roles for tocopherol also exist, such as those of a membrane-stabilizing agent and a potential pro-oxidant compound in some systems when transition metals are present (100, 110, 121, 209, 242, 250, 251, 254).

REDOX PHENOMENA (RESPONSE, REGULATION, AND SIGNALING) AND REDUCING POWER

The redox potential is defined as the ratio between oxidant and reductant, eg, ROS and scavengers; since it is a thermodynamic parameter, it cannot be determined in biological environments, but only under appropriate thermodynamic conditions. It cannot be simply calculated according to the Nernst equation

$$E = E^0 + RT/nF \ln(\text{oxidants})/(\text{reductants}),$$

where E = redox potential in volts; E^0 = standard redox potential; R = universal gas constant; T = temperature in Kelvin; n = number of electrons involved; F = Faraday constant in biological sites (111, 137, 225, 232), and can be determined only under appropriate specific conditions, in reversible systems, where all factors affecting the system are known and can be controlled. Therefore, in biological systems where these conditions cannot be fulfilled, equilibrium does not exist, and the systems are not fully reversible, the redox potential cannot be determined and does not "fit" its classical definition. Nevertheless, a relative definition that describes the steady state approximation of redox can be suggested and should be termed "redox status" or "redox state," rather than biological redox potential (139). Schafer and Buettner (225) suggested that this parameter is a total of the products of the reduction potential and reducing capacity of all the redox couples found in biological fluids, organelles, cells, or tissues. The redox state of a biological system is kept within a narrow range under normal conditions—similar to the manner in which the biological system regulates its pH parameter. Under pathological conditions, the redox state can be changed toward lower (redosis) or higher (oxidosis) values (220, 232). A 30-mV change in the redox status means a 10-fold change in the ratio between reductant and oxidant (202, 217, 220, 256).

In relating to redox state and reducing power, one must carefully distinguish between the two, since they differ. Although redox is a thermodynamic parameter, reducing power is not and therefore can be calculated in biological systems and supply valuable information concerning cellular response to oxidative stress (139, 225). This parameter represents and encompasses the overall capability of the cell, biological fluid, or tissue to donate electrons (oxidation potential) and the overall concentration of the reducing equivalents responsible for this ability. Many compounds contribute to the reducing power of the resting cell. The major players, which can be measured in the creation of reducing power in cellular and biological environments, are the LMWA (139, 141). Other compounds possess reducing properties but are present in low concentrations and not considered contributors, although they may play a role in cellular microenvironments. Evaluation of the reducing power may therefore indicate the total LMWA status of a biological system (131) (See voltammetry measurements of reducing power next). The regulative mechanisms responsible for maintaining the redox state are not yet fully known; however, changes in this state might lead to a cascade of events, some beneficial and some deleterious to the cell (56, 150).

BENEFICIAL EFFECTS OF ROS AND REASONS FOR THE FAILURE OF ANTIOXIDANT THERAPY (ANTIOXIDANT PARADOX)

Although paradoxical, the oxidative stress phenomenon, while associated with deleterious processes and harmful events, is essential to the existence and development of the cell (56). Oxidative conditions are the stimulating force for biochemical processes and events during the life span of the cell. While the resting-cell environment is highly reduced, it becomes more and more oxidized during proliferation and activation of cellular biochemical pathways until apoptosis and necrosis occur. In these latter stages the cellular environment becomes highly oxidized (95). ROS and RNS play crucial roles in gene activation (61, 124), cellular growth (230), and modulation of chemical reactions in the cell (227, 229) and function as major components of the defense against bacteria and viruses provided by neutrophils (phagocytes) and as agents responsible for dilation of blood vessels (eg, $NO^{\cdot}$) (89). They also participate in blood pressure control (264); are important mediators in the biosynthesis of other molecules, such as prostaglandins (145); function in embryonic development (223); and act as signaling molecules within the individual cell and among cells during their life spans (26).

Knowing the beneficial multifunctional role of ROS reveals why many irresponsible attempts to treat ROS-associated diseases with antioxidant supplementations have failed and might have caused deleterious effects to the individual. Indeed, many clinical trials with antioxidants failed to demonstrate protection and in many cases even revealed an acceleration of the disease. The CHAOS study (172, 241) and the beta-carotene cancer prevention study (205) exemplified such failures. Radicals by definition are extremely short-lived species with a high-hazard potential; to scavenge them efficiently, antioxidants must be present at the location of radical formation in order to compete with the biological target on the radical. Because ROS are being produced continually, antioxidants must, therefore, be present at their sites of protection at high concentrations continually. A temporary decrease in antioxidant concentration at the susceptible location, while radical formation occurs constantly and continually, would result in accumulating oxidative damage, which could not be prevented or repaired by the late addition of the antioxidants. On the other hand, loading the cell with a high dose of

antioxidants might scavenge too many of the ROS, prevent them from functioning in essential biochemical pathways, and even preclude their performing their essential beneficial role. For example, too many scavengers in the blood might mask the ROS released by the neutrophils and prevent the killing of invading bacteria. Loading the organism and the cell with scavengers might alter the balance between the oxidant, reductant, and biological redox state and lead the cell to a situation characterized as reductive stress (Figure 1), which, in turn, might trigger deleterious processes in the cell or prevent the occurrence of other, essential processes.

Antioxidant therapy should be designed carefully (49, 54, 75, 120). Taking into consideration the high reactivity of ROS, their short life span, their continuous production in close proximity to biological targets, and their ability to be modified into other more reactive species, one realizes that, in order to cope with these deleterious metabolites, the antioxidant should be administered to the body continuously, in high concentrations, and targeted to the biological site susceptible to oxidative damage. On the other hand, an understanding of the mechanism of the activity of scavengers, including their mutual collaboration, synergistic activity, and interrelationships, prompts the suggestion that the antioxidant be given in combinations, such as preparations of multiscavenger in both oxidized and reduced forms and with no transition metals in the formulations. They should be designed in appropriate pharmaceutical dosage forms such as sustained-release formulations. One has to be aware of their potential side effects and their upper toxic dose, which can easily be reached, because these compounds are widely distributed in our diet.

Methods for Determination of ROS and Radicals

Many approaches allow evaluation and demonstration of the participation of ROS in biochemical events. Indeed, the literature is replete with descriptions of different methodologies and approaches for these purposes (40, 161, 192). In this review we do not intend to outline these numerous methods but, rather, describe their general classification and present in detail two of the approaches important to scientists in toxicological and pharmacological fields (Figure 7, dashed-line boxes). The only technique for direct detection of radicals is electron spin resonance (212), which allows the detection of relatively stable radicals. Another technique is the spin trapping method in which a highly reactive radical, such as $OH^{\cdot}$, reacts with a trap molecule to produce a stable radical product that can be evaluated (163). Other trapping procedures allow a radical to react with a detector molecule to yield a stable product that can then be evaluated using a

