12

OXYGEN IN BIOLOGY AND BIOCHEMISTRY: ROLE OF FREE RADICALS

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12.1 FREE RADICALS AND OXIDIZING AGENTS IN BIOLOGY

A *free radical* is defined as any atom or molecule that contains unpaired electrons and has independent existence (hence, the term *free*). A *dot* is used to represent a free radical species. The abstraction or gain of one electron by a nonradical molecule may (or may not) convert it to a radical species. Free radicals can have positive, negative, or neutral charges [reactions (12.1.1) and (12.1.2)].

$$\text{A} \rightarrow \text{minus one electron} \rightarrow \text{A}^{+\bullet} \qquad (12.1.1)$$

$$\text{B} \rightarrow \text{plus one electron} \rightarrow \text{B}^{-\bullet} \qquad (12.1.2)$$

For most of the nineteenth century, radicals were regarded as parts of molecules and without free existence. In the late nineteenth century, however, it was believed that even if free radicals did exist, their very nature would preclude the possibility of isolating them. Free radicals would have such a fleetingly short existence that the available analytical methods of the period could not detect (or isolate) them. However, the solution-phase studies of Moses Gomberg proved the "free" existence of the triphenylmethyl radical in 1900, which opened the field for much more interesting discoveries. In the mid-twentieth century, the invention of free-radical-based polymerization reactions became very important for the industrial world. Two good examples are the polymerization reactions of styrene and butadiene for the production of synthetic rubber and the polymerization of various vinyl monomers for the production of many plastics.

In the late 1930s Leonor Michaelis proposed that *all* oxidation reactions involving organic molecules would be mediated by free radicals. This *radical* and incorrect prediction prompted interest in the role of free radicals in oxidative biological processes. Up to the early 1970s, there was still very little evidence that free radicals could affect living organisms. Thirty years later, however, the evidence is just too great to be covered in a single book chapter. The following sections will focus on the principles of free radical chemistry and highlight various roles of free radicals in biology and medicine, with selected sections on some key historical events in the study of free radicals in metabolism. Further information connecting free radicals with selected human pathologies are discussed in Chapter 13.

12.1.1 Free Radical Reactions in Biology

In cells, one-electron abstraction of molecules can yield sulfur-, oxygen-, carbon-, and nitrogen-centered free radicals. For example, removal of one electron (and a proton, H^+) from a $-SH$ group of a protein by a radical species ($R^{\bullet}$) yields a sulfur-centered free radical [reaction (12.1.3)]. If $R^{\bullet}$ had just one unpaired electron, the reaction would convert it to a nonradical species. Another example is the one-electron removal from bis-allylic C–H bonds of polyunsaturated fatty acids (PUFAs) that yields a carbon-centered free radical [reaction (12.1.4)]. This reaction can

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initiate lipid peroxidation damage of biological membranes (see Section 12.3).

$$R^{\bullet} + \text{protein-SH} \rightarrow \text{one-electron transfer to } R^{\bullet} \rightarrow \text{protein-S}^{\bullet} + R^{-} + H^{+} \quad (12.1.3)$$

$$R^{\bullet} + \text{(PUFA)-CH(bis-allylic C–H bond)} \rightarrow \text{(PUFA)-C}^{\bullet} + RH \quad (12.1.4)$$

High-energy splitting of nonradical molecules also yields free radicals. A classic reaction is the radiation-induced homolysis of water [reaction (12.1.5)], yielding hydroxyl radical ($^{\bullet}OH$) and a hydrogen atom ($H^{\bullet}$), the simplest kind of free radical. A free radical can also add to a nonradical molecule, yielding a free radical product ($A^{\bullet} + B \rightarrow A\text{-}B^{\bullet}$) or a nonradical product ($A^{\bullet} + B \rightarrow A\text{-}B$); a good example in this case is the hydroxylation of aromatic compounds by $^{\bullet}OH$ [e.g., reaction (12.1.6) shows the hydroxylation of phenylalanine]:

$$H_2O + \text{energy} \rightarrow \text{activated } H_2O \rightarrow H^{\bullet} + {}^{\bullet}OH \quad (12.1.5)$$

$$^{\bullet}OH + \text{phenylalanine} \rightarrow \textit{ortho}\text{-}, \textit{para}\text{-}, \text{ or } \textit{meta}\text{-tyrosine} \quad (12.1.6)$$

Two radicals can also react with each other to yield a nonradical product ($A^{\bullet} + C^{\bullet} \rightarrow A\text{-}C$). When the two reactants are the same free radical species with one unpaired electron each, then a dismutation reaction may take place; one example is the dismutation of the carbon-centered ascorbate radical (ascorbyl$^{\bullet}$), yielding ascorbate (vitamin C) and dehydroascorbate [DHA; reaction (12.1.7)].

$$2\ \text{ascorbyl}^{\bullet} \rightarrow \text{ascorbate (reduced product)} + \text{DHA (oxidized product)} \quad (12.1.7)$$

Radical molecules, with one or more unpaired electrons, can be attracted to a magnetic field; this means that most radicals have paramagnetic characteristics. Application of the correct electromagnetic energy causes absorption of that energy by the unpaired electrons and an absorption spectrum is obtained (usually unique for each radical molecule), generally in the microwave region. This is the basic principle of the well-known technique of electron spin resonance (ESR; also known as electron paramagnetic resonance, EPR), which has been used for the study of biologically important radicals since the 1970s. An example of an ESR signal of a radical molecule (ascorbyl$^{\bullet}$) is depicted in Figure 12.1 (see also Chapter 13 for an example of ESR usefulness in experimental medicine).