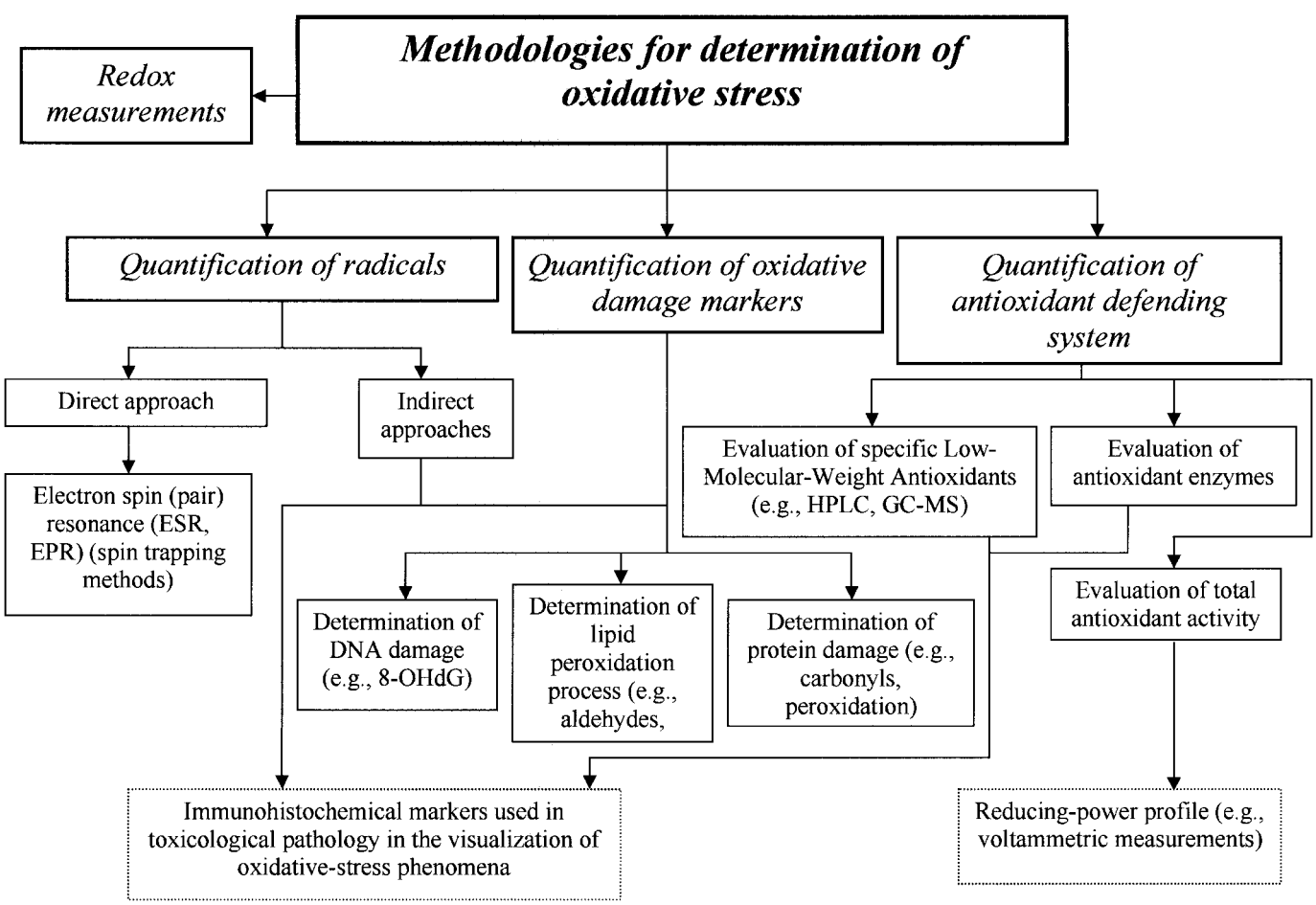


FIGURE 7.—Classification of methods used to determine and quantify oxidative stress.

variety of techniques, such as hydroxylation of salicylic acid (101), the deoxyribose assay (30, 259), the cytochrome *c* reduction assay for detection of superoxide radicals (146), and detection of nitric oxide radicals by colored end-product compounds (5). Figure 7 demonstrates alternative approaches for the direct determination of ROS. Techniques for quantification of oxidative damage markers are often called fingerprinting methods by which specific end products resulting from the interaction of the ROS with biological macromolecules, such as DNA, proteins, lipid, and LMWA are measured. The appearance of these end products serves as proof of the prior existence of ROS that left their footprints in the cell. Among the various sites of interaction, DNA, proteins, and lipids are of the greatest importance (Figure 7). HPLC or GC-MS analysis of 8-OHdG after enzymatic hydrolysis of DNA and assessment of oxidative base damage by the single-cell gel electrophoresis, or comet, assay (65) are 2 of many techniques utilized to detect DNA adducts and base modification. Other methods exist to determine single- and double-strand breaks (245). Different oxidized adducts of DNA can be determined. Examples are DNA-aldehyde adducts, such as deoxyguanosine-malondialdehyd e adducts (268), or the end product of the reaction between DNA and 4-hydroxynonenal, the aldehyde formed following exposure to ROS (238) to generate N_2-ethenodeoxyguanosine.

Damage to lipid deleteriously alters and modifies cellular membranes and, therefore, cellular function. Lipid peroxidation, important in arteriosclerosis, inflammation, and mitochondrial functions, is a complex process consisting of 3 stages—initiation, propagation, and termination (100). For each stage, there are many available methods to quantify the progress of the process and evidence its existence. For example, because lipid peroxidation causes loss of substrates, like unsaturated fatty acid chains, measuring the lipid content prior to and following exposure may indicate peroxidation. In addition, because oxygen is consumed during the propagation stage, measurement of its uptake by oxygen electrodes may serve as a tool for evaluating the progress of oxidation. Another approach is the measurement of peroxide formation during the process. Among the many methods devised for this propose, some reveal total peroxide formation, while some determine specific peroxide, which can indicate the fatty acid undergoing the peroxidation process. Following the abstraction of hydrogen by a reactive species, a rearrangement of the fatty acid radical occurs. This process is characterized by the formation of conjugated diene (100), which can be easily monitored by spectroscopic means.

In the last stage of the peroxidation process, peroxides are decomposed to aldehydes like malondialdehyde (MDA), which can be detected by thiobarbituric acid that gives a pink color easily measurable. The end products of other aldehydes, eg, hexanal, can also be measured. All of these are termed thiobarbituric reactive species (TBARS) (63). This method is one of the most widely used assays to assess peroxidation in the whole organism. Other methods detect these end products, but an awareness of their advantages and problematical disadvantages, such as numerous artifacts in the assays, is necessary. Evidence for the peroxidation process in animals and humans can be obtained by measuring hydrocarbon gases, such as pentane and ethane, which are formed as end products of peroxidation. These gases can be detected in the breath of the tested species using gas chromatography. Measurements of light emission by low-level chemiluminescence (3) and of fluorescence emanating from age pigments produced from the interaction of aldehydes (eg, MDA) with side-chain amino groups of proteins, amino acids, or nucleic acid bases to form Schiff bases (103) serve also as indices of oxidative stress.

Evaluation of protein oxidative damage can be accomplished using the carbonyl assay (155). Carbonyls are produced from the attack of ROS on amino-acid residues in proteins. Several methods evaluate these carbonyls, including a general estimation of the total carbonyl pool; a specific determination using gel electrophoresis techniques; and detections of peroxides, loss of —SH groups, loss of fluorescence (eg, tryptophane), chlorination of proteins, nitration of proteins, and hydroxylation of amino acids.

Many methods exist for evaluating the activity and composition of the antioxidant enzymes, which, along with the LMWA, constitute the 2 major components of the antioxidative system. Some techniques that directly evaluate enzymatic activity utilize spectroscopic measurements or gel-activity procedures; other methods employ immunocytochemistry (see below). Assays of antioxidant enzymes may indicate prior exposure of the cell to oxidative stress, although these enzymes are under regulation, and one might detect an increase, rather than a decrease, in their activity. ROS may serve as a stimulating species for induction of antioxidant enzymes on the one hand and, on the other, may themselves damage the proteins; for example, O_2^- might inactivate catalase. Determination of the fate of LMWA may serve as a better indicator for ROS, because the adduct is specific to these molecules. For example, the attack of ROS on uric acid leads to the production of allantoin (100); similarly the attack of ROS on ascorbic acid causes the production of dehydroascorbic acid. Determination of the ratio between oxidant and reductant (eg, ascorbate/dehydroascorbic acid or GSH/GSSG) may therefore serve an indicator of oxidative damage. One of the approaches most commonly used is the measurement of the total antioxidant activity of a biological site. Depletion of one antioxidant molecule causes changes in the level of overall antioxidant molecules and may be evaluated using a variety of techniques including biochemical, immunohistological, spectroscopical, and electrochemical (201).

The total-antioxidant-activity assay offers many advantages and is considered a useful tool for detecting oxidative stress phenomena in bodily fluids and tissues. It may serve as an appropriate tool for the evaluation of antioxidant therapy. Determinations of total LMWA rather than individual antioxidants are important, because LMWAs work in concert (28), and measurement of only one or a few compounds out of many present at a specific biological location might be misleading. Moreover, measurement of the total LMWA ensures a reliable picture of the physiological situation. We do not know the concentration of a specific compound at a specific location at a given moment. Sometimes we try to detect compounds that are not present in the site under investigation. A few dozen LMWA exist, and usually only a few of them, such as Vitamin E and ascorbic and uric acids, are revealed; thus, many compounds that can be present at the biological site are overlooked. The measurement of the total LMWA is designed to overcome these problems.

Numerous procedures allow measurement of the total LMWA activity. These include indirect and direct methods for measuring total antioxidant activity originating from the LMWA (139, 201). Indirect methods are those that measure consequential factors of redox capacity, such as oxidation products formed or concentrations of major redox couples in the biological environment, by fluorescent or spectrophotometric techniques (139). In this approach one assumes that a biological redox buffer exists in the form of a redox couple that is sensitive to changes in the redox environment. Thus, it reflects changes in the reducing power of the measured sample, which is in correlation with all of the LMWAs. Other indirect techniques are inhibition methods that involve adding a radical species to the sample together with a scavenger that can be monitored with laboratory instruments. The LMWAs present in the sample under investigation can quench the radical and, therefore, interfere in its reaction with the added scavenger. Examples of indirect methods are 1) measurement of electrochemical couples, such as GSH/GSSG (2), NADH/NAD+, and ascorbic acid/ascorbate (144, 175); 2) the Trolox equivalent-antioxidant-capacity (TEAC) assay (211); 3) the total radical-trapping potential (TRAP) assay (159); 4) the chemiluminescence method; and 5) the oxygen-radical absorbance capacity (ORAC) methodology (201).

Direct methods for measuring total LMWA are those that utilize an external probe to measure the reducing or oxidizing capacity of a system. An example of a direct method is an electrode, in which the current is proportional to the concentrations of the scavenger or the redox couple under investigation. These direct methods can be classified into 2 groups—chemical and electrochemical. The chemical methods measure a known redox active couple whose reduced and oxidized states have different physical properties that can be measured as a function of concentration. For example, the ferric-reducing antioxidant power (FRAP) assay is based on the reaction of the redox couple ferric/ferrous with antioxidants in the sample and results in the creation of a blue color that can be measured at 593 nm (27). The electrochemical methods include various techniques, such as potentiometry, electrochemical titration, and voltammetry (237). The latter is described in detail subsequently, because it makes possible the measurement of the reducing power of both lipophilic and hydrophilic antioxidants in biological fluids and tissue homogenates. Measurement of the reducing power by voltammetric methods offers several advantages (84, 85). Such measurements can be performed easily and rapidly, allow the evaluation of numerous samples without sophisticated extraction and treatment, and thus are most suitable for screening a large number of samples. Information derived from these measurements cannot be obtained by other methods. The evaluations provide information concerning all LMWA of both lipophilic and hydrophilic nature and can be conducted in cells, biological fluids, and tissues. A unique characteristic, the reducing-power profile can supply information concerning the type and concentration of LMWA. The profile is specific to the tissue, and each biological site possesses its own characteristic set of data. Changes in the profile can immediately indicate the occurrence of oxidative stress to the system.

Principles and Methodologies of Cyclic Voltammetry (CV) and Examples of Evaluation of Biological Reducing Power

Voltammetric measurements have been conducted for many years to measure electron transfer between molecules and evaluate oxidation/reduction potentials of various redox-active compounds (17). These methodologies can provide information concerning thermodynamic, kinetic, and analytical features of the tested compounds (187). From the many different voltammetric methods, we adapted the cyclic voltammetry (CV) technique to the evaluation of the overall reducing power of a biological sample (133, 139, 141).

Following preparation of the sample for measurement, the sample is introduced into the tested well. A potentiostat with a 3-electrode system, required to conduct the measurement, consists of a working electrode (eg, glassy carbon, mercury film, or platinum electrode), a reference electrode (eg, silver/silver chloride or calomel electrode), and an auxiliary electrode (eg, a platinum wire) (Figure 8A). Following introduction of the sample, the voltage is linearly applied to the working electrode and changed from an initial to a final potential and immediately swept back at the same sweep rate to the initial. This potential is aimed to oxidize or reduce a species present in the solution in the voltammetric cell. The resulting current vs potential is recorded to produce a cyclic voltammogram (Figure 8) (107, 181, 187) that can supply thermodynamic, kinetic, and analytical information concerning the electrochemical couple under investigation (129). The position of the current wave (eg, anodic wave) on the voltage axis (x-axis of the voltammogram) can be determined and is referred to as the potential at which the peak current [peak potential $Ep(a)$] occurs (Figure 8B). This potential can be defined as the oxidation potential of a compound for a given set of conditions.

The anodic current (AC) (Ia) is calculated from the y axis of the voltammogram and depends on a number of parameters (Figure 8B, H), including the area of the electrode and the rate at which the potential is applied. It is directly proportional, at any potential, to the concentration of the compound in the bulk solution. Analytically, it is used to monitor concentration. When several compounds have the same or close oxidation potentials, the anodic wave obtained is composed of all of these compounds, and the peak potential is evaluated for the whole group. In this case the anodic current describes the concentration of the various compounds composing the wave, and changes in the anodic current values indicate changes in the concentrations of these compounds. This pattern is usually seen in voltammetric measurements of biological samples. Although the voltammogram cannot provide specific information on the exact nature of the LMWA, it can supply data concerning the reducing power of the sample under investigation.

Examples of Evaluation of Total Reducing Power in Some Clinical and Pathological Cases

Since this methodology for quantification of the overall LMWA was first introduced (131, 140), it has been used in a variety of clinical situations and pathological disorders,

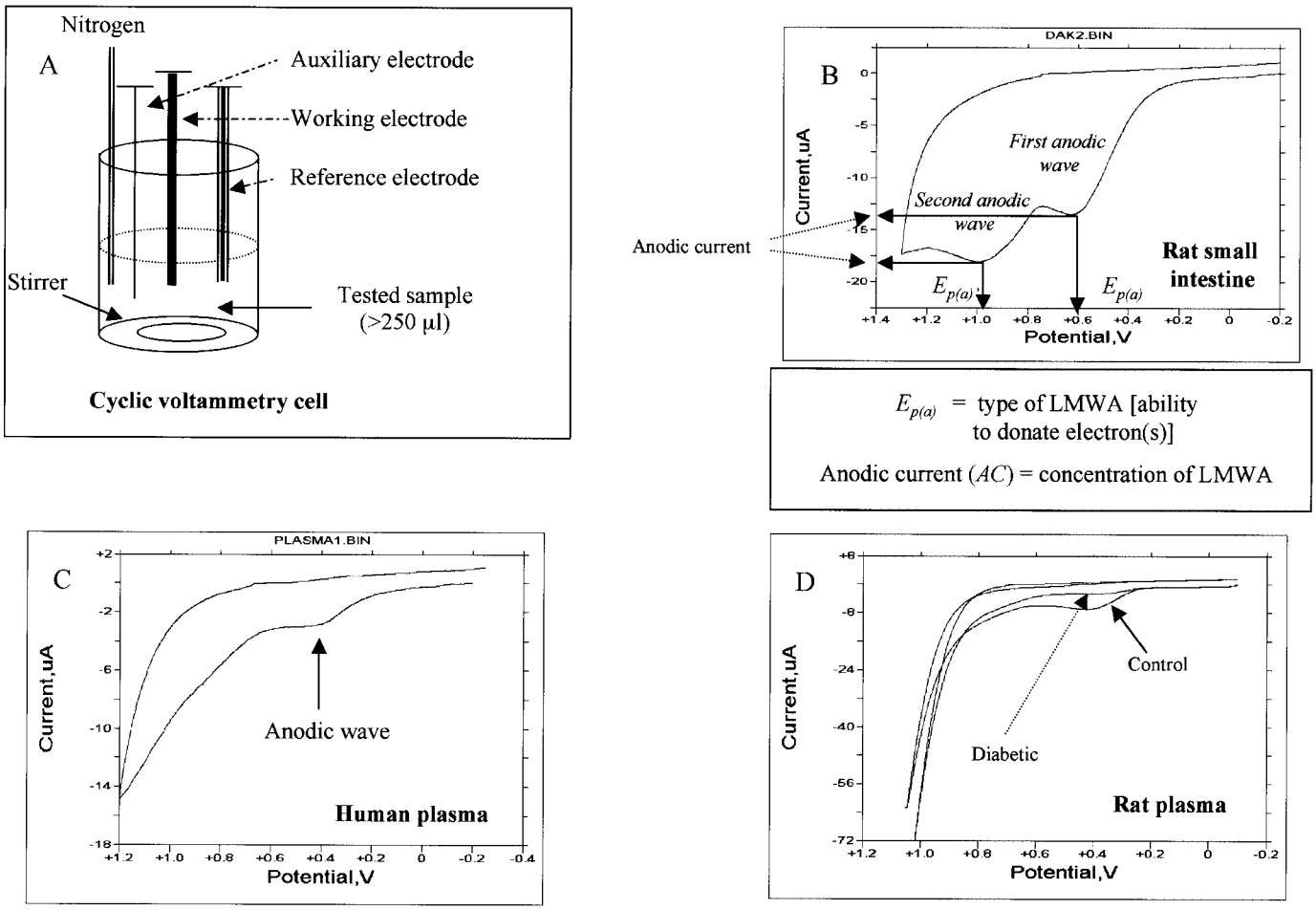
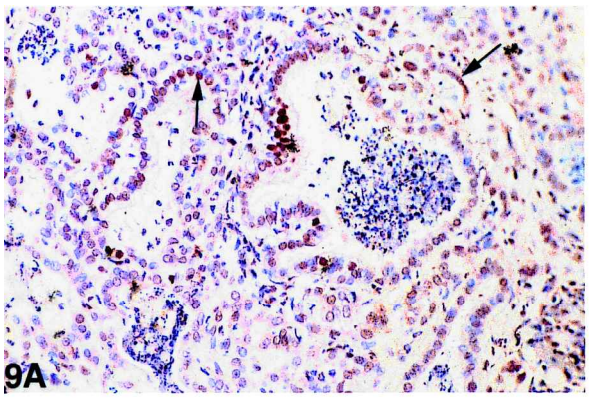


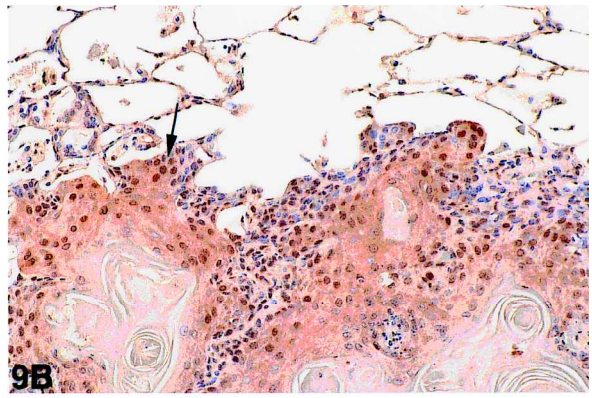
FIGURE 8.—Typical cyclic voltammogram (CV)s of some biological samples. Panel A shows the cyclic voltammeter cell, which contains 3 electrodes: glassy carbon as working electrode, Ag/AgCl as reference electrode, and platinum wire as auxiliary electrode. The working electrode is polished prior to the measurement. Panel B presents a typical voltammogram of the mucosa of rat small intestine. Two anodic waves are shown (each anodic wave may contain several LMWA possessing similar or close oxidation potentials). The $E_{p(a)}$ indicates the types of LMWA by their ability to donate electrons. In panel C, a voltammogram of human plasma, only 1 group of LMWA can be detected, as indicated by the anodic wave. Panel D contains voltammograms obtained from rat plasma. The control animal shows 1 major anodic wave. CV from streptozotocin-induced diabetic rats, 3 weeks following injection (58), does not show any anodic wave indicating significant decrease in plasma LMWA profile. Panel E exhibits a voltammogram, obtained following lipophilic extraction of rat brain, in which three anodic waves can be detected. Panel F presents a CV of rat heart, while panel G reveals a typical CV of rat kidney. Panel H shows a typical CV of rat lung and calculation of the anodic current. All tissue homogenizations were conducted in phosphate-buffered-saline at pH 7.4, as described in the text.