Due to the instability of many radicals (which preclude their direct detection), *spin trapping* techniques are used to study them. This is based on the reaction of radicals with nitroso molecules (R-NO), giving stable nitroxide radicals. Each nitroxide radical has a characteristic ESR spectrum, which can be properly studied. The most commonly used nitroso compounds for biochemical studies are 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-phenyl-*N*-*tert*-butylnitrone (PBN) (see Section 12.1.2.5). These spin traps are also useful for *in vivo* studies because the nitroxide radical products can be quantified, for example, in plasma samples.

In the following subsections, we will discuss the biological role of oxygen radicals and related species, including nitrogen radicals and other reactive oxygen/nitrogen nonradical species (Table 12.1). The literature often calls

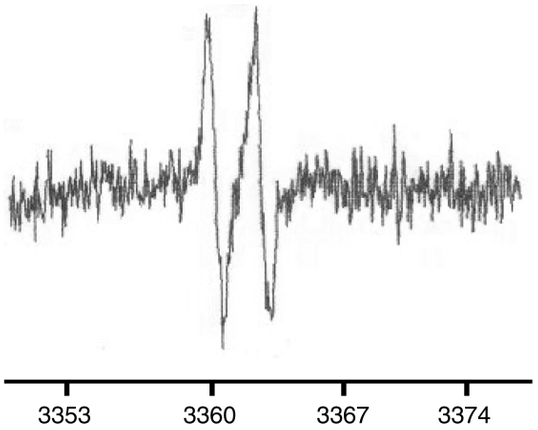

Figure 12.1 A typical two-lined ESR spectra of ascorbyl radical. The spectral data was obtained in an associated Brazilian laboratory after incubation of 5 μM Fe(III)–EDTA with 1 mM ascorbate, in neutral pH. Values on the *x*-axis represent the magnetic field (in Gauss).
Note: All illustrations in this chapter were prepared by Guilherme Pintarelli, University of Brasilia Medical School.

TABLE 12.1 Reactive Oxygen and Nitrogen Species (ROS and RNS)

Radicals	Nonradicals
Superoxide (O_2^{-})	Hydrogen peroxide (H_2O_2)
Hydroperoxyl ($HOO^{\bullet}$)	Alkyl hydroperoxides (LOOH)
Peroxyl ($LOO^{\bullet}$)	Singlet oxygen ($^1O_2$)
Alkoxyl ($LO^{\bullet}$)	Ozone (O_3)
Hydroxyl ($^{\bullet}OH$)	Hypochlorous acid (HOCl)
Nitric oxide (nitrogen monoxide; $^{\bullet}NO$)	Peroxynitrite ($ONOO^{-}$)
Nitrogen dioxide ($^{\bullet}NO_2$)	

these species *reactive oxygen species* (ROS), a term that often includes nitric oxide, peroxynitrite, and other reactive nitrogen species (RNS). For the present discussion, we have decided to differentiate between ROS and RNS for didactic purposes (e.g., nitric oxide is considered an RNS in this chapter). The biological roles of sulfur- and carbon-centered radicals are also discussed in Sections 12.2 and 12.3.

12.1.2 Reactive Oxygen Species

Oxygen gas, which is also known as dioxygen or diatomic oxygen, is a free radical species. In the ground state it contains two unpaired electrons located in different π-antibonding orbitals; these electrons have parallel spins ($\uparrow\uparrow$). The spin restriction rule makes it difficult for oxygen, in the absence of a catalyst, to receive a pair of electrons with parallel spins when oxidizing a molecule. Thus, oxygen must receive one electron at a time.

Several biologically relevant donors (Table 12.2) are able to induce one-electron reduction of oxygen, yielding another radical species: the superoxide ion radical ($O_2^{-\bullet}$). Superoxide is usually represented in the literature without the dot (O_2^-), and we will do the same. Further one-electron reduction reactions yield hydrogen peroxide (H_2O_2, a nonradical species), hydroxyl radical ($^\bullet OH$), and finally hydroxy ion (represented below as OH^-, *which is not the same as* $^\bullet OH$):

$$O_2 \underset{e^-}{\rightarrow} O_2^- + 2H^+ \underset{e^-}{\rightarrow} H_2O_2 \underset{e^-}{\rightarrow} OH^- + {}^\bullet OH \underset{e^-}{\rightarrow} OH^- \quad (12.1.8)$$

12.1.2.1 Singlet Oxygen and Ozone Ground state oxygen can also be converted to singlet oxygen ($^1O_2$) by interaction with triplet-excited molecules, such as excited-protoporphyrin IX. Singlet oxygen is a relatively long-lived (microseconds) nonradical species with outer electrons in antiparallel spins ($\uparrow\downarrow$). Due to the lack of spin restriction $^1O_2$ has high oxidizing power and is able to attack membrane PUFAs, amino acid residues in proteins, deoxiribonucleic acid (DNA), and carotenoids. Skin damage in some types of porphyrias is attributed to the oxidizing effects of $^1O_2$ (see Text Box 13.3). Measurement of light emission from the decay of $^1O_2$ (e.g., at 1270 nm in the infrared region) is often used to quantify $^1O_2$.

Ozone is also a nonradical triatomic species. In the high atmosphere, ozone is Earth's protector against ultraviolet (UV) radiation. However, it is also formed in urban smog and is extremely reactive and a lung poison; notably, some large cities like São Paulo, Brazil, have ozone meters along with normal traffic signs! The $E^{0\prime}$ value of ozone makes it a powerful oxidizing agent that can react with proteins, DNA, PUFAs (see Section 12.3.1), and with small antioxidants, such as vitamin C and uric acid [reaction (12.1.9)]:

$$O_3 + \text{target} \rightarrow O_3^{-\bullet} + \text{oxidized target} \quad (E^{0\prime} = +0.89\ \text{V}) \quad (12.1.9)$$

Ozone is also highly toxic to crops and forests. Interestingly, transgenic tobacco plants overexpressing superoxide dismutase, an antioxidant enzyme (see Section 12.2.1), in chloroplasts have high resistance to O_3.