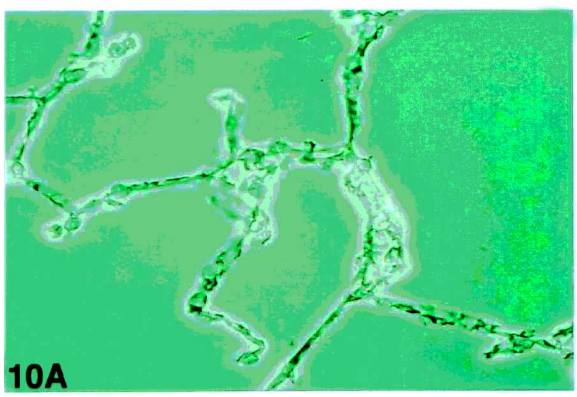
including diabetes (57, 133, 190), ulcerative colitis (33), brain degenerative diseases and head trauma (234, 235), skin status and pathologies (64, 135, 136, 134), and irradiation therapy (140), as well as study of the aging process (134, 135), and stages of embryonic development (190, 267). Biological fluids (166), such as seminal fluid, cerebrospinal fluid, saliva, sweat, urine, plasma, and gastric juice, possess reducing power derived from their LMWA content (Samples are shown in Figure 8B–H.). Before measurements, the samples are diluted 1:1 with phosphate-buffered saline to maintain constancy of the pH and ensure sufficient levels of electrolytes in solution. To extract the lipid-soluble compounds for voltammetric analysis, the tissue homogenate or biological fluid is combined with a mixture of organic extraction solution (ethanol:hexan, 1:5, or other suitable combinations of extraction solvents). Following evaporation of the organic solvents, the residue is dissolved in an acetonitrile solution containing 1% *tert*-butylamonium perchlorate as a supporting electrolyte (Figure 8) (135, 137).

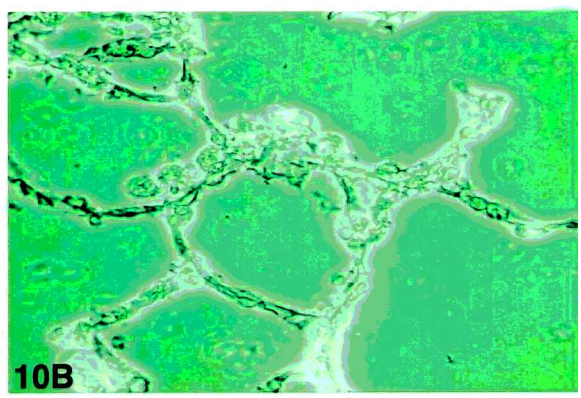
CV Measurements During the Aging Process (132–136)

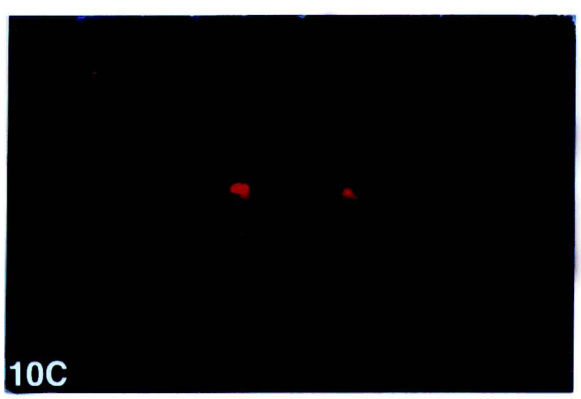
Among the many speculations concerning this process, the free-radical theory of aging has emerged as a dominant one. To clarify the assumption raised by this theory, which suggests that the level of antioxidants decreases with age, we measured the total LMWA capacity of various rat organs during the aging process. We found that the reducing power changes during aging in a bell-shaped manner in most of the tissues tested. Brain tissue, however, did not demonstrate a similar pattern, for the reducing power of rat brain, for example, does not change during the life span. Skin also demonstrated a different behavior (134). We evaluated the total capacity of the hydrophilic LMWA in the skin at various

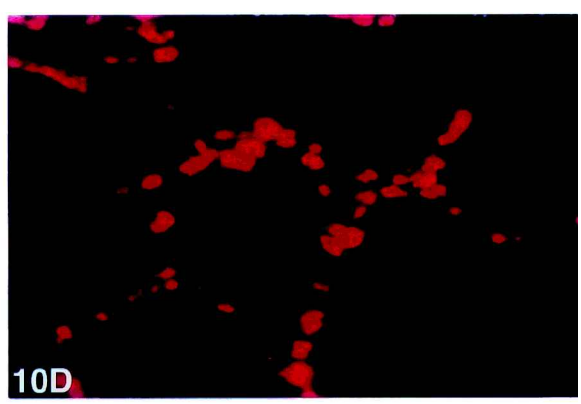


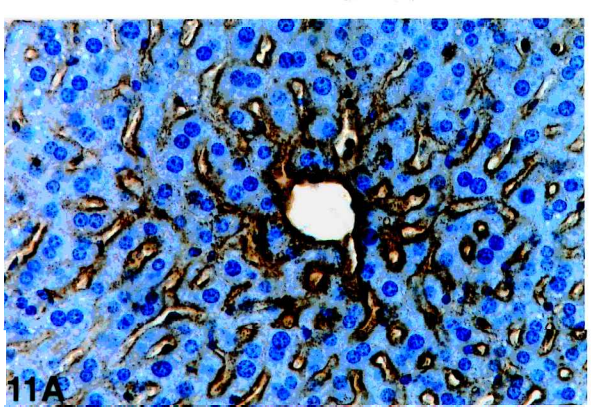


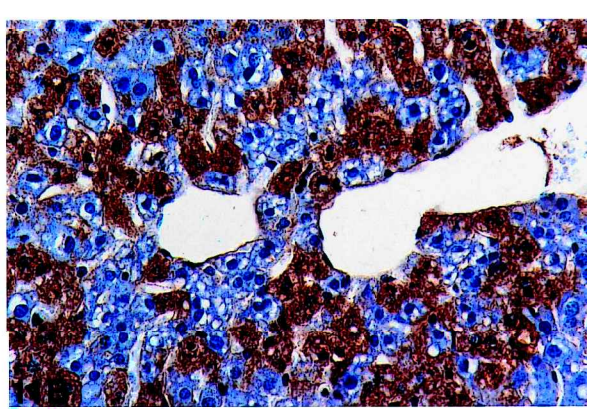












Figures 9–11

ages by CV. We found during the aging process a similar peak potential of the 3 waves (3 groups of LMWA), indicating that the nature, or type, of the reducing LMWA was not altered. By measuring the anodic current of the waves that related directly to the concentration of the LMWA creating the waves, we observed a significant decrease in the overall concentration of the LMWA of the various waves. These findings support the general claim in the free-radical theory of aging that a decrease in antioxidant capacity occurs in old age.

CV of Diabetic Rat Tissues

Oxidative stress plays a major role in diabetic complications (58, 190); however, the importance of the antioxidant system in protecting biological sites from deleterious processes induced by diabetic conditions is not fully understood. To clarify some of the inconsistencies in the scientific literature, we evaluated, using CV, the overall LMWA capacity, or reducing equivalents, of plasma and tissues of streptozotocin (STZ)-induced diabetic rats (1–4 weeks) and insulin-treated diabetic rats by measuring reducing power. The levels of both water- and lipid-soluble LMWA progressively decreased in diabetic plasma (Figure 8D), kidney, heart, and brain; however, a significant elevation in the antioxidant capacity of the liver was detected at 2, 3, and 4 weeks after induction of the diabetes as reflected by an increase in the anodic current of the lipophilic extraction (58). Several other studies were performed to determine the reducing-power profile in diabetic rats under additional stress, such as brain injury (58) and development of abnormalities in the embryo (190).

CV Measurements of Brain Injury (25, 174, 234, 235)

That ROS play a role in the pathophysiology of brain damage has been suggested. In an experimental rat and mouse model of closed-head injury, we have studied the overall reducing power of rat brain following head injury and discovered that the LMWA activity in the brain changes significantly at 5 minutes, 1 hour, and 24 hours following cerebral head injury. Only at 48 hours following the induction of the injury did the anodic current values representing LMWA concentrations return to their basal levels. We suggested that the lower LMWA levels following the injury were due to LMWA consumption. The CV methodology allowed us to describe the temporal changes in the brain LMWA, following induction of injury, as part of the response of the tissue to high levels of ROS. It also made possible the establishment of the correlation between the ability of the brain to elevate its LMWA and clinical recovery from the injury.

CV Measurement of the LMWA Along the Gastrointestinal Tract (33, 35)

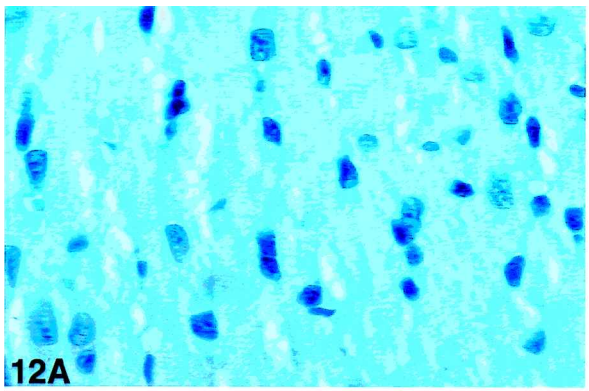
The overall LMWA capacity in the mucosa/submucosa and muscularis/serosa of various sections along the small intestine and colon of rat was evaluated by measuring the total reducing power with the CV methodology. Reducing power was higher in the mucosa and submucosa of the small intestine as compared to these same regions of the colon. Differences were also observed for the reducing power in the muscularis/serosa of the rat small intestine compared to the colon. The detailed procedures and experimental conditions have been described previously (35). The observed relatively lower reducing power—lower LMWA capacity—of the colon may provide a possible explanation for the reduced ability of this organ to cope with oxidative damage, which leads to inflammatory diseases, such as ulcerative colitis. At the same time the small intestine demonstrated a higher reducing power and thus a higher ability to manage tissue oxidative stress; it is, in fact, less susceptible to inflammatory diseases.

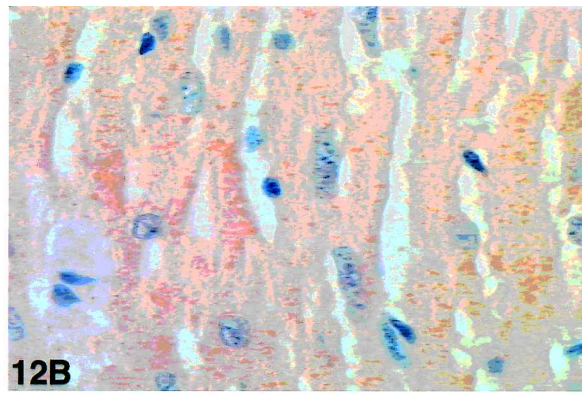
The use of this technique assists in the clarification of several hypotheses concerning the role of antioxidants and their regulation in biological environments. Recently, we suggested a new hypothesis based on the existence of many different LMWAs widely distributed among various organs and results obtained from voltammetric measurements. We hypothesized that the cell requires a certain level of antioxidant activity. Which LMWAs contribute to this purpose is inconsequential to the cell. Whenever a decrease in the level of a specific antioxidant molecule occurs, the cell can cope by increasing the level of another antioxidant possessing a similar oxidation potential. Using voltammetric measurements, we were able to demonstrate that, in many cases, the overall reducing-power profile is not affected, while specific changes in the concentration of specific LMWAs may be detected using laboratory techniques, such as HPLC-ECD. In specific pathological cases and defined clinical situations, however, the alteration of the overall profile can be easily detected by voltammetric determination; such detection may imply a possible intervention using LMWA.

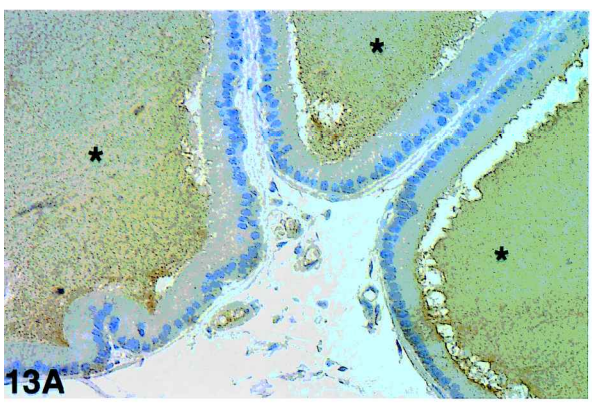
IMMUNOHISTOCHEMICAL MARKERS USED IN TOXICOLOGIC PATHOLOGY IN VISUALIZATION OF OXIDATIVE-STRESS PHENOMENA

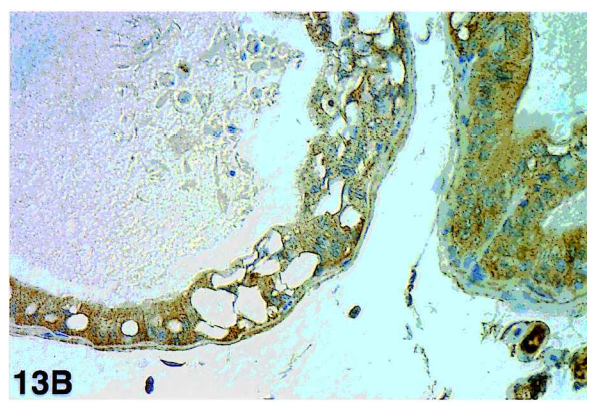
Because ROS and RNS are involved in a variety of biological phenomena, such as mutation, carcinogenesis, aging, and inflammation, evaluating the cellular status of oxidative stress

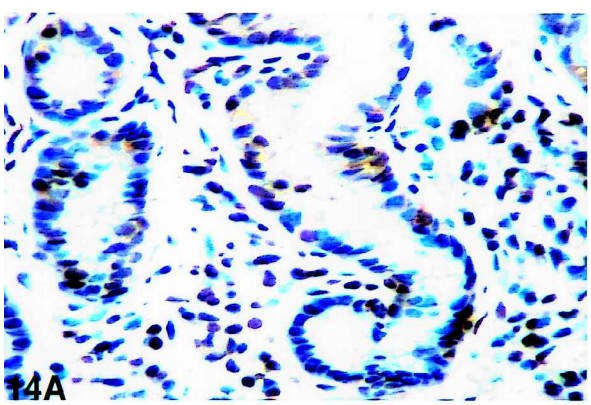
FIGURE 9.—Photomicrographs demonstrating localization of 8-hydroxydeoxyguanosine (8-OHdG) in pulmonary atypical hyperplasia and squamous cyst induced in female rats exposed to 0.3 mg/m^3 IP in a 2-year inhalation-exposure study. Staining was performed on formalin-fixed tissues using specific monoclonal antibody (82, 253). Strong brownish nuclear staining indicates presence of antigen. Bar = 100 μm. A) Atypical hyperplasias vary in size and have rounded outlines and central fibrous cores containing dispersed alveolar structures lined by uniformly cuboidal epithelial cells. Aggregates of mostly necrotic inflammatory cells can be seen within adjacent alveoli and, often, glandular structures. Peripherally, fibroproliferative lesions have one to several epithelial layers, frequently forming papillary projections. B) Squamous cysts are characterized by variably thick band of viable squamous epithelium with large central core of keratin. From Gottschling et al (82) with permission from Oxford University Press. 10.—Fluorescence visualization of 8-OHdG immunopositivity in frozen sections of rat lung exposed to 1.2 mg quartz for 90 days. A) Phase contrast; control (physiological saline) exposure. B) Phase contrast; quartz exposure. C) Fluorescence; control (physiological saline) exposure. D) Fluorescence; quartz exposure. From Seiler et al (226) with permission from *American Journal of Respiratory Cell and Molecular Biology*, official journal of the American Thoracic Society. 11.—Immunohistochemical staining for nitrotyrosine of paraffin-embedded rat liver tissue 1 hour (A) and 4 hours after 300 mg/kg acetaminophen exposure. In A, staining is limited to the vascular-lining cells, while, in B, intensely stained hepatocytes are scattered among unstained cells in centrilobular areas. From Knight et al (130) with permission from Oxford University Press.