12.1.2.2 Superoxide and Hydrogen Peroxide About 1 to 4% of all oxygen consumed by vertebrates (and possibly by higher invertebrates) produces O_2^- radicals. Mitochondria are the main site of O_2^- production in cells, by means of the "leaky" electron transport system (see details in

TABLE 12.2 Endogenous Sources of Superoxide and Hydrogen Peroxide

Superoxide
Autoxidation of small molecules[b] (e.g., adrenalin, noradrenalin, cysteine, reduced pteridines, L-DOPA,[b] dopamine,[b] 5-aminolevulinic acid[b])
Autoxidation of aqueous Fe^{2+} and certain ferrous iron complexes (e.g., Fe^{2+}–citrate, Fe^{2+}–ATP, Fe^{2+}–ADP)
Autoxidation of oxyhemoglobin, oxymyoglobin, and *Escherichia coli* flavohemoglobin
Mitochondrial electron chain (at the ubiquinone and NADH-dehydrogenase sites)[b]
Cytochrome P450 system of endoplasmic reticulum
Electron chain of chloroplast (at photosystems I and ferredoxin sites)
NADPH oxidase of phagocytes and some endothelial cells[b]
Xanthine oxidase,[b] aldehyde oxidase, and fungi galactose oxidase
Cyclooxygenases and lipoxygenases[c]
Hydrogen Peroxide
Dismutation of O_2^- and/or $HOO^\bullet$ (spontaneous or catalyzed by SOD)
Microbial glucose oxidase and glycoate oxidase from plant peroxisomes
Other peroxisomal oxidases (e.g., amino acid oxidase, urate oxidase)
Amine oxidases (e.g., monoamine oxidase, diamine oxidase, lysyl oxidase)

[a]These reactions are usually catalyzed by transition metals.
[b]See Chapter 13 for more details.
[c]See article by Kühn and Borchert (*Free Radic Biol Med* **33**:154–172, 2002) for more details.

Chapter 13); other sources are listed in Table 12.2. However, there is concern that the estimates of mitochondrial O_2^- generation (mostly done *in vitro* with isolated mitochondria) are about 10-fold higher than the amount of O_2^- formed *in vivo*. In any case, even if "only" 0.1 to 0.4% of consumed oxygen produces O_2^-, it is still an enormous production of free radicals.

Superoxide is a good example of a free radical species that can act as either an oxidizing [it can oxidize the reduced form of nicotinamide adenine dinucleotide (NADH) to the oxidized form of NAD (NAD^+)] or reducing agent, the later action being much more relevant in biology. Superoxide is able to reduce Fe^{3+} bound to citrate [reaction (12.1.10)], cytochrome C, ferritin (an iron storage protein; see Text Box 12.1), and enzymes containing iron–sulfur clusters (such as aconitase), but not iron bound to transferrin (a plasma iron transporting protein; see Text Box 12.1). The rates of reaction of O_2^- with lipids, proteins, and DNA are too slow to have biological significance:

$$Fe^{3+} - \text{citrate} + O_2^- \rightarrow Fe^{2+} - \text{citrate} + O_2 \quad (12.1.10)$$

The protonated form of superoxide, hydroperoxyl radical ($HOO^\bullet$; also represented in the literature as $HO_2^\bullet$), has a lower reduction potential than O_2^- [reactions (12.1.11a) and (12.1.11b)] and is able to abstract hydrogen from PUFAs. The pK_a value of $HOO^\bullet$ is 4.8 and the acid microenvironment near biological membranes favors its formation:

$$O_2 + e^- \rightarrow O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \quad (E^{0\prime} = -0.33 \text{ and } +0.94\,\text{V, respectively}) \quad (12.1.11a)$$

$$O_2 + e^- + H^+ \rightarrow HOO^\bullet + e^- + H^+ \rightarrow H_2O_2 \quad (E^{0\prime} = -0.46 \text{ and } +1.06\,\text{V, respectively}) \quad (12.1.11b)$$

Moreover, the reaction of O_2^- with "free Fe^{3+}" yields a perferryl intermediate [reaction (12.1.12)], which may react with PUFAs and induce lipid peroxidation. Superoxide also reacts with nitric oxide at a very high rate and produce peroxynitrite (see Section 12.1.3):

$$Fe^{3+} + O_2^- \leftrightarrow [Fe^{2+} - O_2 \leftrightarrow Fe^{3+} - O_2^-] \leftrightarrow Fe^{2+} + O_2 \quad (12.1.12)$$

One electron reduction of either O_2^- or $HOO^\bullet$ yields H_2O_2 [reactions (12.1.11a) and (12.1.11b)]. Dismutation of superoxide [reaction (12.1.13)], either spontaneous or catalyzed by the enzyme superoxide dismutase (SOD; see

TEXT BOX 12.1 IRON IN HEALTH AND DISEASE*

Iron is a relatively abundant element on our planet and is vital for almost all known organisms. In humans, iron is present at 35 to 50 mg/kg of body mass. It plays a role in important cellular processes such as DNA synthesis, electron transport, and oxygen transport. Iron is a component of many cellular proteins and enzymes, including hemoglobin (containing 67% of the body iron), myoglobin, catalase, peroxidase, cytochromes, and nitric oxide synthase. It also influences its own homeostasis by regulating the expression of iron-metabolism-related proteins in response to changes in intracellular iron concentration. The wide usefulness of iron in metabolism is due to its redox chemistry: the capacity for donating or accepting electrons in the ferric (Fe^{+3}) or ferrous (Fe^{+2}) oxidation states (iron ions are free radicals since they contain unpaired electrons in the 3*d* orbital). However, this capability can also be harmful because it promotes the generation of oxygen radicals via the Fenton reaction [see reaction (12.1.16)] or via autoxidation reactions. Because iron is a crucial element in multiple cellular processes, a closely regulated balance must be maintained between iron uptake, transport, utilization, and storage.