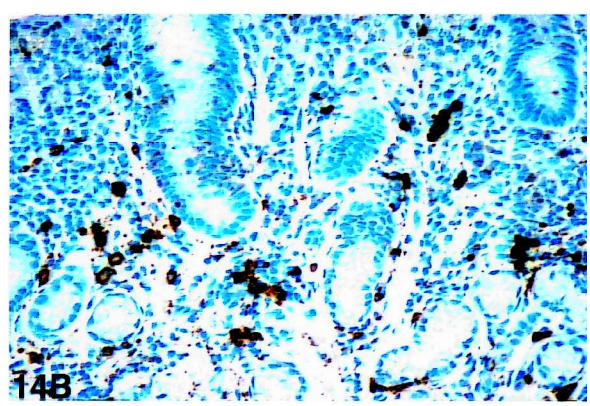


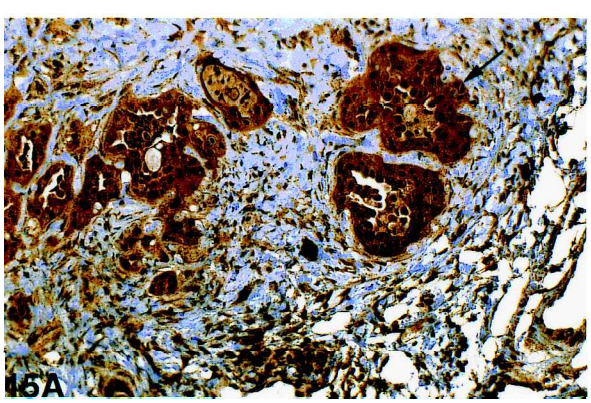


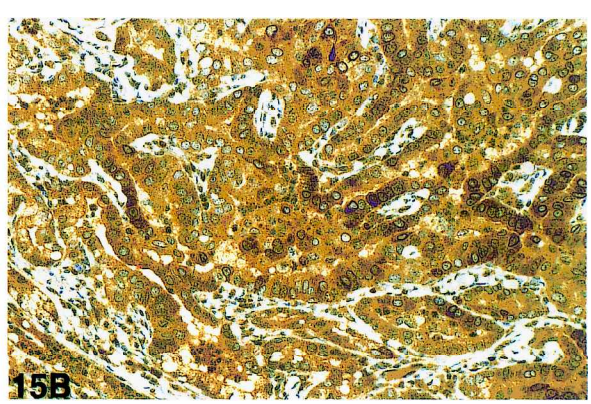












Figures 12–15

is essential. Direct detection of the production of ROS and other free radicals is difficult, as these molecules are short-lived and highly reactive in a nonspecific manner. Thus, ongoing oxidative damage is generally analyzed by measurement of secondary products (252), including derivatives of amino acids, nuclei acids, and lipid peroxidation (188).

Oxidative-stress markers have been divided into 3 categories. First, molecules modified by free radicals, such as 4-hydroxy-2-nonenal, malondialdehyde, and 8-oxo-2′-deoxyguanosine (8-oxo-dG) are derivatives respectively of proteins, lipids, and nucleic acids. The amounts of these products are proportional to dose, and they are detected at the sites where free-radical attacks occur. Second, antioxidant enzymes and molecules are associated with the metabolism of radicals, such as GSH and catalase. Finally, transcriptional factors are included, such as nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$) and c-myc, which are modulated by these radicals. Because tissues collected during toxicity studies are fixed chiefly in formalin, investigators must focus on well-defined products that are stable in this fixative and unlikely to share homology with formalin-induced modifications (203).

Reviewing histochemical and immunohistochemical approaches to the study of oxidative stress, Raina et al (203) stated that "the importance of in situ methods over bulk analysis cannot be overstated when considering the structural and cellular complexity of tissues and the effects of diseases thereof. Indeed, in situ detection allows detection of specific cell types affected or specific localization such that a process affecting only a small fraction of the tissue or cells can be readily visualized."

In this article we feature several cases in which immunohistochemical markers directed to specific enzymes, factors, or damage products suggestive of oxidation stress are used to support investigation of the pathophysiology of toxic or cancerous diseases or are applied to drug development. To discuss all types of existing markers suggestive of oxidation stress is beyond the scope of this review. Rather, the intention is to encourage the toxicological pathologist to adopt these markers whose reliability has been confirmed and apply them to paraffin-embedded tissues.

8-Hydroxydeoxyguanosine

8-hydroxydeoxyguanosine (8-OHdG), an oxidized form of guanine, is a major oxidative DNA-damage product that can produce mutation. This compound causes A:T to C:C or G:C to T:A transversion mutations because of its base pairing with adenine as well as cytosine. According to Toyokuni (252, 253), most reported polyclonal or monoclonal antibodies for 8-OHdG show a relatively high cross-reactivity with 8-hydroxyguanosine (8-OHG), except for the monoclonal antibody N45.1 that is more specific and useful in paraffin-embedded sections (253). Takahashi et al (248) confirmed, by using a carbon tetrachloride-induced, acute-liver-damage model in rat, that the anti-8-OHdG antibody detects cells targeted by free radicals in paraffin-embedded sections. Immunohistochemical accumulation of high levels of 8-OHdG was reported to occur in various human tumors, like high-grade glioma, compared to adjacent, normal tissue or low-grade glioma (113). These studies suggested that oxidative stress may play a role in tumor progression.

Gottschling and colleagues (82) applied the 8-OHdG N45.1 antibody to a range of paraffin-embedded lesions induced in rats exposed to indium phosphide (IP) by inhalation for 3 months and 2 years. Results indicated increased 8-OHdG expression in cells of carcinoma epithelium, atypical hyperplasia (Figure 9A), and squamous cyst (Figure 9B). The findings suggested that IP inhalation causes pulmonary inflammation associated with oxidative stress, resulting in progression to atypical hyperplasia and neoplasia. According to Upham and Wagner (255), while 8-OHdG is implicated in G-C to T-A transversions, this marker does not always correlate with genotoxicity. Despite its weak link to tumorigenicity, however, 8-OHdG is likely a good indicator of oxidative stress.

Seiler et al (226) applied immunohistochemical staining for 8-OHdG on frozen lung sections and tested the time- and dose-dependent biologic effects of different doses of quartz. Morphometric analysis of the fluorescent signals indicated that doses of quartz higher than 0.3 mg/day induced significantly increased 8-OHdG levels in lung cells, following 21 and 90 days of exposure (Figure 10 A–D). The 8-OHdG expression was particularly associated with the presence of *p53*-immunopositive cells when a higher dose (7.5 mg/day) of quartz for 90 days was tested. The results suggested that quartz-induced tumor formation in the rat lung is triggered only by a significant and permanent inflammatory response in the lungs provoked by exposure to high doses of this compound.

FIGURE 12.—Photomicrographs of cardiac left ventricular sections from vehicle control (A) and doxorubicin (DOX)-treated (B) (20 mg/kg DOX, ip) mice immunostained for nitrotyrosine (5 days posttreatment). Brown nitrotyrosine immunopositivity indicates association of cardiotoxicity with increased cardiac protein nitration. From Mihm et al (171) with permission from Nature Publishing Group. 13.—Photomicrographs demonstrating localization of nitrotyrosine in paraffin-embedded ventral lobe of prostate of Nobel rat carcinogenesis model. In control rat (A), nitrotyrosine immunostaining is expressed only by the intraluminal secretion (asterisks). In contrast, 8 weeks following implantation with two 2-cm testosterone-filled capsules and one 1-cm diethylstibestrol-filled capsule, intense cytoplasmic staining occurred in dysplastic as well as morphologically unaltered acini (B, arrow). No staining was observed in luminal secretions (asterisk). Figures generously contributed by Drs. N. Tam and S.M. Ho, Division of Urology, Department of Surgery, University of Massachusetts Medical School, Worchester, Massachusetts. 14.—Photomicrographs demonstrating localization of inducible nitric oxide synthase (iNOS) (A) and nuclear factor κB (NF-κB) (B) in Helicobacter pylori-infected gastric antral biopsies before antimicrobial treatment. Note positive immunoreactivity (brown color) for iNOS in cytoplasm of inflammatory cells present in the mucosa and lamina propria of the mucosa. (iNOS immunoreactivity was dramatically reduced after antimicrobial therapy.) Nuclear immunoreactivity (brown color) for NF-κB is present in the glandular epithelial cells and lamina propria inflammatory cells. (After antimicrobial therapy, staining was greatly diminished in the epithelial cells.) From Zhang et al (269), with permission from Elsevier Science. 15.—Photomicrographs demonstrating localization of cyclooxygenase-2 (COX-2) in pulmonary atypical hyperplasia (A) and bronchiolo-alveolar carcinoma (B) induced in female rats exposed to 0.3 mg/m^3 IP in a 2-year inhalation-exposure study. Staining was performed on formalin-fixed tissues using specific monoclonal antibody. Strong brownish cytoplasmic staining indicates presence of antigen. Note in A) COX-2 immunopositivity lining atypical epithelium and in macrophages within the alveoli. Bar = 100 μm. From Gottschling et al (82), with permission from Oxford University Press.

Nitrotyrosine

The toxicity of NO is enhanced by its reaction with a superoxide to form $ONOO^-$ (22). It or secondary metabolites can cause tyrosine nitration in protein, creating nitrotyrosine, a footprint detectable in vivo. The presence of nitrotyrosine has been demonstrated in various human diseases like atherosclerosis, myocardial ischemia, inflammatory bowel disease, and amyotrophic lateral sclerosis, as well as in toxic and carcinogenic models (22, 130, 263). Immunostaining for nitrotyrosine has been used to investigate the pathophysiology of acetaminophen-induced hepatotoxicity. Initial formation of nitrotyrosine in the sinusoidal endothelial cell (EC)s was demonstrated (Figure 11A), with later progressive accumulation within hepatocytes, indicating oxidative stress in both types of cells (Figure 11B) (130). That early $ONOO^-$ formation within ECs may also contribute via EC injury and hemorrhage to later development of hepatocellular necrosis was suggested.