Iron is taken up from the diet in the form of both heme and nonheme iron. Heme iron is internalized via a heme-receptor process at the cell surface of absorptive enterocytes. Within the cell, heme degradation occurs via a heme oxygenase, releasing inorganic iron to be captured by the ferritin storage protein or transported across the basolateral membrane into the plasma. In the human intestine, the absorption of nonheme inorganic iron is more efficient as the ferrous iron. A mucosal ferrireductase enhances the availability of ferrous iron, which is absorbed at the apical enterocyte membrane by a divalent cation transporter, termed Nramp2. Ferric iron also can be internalized by a membrane complex called paraferritin with subsequent reduction intracellularly.

Iron that is diverted to storage is complexed with ferritin, a 450-kDa protein that can store up to 4500 atoms of iron. The synthesis of ferritin responds to intracellular iron concentration, high iron concentration inducing ferritin expression whereas low iron concentration inhibits expression. This iron-mediated feedback of ferritin expression is regulated, not at the transcriptional level but by control over mRNA translation, as are many

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other proteins involved in iron metabolism (e.g., transferrin receptor, Nramp2, ferritin, and 5-aminolevulinic acid synthase). Two elements are crucial to this process, namely iron response elements (IRE) and iron regulatory proteins (IRP).

Iron response elements are stem-loop structures, located in the 5′ (e.g., ferritin) or 3′ (e.g., transferrin receptor) untranslationed regions of mRNA transcripts encoding proteins of iron metabolism. These structures serve as a binding site for IRP. Depending on the location of the IRE, at the 5′ or 3′ region, the binding complex IRE–IRP represses or enhances translation. In iron-deficiency status, the binding of IRP to the IRE in the 5′ region of ferritin mRNA blocks its translation. On the other hand, the binding of IRP to the IRE in the 3′ region of the transferrin receptor mRNA stabilizes the mRNA and enhances its translation to increase the number of transferrin receptors at the cell surface. In high intracellular iron status, IRP shows an aconitase activity (an enzyme of the tricarboxylic acid cycle) and does not bind to the IRE of ferritin mRNA; hence, translation of ferritin mRNA is enhanced. By contrast, transferrin receptor mRNA is probably degraded by an endonuclease, due to the lack of formation of an IRP–IRE complex.

Iron exits enterocytes via an iron transporter called ferroportin located at the basolateral membrane. The metal is transferred to the plasma protein, transferrin, an 80-kDa glycoprotein. The protein has two specific binding sites, occurring as monoferric or diferric transferrin, and its affinity for iron is pH dependent with low affinity at acidic pH. Ferrous iron exported into the plasma undergoes a ferroxidase reaction via the copper-containing oxidase ceruloplasmin. Uptake of iron from the plasma into other tissues is mediated through the binding of the transferrin–iron complex to the transferrin receptor, a dimeric glycoprotein of 80 kDa on the plasma membrane, followed by endocytosis. In the cytosol, the endosomal pH is reduced by a proton pump-mediated influx of H^+. Low pH causes iron to be released from the transferrin (still bound to its receptor) followed by export into the cytoplasm by the Nramp2 transporter where inorganic iron is again stored into ferritin or associated with a diverse group of ligands including organic anions (e.g., phosphates and carboxylates) and polypeptides.

A large number of diseases are accompanied by iron overload due to errors in iron metabolism. One such condition is hemochromatosis, an autosomal recessive iron overload disease where a mutation in the Hfe protein (the hemochromatosis protein that exerts an inhibitory effect on the transferrin receptor, causing increase in iron absorption) results in an accumulation of iron, mainly in the liver. Tissue iron accumulation can lead to morphological alterations, such as cirrhosis, probably due to the iron-mediated generation of ROS [for more details see Miret et al. (2003) *Annu. Rev. Nutr.* **23**:283–301]. Iron overload also occurs in other diseases such as β-thalassemia where a defect in the production of hemoglobin β-chains results in mild to severe anemia and chronic iron deposition in liver and heart. Other diseases result from defects in iron transport (e.g., aceruloplasminemia and atransferrinemia) and from secondary iron disorders, such as chronic inflammation-associated anemia. Iron is also involved in neurodegenerative pathologies, including Alzheimer's and Parkinson's diseases (see Chapter 13).

To avoid the deleterious effects of iron overload, drugs are used to reduce intracellular iron content. These drugs are called chelators (from the Greek *khele*, pincers) and they bind to metal ions to form, in some cases, an inert complex. An ideal iron chelator has an easy oral absorption, low production costs, a high and selective affinity for iron, low toxicity, the ability to cross membranes, and a high antioxidant capacity to inhibit metal-mediated oxyradical formation.

Deferoxamine is an iron chelator that is used clinically in the treatment of β-thalassemia and in some hemochromatosis patients. However, it has some disadvantages such as poor oral absorption, high costs, and a short half-life. Other compounds under evaluation for possible use in the treatment of iron overload diseases include L1 (1,2-dimethyl-3-hydroxypyrid-4-one; still highly controversial), certain plant polyphenols with metal chelating activity (see Text Box 12.4), and pyridoxal isonicotinoyl hydrazone (PIH). PIH was developed in 1970s by Prem Ponka (McGill University, Montreal) and is currently studied by our laboratory in Brazil. It is well-absorbed orally, economical, and effective in inhibiting *in vitro* lipid peroxidation, oxidative DNA damage, ascorbate oxidation, and 2-deoxyribose degradation through the formation of an inert complex of PIH with Fe^{3+} that does not catalyze oxyradical formation (see *Biochim Biophys Acta* **1620**:15–24, 2003). Further studies are needed to uncover other drugs (including new PIH analogs) that meet the requirements for an ideal iron chelator.

Section 12.2.1), is a main source of H_2O_2 *in vivo*. It is postulated that the fast reaction of O_2^- with $HOO^\bullet$ is a main source *in vivo* of noncatalyzed H_2O_2 formation [reaction (12.1.14); rate constant of $8 \times 10^7\ M^{-1}\ s^{-1}$]. Moreover, the speed of the dismutation reaction increases with the decrease in pH. The "overall" rate of spontaneous dismutation of superoxide is considered to be $4.5 \times 10^5\ M^{-1}\ s^{-1}$. Superoxide can also oxidize ascorbate, yielding H_2O_2 at a

rate constant of about $3 \times 10^5\ M^{-1}\ s^{-1}$ [reaction (12.1.15)]:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (12.1.13)$$

$$O_2^- + HOO^\bullet + H^+ \rightarrow H_2O_2 + O_2 \quad (12.1.14)$$

$$O_2^- + \text{ascorbate} \rightarrow \text{ascorbyl}^\bullet + H_2O_2 \quad (12.1.15)$$

Hydrogen peroxide is a relatively poor oxidizing agent, having an $E^{0\prime}$ value (+0.32 V for the pair $H_2O_2/{}^\bullet OH$) lower than ozone, $HOO^\bullet$, or superoxide. For example, H_2O_2 cannot abstract hydrogen from PUFAs. What makes H_2O_2 a potentially dangerous species is its ability to easily cross biological membranes and its high stability. Thus, H_2O_2 produced in mitochondria (which actually arises from O_2^- formation that is followed by dismutation into H_2O_2), for example, could move throughout the rest of the cell to produce effects in multiple other compartments such as in the nuclei. However, in the intracellular environment, the presence of many enzymatic and nonenzymatic processes of H_2O_2 decomposition (e.g., transition metals, selected enzymes, and small molecules) keeps H_2O_2 at a low concentration, ranging from 10^{-9} to 10^{-8} M (notably, the intracellular O_2^- concentration is even lower, at around 10^{-11} to 10^{-10} M).

12.1.2.3 Hydroxyl Radical Many of the oxidizing effects of H_2O_2 on DNA, lipids, and proteins that were observed in the past are now known to have been caused by the interaction of H_2O_2 with transition metals, mainly $Fe^{2+}$and Cu^+, yielding ${}^\bullet OH$ radicals [reaction (12.1.16) shows the *Fenton reaction*] or other highly reactive oxometallic species, such as ferryl ($Fe^{4+}{=}O$). One electron reduction of H_2O_2 splits the O–O bond, forming ${}^\bullet OH$ and OH^- [see reactions (12.1.8)]. Some small organic peroxides can also undergo Fenton reactions. Hydroxyl radical can also be formed by radiation-induced homolysis of water [see reaction (12.1.5)] or H_2O_2 (reaction 12.1.17), and by the reaction of hypochlorous acid with O_2^- [reaction (12.1.18)].

$$H_2O_2 + Fe^{2+}(\text{or } Cu^+) \rightarrow Fe^{3+}(\text{or } Cu^{2+}) + OH^- + {}^\bullet OH \quad (12.1.16)$$

$$H_2O_2 + \text{energy} \rightarrow 2{}^\bullet OH \quad (12.1.17)$$

$$HOCl + O_2^- \rightarrow Cl^- + O_2 + {}^\bullet OH \quad (12.1.18)$$

The oxidizing power of a mixture of H_2O_2 with Fe^{2+} salts (but not with Fe^{3+} salts) on tartaric acid was first reported in the form of a note by 22-year old Henry J.H. Fenton, who was still a Cambridge undergraduate student at that time (*Chemical News* **33**:190, 1876). Fenton never mentioned the formation of ${}^\bullet OH$ as the intermediate species in tartaric acid oxidation, even in later publications (see also: Fenton 1984), but nonetheless, the reaction was later named after him. The concept of ${}^\bullet OH$ radical production appeared only in the early 1930s, soon after Fenton's death. German chemists Haber and Weiss (*Proc R Soc Lond [A]* **147**:332–351, 1934) proposed that ${}^\bullet OH$ radicals are formed from the reaction of superoxide with H_2O_2 in the presence of iron. Rediscovery of these reactions, in connection with a biological role occurred only in the late 1970s.

It is widely accepted that the Fenton reaction may be the most relevant source of ${}^\bullet OH$ formation in biology. The reaction of H_2O_2 with aqueous Fe^{2+} is very slow (rate constant of $10^2\ M^{-1}\ s^{-1}$) and is considered of limited biological significance by many current authors. However, iron bound to low-molecular-weight compounds such as adenosine 5′-triphosphate (ATP) or citrate increases the reaction rate by two orders of magnitude. On the other hand, the Fenton reaction with copper salts proceeds at a rate constant of $5 \times 10^3\ M^{-1}\ s^{-1}$.