Investigating the mechanism of doxorubicin-induced cardiotoxicity in mice and using immunostaining for inducible nitric oxide synthase (iNOS) and nitrotyrosine, Weinstein and collaborators (263) and Mihm et al (171) showed that the extent of left ventricular dysfunction was highly correlated with the extent of nitrotyrosine immunoreactivity rather than iNOS staining (Figure 12 A–B). The results suggested that $ONOO^-$ may play a pivotal role in doxorubicin-related dysfunction. Tam, Ho, and Ghatak (personal communication, 2001), performing immunostaining for nitrotyrosine and using the method of Oberley et al (185), investigated whether treatment of carcinogenesis-model Nobel rats with sex hormone exerts an effect on prostatic nitrosative stress, causing perturbations in pro- and antioxidant balance. They found that, in the untreated ventral lobe of the prostate (VP) (Figure 13A), an intense nitrotyrosine staining occurred only in luminal secretions and was negative or relatively weak in both glandular epithelial cells and periacinar smooth muscle cells. In the VP of testosterone- + diethylstibestrol-treated rats, intense cytoplasmic staining was localized in dysplastic as well as morphologically unaltered acini (Figure 13B). Focal staining was occasionally seen in periacinar smooth muscle cells, but positive staining was rarely observed in luminal secretions. The authors' conclusion was that ROS and RNS might be involved in hormone-induced prostatic carcinogenesis.

Nuclear Factor κB

Nuclear factor κB (NF-κB) is a transcriptional factor implicated in inflammation and immune activation and activated by oxidants and cytokines (19). This factor normally resides in an inactive form in the cytoplasm and has been shown to enhance iNOS gene expression in different types of cells, like macrophages (112). Zhang et al (269) applied immunostaining for iNOS and NF-κB to investigate their involvement in the pathogenesis of Helicobacter pylori-induced gastritis. The authors showed that NF-κB was expressed in the glandular epithelial cells (Figure 14A), while the cytoplasm of inflammatory cells infiltrating the lamina propria was immunopositive for iNOS (Figure 14B). To prove their hypothesis, they showed that use of antimicrobial therapy was effective in reducing immunostaining for both markers in correlation with reduced mucosal inflammation. The findings corroborated the supposition that a NF-κB-dependent, epithelia-derived mediator may be responsible for the induction of iNOS expression and suggested a therapeutic approach targeting NF-κB.

Cyclooxygenase-2

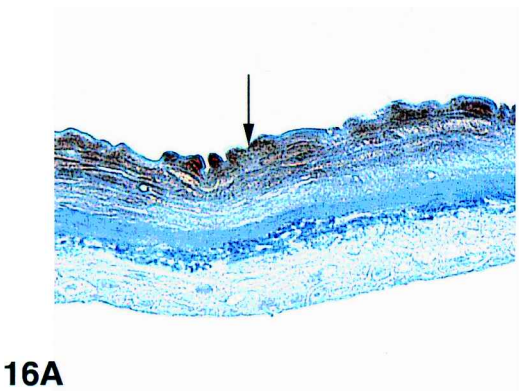
Cyclooxygenase catalyzes the formation of prostaglandins and other eicosanoids from arachidonic acid. Cyclooxygenase-2 (COX-2) is induced at the site of inflammation following stimulation with proinflammatory agents, such as interleukin-1 (IL-1), tumor necrosis factor-α, and lipopolysaccharides (57). Investigators have suggested that the release from inflammatory cells of $NO^{\cdot}$, which is synthesized from L-arginine by the action of iNOS, increases COX-2 activity (221). COX-2 overexpression is involved in cellular proliferation and carcinogenesis in different organs (186, 270), and COX-2-specific inhibitors prevent lung carcinogenesis (215).

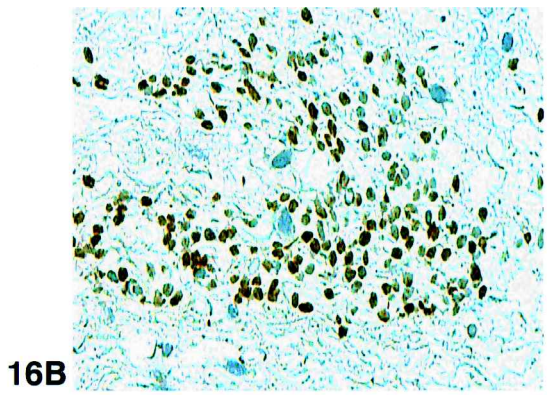
Paraffin-embedded tissues from rats exposed by inhalation for 3 months and 2 years to the lung carcinogen IP were used in an immunohistochemical investigation of expression of oxidative stress markers (82). Following 3 months of exposure, IP inhalation resulted in severe pulmonary inflammation that correlated with the infiltration of macrophages exhibiting strong immunopositivity for iNOS and COX-2. After 2 years of exposure, i-NOS was strongly expressed in lesions containing macrophages, including chronic inflammatory foci, atypical hyperplasias, adenomas, and carcinomas. COX-2 was expressed in all of these lesions, both in the macrophages and epithelium (Figure 15A, B). The results support the hypothesis that IP inhalation, effecting uptake of foreign particles into the lung, causes oxidative stress progressing to induction of pulmonary cancer. Within the IP-induced pulmonary lesions, the macrophage-derived prostanoids might have contributed to the progression of hyperplasia in nearby epithelial cells.

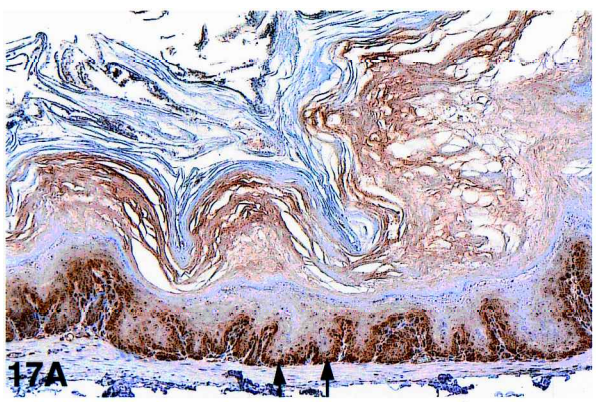
Evaluation of the immunoexpression of COX-2 was applied in 2 rat models of acute skin inflammation—carrageenan-induced paw edema and mechanical hyperalgesia (180). The authors demonstrated that the extent of tissue inflammation, COX-2 staining, and blockage of COX-2 induction using a nonselective, nonsteroidal anti-inflammatory drug (NSAID) correlated with the dose of carrageenan used to induce the model (Figure 16A, B). The study suggested that a prostanoid-dependent positive feedback loop existed only in the paw-edema model in which COX-2 induction was completely abolished by the NSAID.

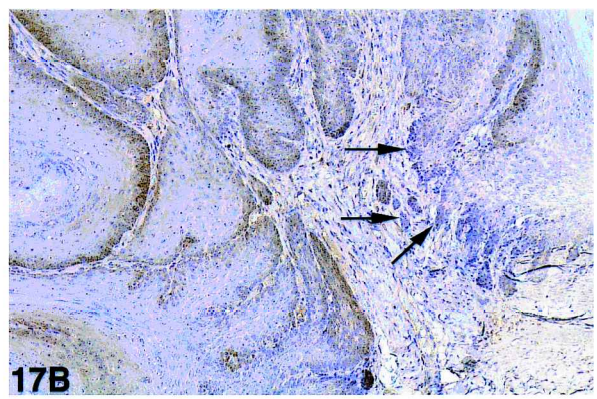
Glutathione S-Transferase-pi

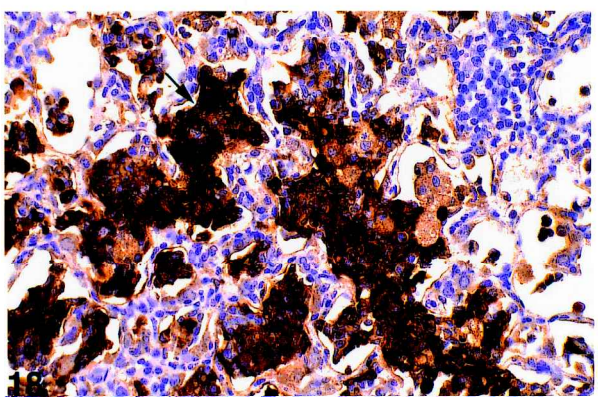
Drug-metabolizing enzymes, such as glutathione S-transferase (GST)s, and antioxidant systems, such as glutathione, vitamins, catalase, and superoxide dismutase, function concertedly as the 2 major inducible defense systems against electrophiles and xenobiotic toxicity (62). The expression of these 2 systems occurs through a common regulatory region called the antioxidant responsive element