For the Fenton reaction to continue, Fe^{3+} must be converted back to Fe^{2+} by reducing agents such as O_2^- [see reactions (12.1.10) and (12.1.19b)] and ascorbate. The scheme below shows the so-called *Haber–Weiss reactions*:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^\bullet OH \quad (12.1.19a)$$

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (12.1.19b)$$

$$\overline{H_2O_2 + O_2^- \rightarrow O_2 + OH^- + {}^\bullet OH} \quad (12.1.19c)$$

Hydroxyl radical is one of the most reactive species known. It reacts with almost all kind of molecules with reaction rate constants ranging from 10^7 to $10^{10}\ M^{-1}\ s^{-1}$; indeed, about half of these rate constants are higher than $2 \times 10^9\ M^{-1}\ s^{-1}$. This is so fast that the reaction is controlled by the diffusion rate of ${}^\bullet OH$ (and the presence or absence of a specific target near the site of ${}^\bullet OH$ formation!). The $E^{0\prime}$ value for ${}^\bullet OH$ (+2.31 V) is also the highest among the biologically relevant free radicals (see Table 12.3).

Hydroxyl radicals are involved in the initiation of lipid peroxidation in biological membranes as well as oxidation and damage to proteins, nuclear DNA, and mitochondrial DNA (see Sections 12.3 to 12.5). Carbohydrates and ribonucleic acid (RNA) are also relevant targets of ${}^\bullet OH$. The ${}^\bullet OH$ radical participates in the etiology or worsening of multiple human diseases and pathological/stress conditions including cancer, neurological disorders, chronic inflammatory diseases, atherosclerosis, myocardial infarction, iron-overload diseases, and even muscle fatigue in extenuating exercise (see Chapter 13). Moreover, ${}^\bullet OH$ is also involved in the natural aging process by causing cumulative damage to DNA and proteins in cells.

TABLE 12.3 Standard Reduction Potentials ($E^{0\prime}$) of Selected Substances[a]

Half-cell	Volts	Half-cell	Volts
H_2O/hydrated electron (e_{aq}^-)	−2.87	Fe^{3+}/Fe^{2+} (aqueous)	0.11
$CO_2/CO_2^{\bullet -}$	−1.80	Fe^{3+}–EDTA/Fe^{2+}–EDTA	0.12
O_2, $H^+/HOO^\bullet$	−0.46[b]	$CoQH^\bullet$, $H^+/CoQH_2$ (ubiquinol)	0.20
Fe^{3+}–deferoxamine/Fe^{2+}–deferoxamine	−0.45	Ascorbyl$^\bullet$, H^+/ascorbate	0.28
Fe^{3+}–transferrin/Fe^{2+}–transferrin	−0.50[c]	H_2O_2, $H^+/{}^\bullet OH$, H_2O	0.32
$O_2/O_2^{-\bullet}$	−0.33,[b] −0.16[d]	Trolox$^\bullet$, H^+/Trolox	0.48
NAD^+, H^+/ NADH	−0.32	α-Toc$^\bullet$, H^+/α-TocH	0.50
Lipoic acid, $2H^+$/DHLA	−0.32[e]	Urate$^\bullet$, H^+/urate	0.59
GSSG, $2H^+$/2GSH	−0.24[e]	PUFA$^\bullet$/PUFA	0.60
Fe^{3+}–ferritin/Fe^{2+}–ferritin	−0.19	$O_3/O_3^{-\bullet}$	0.89
DHA/ascorbyl$^\bullet$	−0.17	$O_2^{-\bullet}$, $2H^+/H_2O_2$	0.94
CoQ (ubiquinone), $H^+/CoQH^\bullet$	−0.04	ROO$^\bullet$, H^+/ROOH	0.77–1.44[f]
		Fe^{3+}-(*o*-phen)$_3$/Fe^{2+}-(*o*-phen)$_3$	1.15
		HOO$^\bullet$, H^+/H_2O_2	1.06
		$^\bullet$OH, H^+/H_2O	2.31

[a]Standard reduction potentials at pH0 and 25°C (E^0) were recalculated for pH 7.0; these values are then $E^{0\prime}$. Basic instructions: Electrons tend to flow from the half-cell of a lower $E^{0\prime}$ to the half-cell of a higher $E^{0\prime}$. For example, it is predicted that the following reaction is possible: DHLA + GSSG → 2 GSH + lipoic acid (electrons flow from DHLA to GSSG). This is so because the $E^{0\prime}$ value of the pair lipoic acid/DHLA is −0.32 V and the $E^{0\prime}$ value for GSSG/2 GSH is −0.24 V.
[b]Calculated at 1 atm of oxygen.
[c]Currently accepted value.
[d]Calculated using an aqueous oxygen concentration of 1 M.
[e]Two-electron reduction.
[f]$E^{0\prime}$ value depends on the type of peroxyl radical.
Source: Data from Garry R. Buettner (1993). *Arch Biochem Biophys* **300**:535–543.

12.1.2.4 Reactive Oxygen Species Formed from PUFAs The process of lipid peroxidation (see Section 12.3 for details) results in the formation of three important reactive oxygen intermediates from PUFAs: alkyl hydroperoxides (LOOH), alkyl peroxyl radicals (LOO$^\bullet$), and alkoxyl radicals (LO$^\bullet$).

As in the case of H_2O_2, alkyl hydroperoxides are not radical species, but they are unstable in the presence of transition metals. Their decomposition causes the formation of an array of different molecules, including aldehydes and alkanes. Some antioxidant enzymes are involved in the conversion of alkyl hydroperoxides into their respective alcohols; these include glutathione peroxidases, glutathione *S*-transferases, and alkyl peroxidases (see Section 12.2 for details).

Alkyl peroxyl radicals (and alkoxyl radicals) are very reactive species and also participate in the process of propagation of lipid peroxidation. The $E^{0\prime}$ values for the various LOO$^\bullet$ species vary between +0.77 and +1.44 V, which is the range of the $E^{0\prime}$ values for HOO$^\bullet$, ozone, and superoxide. Vitamin E is a very important biological reducing agent for alkyl peroxyl radicals (converting LOO$^\bullet$ to LOOH); however, it reacts very slowly with LO$^\bullet$ species (see Sections 12.2.2.3 and 12.3.4). Other biologically important organic peroxides (and peroxyl radicals as well) not derived from PUFAs include those produced from thymine, cholesterol, and certain amino acid residues.

12.1.2.5 Quantitative Determination of O_2^-, H_2O_2, and $^\bullet OH$ Determination of ROS in simple chemical systems or in complex biological preparations can be done in various ways. Some techniques are supposed to be specific for certain species, such as $^\bullet$OH, but there is wide disagreement about this. Assays based on ESR techniques are of great value for the detection of O_2^- and $^\bullet$OH and will be discussed later in this section.

Superoxide radicals, when generated in high quantities from pulse radiolysis or from KO_2 salt, and in simple chemical/biochemical systems, can be detected by UV absorption at 245 nm (whereas HOO$^\bullet$ absorbs at 225 nm). Several molecules can also be used to indirectly quantify O_2^- spectrophotometrically, including cytochrome C (the ferric form is reduced by O_2^-, measured at 550 nm), acetylated cytochrome C (a way to avoid interference from cytochrome oxidase), adrenalin (oxidized by O_2^- to form adrenochrome), and nitroblue tetrazolium (NBT; reduced by O_2^- to the blue-colored formazan). These are the traditional or popular reagents used. A

problem with the use of NBT and adrenalin is that both can produce O_2^- when they are subjected to ROS attack. Ferric-cytochrome C (as well as acetylated cytochrome C) can also be reduced by other molecules that might be present in the tissue extracts, such as ascorbate. Thus, addition of purified CuZn–SOD to the reaction mixture is a necessary control to determine whether reduction of the target molecule is really due to O_2^-. Quantification of light emission from luminol (after reaction with O_2^-) is also a popular technique, but other ROS can also cause light emission from luminol. Formation of formazan precipitates can also be useful for histochemical determination of O_2^-.

Determination of H_2O_2 can be done by various ways. At millimolar concentrations, H_2O_2 can be directly measured at 240 nm or by reaction with $KMnO_4$. In simple chemical systems, it can also be measured in micromolar concentrations by an oxygen electrode after the addition of catalase (which decomposes H_2O_2 to H_2O and O_2). Spectrophotometric or fluorometric methods employing horseradish peroxidase (HRP) plus a substrate that can be oxidized by H_2O_2 (such as scopoletin) are also very common.

In another method, 2′,7′-dichlorofluorescin diacetate can be enzymatically deacetylated to 2′,7′-dichlorofluorescin (DFCH) and then oxidatively converted to the fluorescent compound 2′,7′-dichlorofluorescein DFC; (excitation at 488 nm, emission at 525 nm). Conversion of DFCH to DFC can be done by HRP plus H_2O_2 or by direct reaction with strong oxidants such as peroxynitrite (see below), HOCl, or $^{\bullet}OH$. DFC imaging techniques became popular in the late 1990s for overall determination of oxidative stress in cell culture.

Determination of $^{\bullet}OH$ can be made through hydroxylation reactions, such as the fluorometric determination of benzoate hydroxylation (good for *in vitro* assays) or high-pressure ligand chromatography (HPLC) quantification of salicylate hydroxylation. The latter is often used for *in vivo* studies, where hydroxylation products (2,3- and 2,5-dihydroxybenzoate and catechol) can be detected in blood or urine. Ortho-tyrosine (as well as the *meta*- and *para*-isomers) is a product of $^{\bullet}OH$ attack on phenylalanine residues; *o*-tyrosine is a "classic" biomarker of protein oxidation under oxidative stress conditions (see Section 12.4).

The reaction of $^{\bullet}OH$ reaction with dimethyl sulfoxide (DMSO) (forming methanesulfinic acid and $^{\bullet}CH_3$) and 2-deoxyribose (producing malondialdehyde) has been used for the development of useful colorimetric techniques. However, other ROS (such as ferryl and other $^{\bullet}OH$-like species) are also capable of oxidizing DMSO and 2-deoxyribose; and, therefore, these assays cannot be viewed as specific for $^{\bullet}OH$ detection. Despite this, the 2-deoxyribose assay is still the most popular spectrophotometric assay for $^{\bullet}OH$ (and for the study of antioxidants that react with $^{\bullet}OH$) due to its simplicity and low cost. The product of the reaction, malondialdehyde, forms a pink adduct with thiobarbituric acid (TBA) that is measurable at 532 nm.

Determination of $^{\bullet}OH$ by quantifying the hydroxylation of the nitroso compound DMPO (at 10 to 100 mM) in ESR experiments is a very popular approach. The DMPO–OH radical molecule, with a typical four-lined ESR signal, is reasonably stable for several minutes, making it good for quantification of $^{\bullet}OH$ formation, mostly for *in vitro* studies. However, other reactive species may also produce DMPO–OH and thus the method cannot be considered as specific for $^{\bullet}OH$ detection (although mechanisms exist to confirm $^{\bullet}OH$ involvement). Superoxide radicals also react with DMPO to form a DMPO–OOH adduct, also with a well-defined ESR signal. However, the DMPO–OOH adduct decays quickly (within 1 to 2 min) to DMPO–OH, which needs to be taken into account when interpreting results. Another nitroso compound that is frequently used for $^{\bullet}OH$ quantification is PBN. The low toxicity of PBN makes it useful for *in vivo* studies.