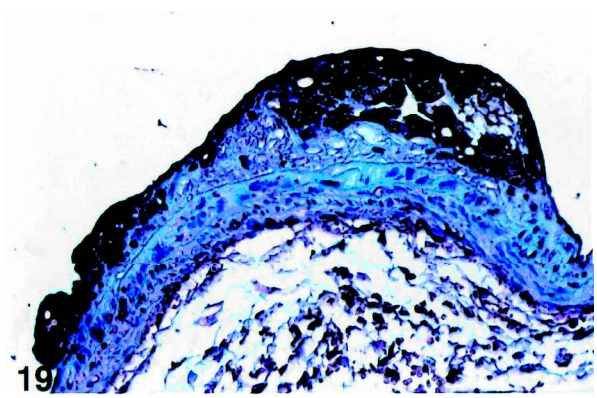












Figures 16–19

FIGURE 16.—Photomicrographs of cyclooxygenase-2 (COX-2) immunoreactivity in rat paw 3 hours following injection of carrageenan in hyperalgesia model. Note COX-2 positivity involves the stratum corneum (A) of the epidermis and small infiltrated cells in the connective tissue (B). From Nantel et al (180) with permission from Nature Publishing Group. 17.—A) Photomicrograph of forestomach hyperplasia of rat treated with 2,4-hexadienal (Hx), 90 mg/kg per day, for 2 years; glutathione S-transferase-pi positive staining (moderate to marked grade) seen in ridges of basal epithelium (arrows). B) Squamous cell carcinoma of rat treated with 90 mg/kg per day of 2,4-Hx for 2 years; atypical submucosa-infiltrating cells express minimal or no immunoreactivity (arrows). From Nyska et al (183) with permission from Springer-Verlag GmbH & Co KG. 18.—Photomicrograph demonstrating localization of inducible nitric oxide synthase (iNOS) within macrophages (arrow) in pulmonary chronic active inflammation induced in female rats exposed to 0.3 mg/m^3 IP in a 3-month inhalation-exposure study. Staining was performed on formalin-fixed tissues using specific monoclonal antibody. Strong brownish cytoplasmic staining indicates presence of antigen. Bar 50 μm. From Gottschling et al (82) with permission from Oxford University Press. 19.—Photomicrograph of immunohistochemical staining for heme oxygenase-1 (HO-1) of atherosclerotic lesion of LDL-receptor knockout mouse fed a western (high-fat) diet for 6 weeks. Note a significant accumulation of HO-1-positive macrophages (arrow) within the subintima of the ascending aorta. From Ishikawa et al (116) with permission from Lippincott, Williams & Wilkins, a Wolters Kluwer Company.

(ARE) (199). Nuclear factor 2 (Nrf2) has been shown to be a key molecule that responds to reactive electrophiles by activating ARE-mediated gene expression (62). Glutathione S-transferase-pi (GST-pi), a member of this family of phase II detoxification enzymes, catalyzes intracellular detoxification reactions, including the inactivation of electrophilic carcinogens by catalyzing their conjugation with glutathione (109). In addition, GSTs have endogenous substrates, such as lipid and nucleic acid hydroperoxides and alkenals, which result from the decomposition of lipid hydroxyperoxides (47).

Investigating the mode of action of the forestomach carcinogen 2,4-hexadienal in rats, Nyska et al (183) used

immunostaining for GST-pi applied to paraffin-embedded tissues from control and high-dose rats from the 13-weeks and 2-years experiments. Results indicated that the simple basal epithelium of control rats expressed strong immunopositivity. In cases of basal cell hyperplasia from the 13-weeks and 2-years' studies, the basal cells usually expressed strong immunopositivity for GST-pi (Figure 17A). In the 2-years treated animals only, occasional focal reduction in immunoreactivity for GST-pi was noted in the basal cell hyperplasia lesions. In papillomas and squamous cell carcinomas, a wide range of GST-pi expression was observed (Figure 17B), perhaps indicating irregularities in its induction or change in the phenotype of these cells, compared to normal or hyperplastic ones. The reduced expression of GST-pi by the foci of basal cell hyperplasia and in tumor cells suggests an altered phenotypic differentiation, different from that of normal cells, and that not all cells are protected from incurred oxidative or electrophilic DNA damage. Cells expressing lower levels of GST-pi may also be the result of inactivation by hypermethylation of regulatory sequences at a locus on the GST-pi gene (152). Jeronimo et al (119) recently analyzed cystidine methylation in the GST-pi promoter in human cases of prostatic hyperplastic and malignant lesions, applying a fluorogenic, real-time, methylation-specific, polymerase-chain-reaction assay. The authors concluded that quantification of GST-pi methylation accurately discriminates between normal hyperplastic tissue and prostatic carcinoma.

Inducible Nitric Oxide Synthase

Nitric oxide is synthesized in a variety of tissues via the catalytic activity of nitric oxide synthase (NOS). The inducible form, iNOS, is found predominantly in mononuclear phagocytes where it may be induced by endotoxins and/or cytokines; it is capable of producing high levels of NO (173). Although the role of NO in tumor biology remains controversial, most data indicate that it promotes tumor progression (191). Increased iNOS expression may play a role in human tumorigenesis, as, for example, in the prostate where high-grade prostatic intraepithelial neoplasia (PIN) and carcinoma display more intense iNOS immunoreactivity than benign prostatic hyperplasia and low-grade PIN samples (16).

Extensive iNOS immunoexpression (average grade, severe) has been noted within infiltrating macrophages at sites of chronic active inflammation, the major lesion in rats exposed by inhalation for 3 months and 2 years to the lung carcinogen IP (Figure 18) (82). Lesion progression suggested that the foreign bodies (IP) introduced into the lungs attracted macrophages for their digestion and removal and induced severe inflammation, which further enhanced NO production. The change in this and other markers analyzed in this investigation lends strong credence to the supposition that oxidative stress plays a major role in the development of lung cancer from IP inhalation (82).

Haem Oxygenase I

Haem oxygenase (HO)-1, a heat shock protein, is the inducible isoform of the rate-limiting enzyme of haem degradation (114). It is induced by various stimuli, including heat shock, hyperoxia, and oxidative stress and represents a powerful endogenous protective mechanism against free radicals in a variety of pathological conditions. Liu and coworkers (160), studying in frozen tissues the immmunohistochemical localization of HO-1 in experimental autoimmune encephalomyelitis, which serves as a model for multiple sclerosis (MS) in human, noted a high expression in scattered macrophage-like and perivascular cells in inflamed lesions of the spinal cord. Repeated intraperitoneal injection of the HO-1 inducer, hemin, was associated with attenuation of spinal cord inflammation and reduced HO-1 immunoexpression. The latter was probably attributable to fewer macrophages, known to be the main source of ROS production. The results suggested that pharmacological modulation of HO-1 expression may serve as a novel approach to therapeutic intervention in MS.

Immunostaining for HO-1 was recently applied to the aorta of LDL (low density lipoprotein)-receptor knockout mice in an effort to confirm the role of oxidized low density lipoprotein (oxLDL) during early phases of atherogenesis. Its proinflammatory properties and the potential function of HO-1 in inhibiting oxLDL-dependent monocytic chemotaxis (116) were studied. By using this knockout mouse, HO-1 activity was modulated with HO-1 inhibitor and inducer using 2 different kinds of high-fat diets. The HO-1 inhibitor enhanced the size of the atherosclerotic lesion, while opposing results were noted when the HO-1 inducer was administered. Immunohistochemistry performed on frozen tissue revealed colocalization of HO-1 and oxidized phospholipids within macrophages in the atherosclerotic lesions, and the immunoexpression and size of these lesions in the ascending aorta were significantly correlated (Figure 19). That HO modulation affected plasma lipid hydroperoxide and nitrite and nitrate levels suggested the possibility that HO-1 influences atherosclerotic lesion formation and development as an intrinsic antioxidant system. The same group of researchers (115) also described the use of HO-1 immunohistochemical staining on paraffin-embedded tissue and tested the effect of inhibition of HO-1 on atherosclerotic lesion formation in Watanabe heritable hyperlipidemic rabbits.

SUMMATION

The significance of the damage inflicted upon biological systems by ROS cannot be overestimated, as they have been implicated in numerous disease processes, including inflammation, degenerative diseases, and tumor formation and involved in physiological phenomena, such as aging and embryonic development. The dual nature of these species with their beneficial and deleterious characteristics implies the complexities of their specific functionings at a biological site and the difficulties in establishing appropriate intervention procedures to treat ROS-related diseases. Their detection using chemical and immunohistochemical methodologies is, therefore, essential to elucidate their exact mechanisms of activity and may allow development of antioxidant intervention strategies leading to reduction in diseases associated with oxidative stress. Such strategies may delay age-related degenerative diseases and enhance the quality of life, particularly in the later years.

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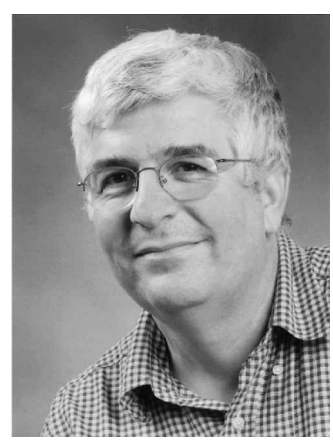

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