12.1.3 Reactive Nitrogen Species

Nitric oxide is a gas (and a free radical species) that was identified in the 1980s as the endothelium-derived relaxing factor (EDRF). The relaxation of vascular smooth muscle cells by $^{\bullet}NO$, which is produced in nearby endothelial cells, was the very first function of $^{\bullet}NO$ to be discovered. Nitric oxide interacts with the heme prosthetic group of the soluble guanylate cyclase, prompting cyclic guanosine 5′-monophosphate (cGMP) formation and activating cGMP-dependent ion channels and kinases. Other functions of $^{\bullet}NO$ are discussed elsewhere in this book (see Section 12.6 and Chapters 4 and 6).

Nitric oxide synthase (NOS) catalyzes the formation of $^{\bullet}NO$. There are three classic homodimeric isoforms of NOS, named after the tissues/conditions where they were characterized and cloned: neuronal NOS (nNOS, or NOS1), inducible NOS (iNOS, or NOS2), and endothelial NOS (eNOS, or NOS3). These isozymes use the same cosubstrates—oxygen and NADPH (reduced nicotinamide adenine dinucleotide phosphate) [reaction (12.1.20)]—and the same cofactors [flavin adenine dinucleotide (FAD), tetrahydrobiopterin, heme, and calcium/calmodulin] but differ with respect to their mode of regulation and tissue expression. NOS2 has transcriptional and post-transcriptional inducibility and regulation of messenger RNA (mRNA) levels by cytokines; cytokines also mediate the mRNA expression of NOS1 and NOS3. Moreover, very recent evidence has indicated the existance of a fourth type of NOS, the mitochondrial one (mtNOS;

see Lacza et al. *Free Radic Biol Med* **35**:1217–1228, 2003).

$$\text{L-arginine} + \text{NADPH} + \text{O}_2 \rightarrow \text{L-citrulline} + {}^{\bullet}\text{NO} + \text{NADP}^{+} \quad (12.1.20)$$

Nitric oxide contains an unpaired electron in the last orbital, making it a free radical molecule. It reacts relatively slowly with O_2 producing the orange-brown gas nitrogen dioxide ($^{\bullet}NO_2$), a very reactive radical species [reaction (12.1.21)]. Further reactions of $^{\bullet}NO$ with $^{\bullet}NO_2$ will eventually produce nitrite (NO_2^-), which is the major decomposition product of $^{\bullet}NO$ [reactions (12.1.22) and (12.1.23)]. The calculated half-life of $^{\bullet}NO$ due to its reaction with O_2 is about 10 min and 15 h at 1 and 0.01 μM, respectively (monitoring of $^{\bullet}NO$ can be done with specific electrodes). The facts that $^{\bullet}NO$ does not have net charge and is high permeable across biomembranes makes it a good signal transduction molecule.

$$2\,{}^{\bullet}\text{NO} + \text{O}_2 \rightarrow 2\,{}^{\bullet}\text{NO}_2 \quad (12.1.21)$$

$${}^{\bullet}\text{NO} + {}^{\bullet}\text{NO}_2 \leftrightarrow \text{N}_2\text{O}_3 \quad (12.1.22)$$

$$\text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow 2\text{NO}_2^- + 2\text{H}^+ \quad (12.1.23)$$

$${}^{\bullet}\text{NO} + \text{O}_2^- \rightarrow \text{ONOO}^- \quad (12.1.24)$$

Even though $^{\bullet}NO$ is quite stable under certain conditions *in vitro*, it disappears within seconds *in vivo*. Nitric oxide reacts very quickly with the heme group of hemoglobin (see Chapter 18); it also reacts with O_2^- at near diffusion rates ($6.7 \times 10^9\ M^{-1}\ s^{-1}$), yielding peroxynitrite [O═NOO$^-$, represented usually as ONOO$^-$; reaction (12.1.24)]. It is noteworthy that $^{\bullet}NO$ controls the intra- and the extracellular concentrations of O_2^- (by forming peroxynitrite) and vice versa. The antioxidant enzyme superoxide dismutase (see Section 12.2.1.2) plays a key role in extending the physiological lifetime of $^{\bullet}NO$ by limiting the amount of O_2^- available to react with $^{\bullet}NO$.

Peroxynitrite is a highly stable and toxic nonradical anion. Molar concentrations at pH 13 can be stored for days in the refrigerator. Moreover, peroxynitrite-mediated oxidations can be mediated by both ground state peroxynitrous acid (ONOOH) and by the activated form of the acid (ONOOH*). (*Note*: The asterisk in ONOOH* means that the molecule contains electrons in the activated state rather than in the ground state.) The base (ONOO$^-$) is also a powerful nucleophile. It has been estimated that about 20% of peroxynitrite is in the acid form at pH 7.4; ONOOH (and/or ONOOH*) is highly reactive, with an $E^{0\prime}$ value (+2.10 V) approaching that of $^{\bullet}OH$. Peroxynitrite (and/or the acid form) is able to oxidize most biological molecules including DNA, RNA, proteins, and lipids.

Most of the damaging effects that were attributed to $^{\bullet}NO$ in the early 1990s are now attributed to peroxynitrite and/or $^{\bullet}NO_2$. Important biological markers of RNS action include 3-nitrotyrosine (a nitration product of tyrosine; see Section 12.4) and 8-nitroguanine (nitration product of the DNA base guanine; see Section 12.5).

Nitrosothiols (or thionitrates) are other important nonradical RNS. They are found in plasma at about 1 μM concentrations (mostly as *S*-nitrosoalbumin and nitrosohemoglobin). For example, the reaction of the radical form of glutathione (a thiyl radical; RS$^{\bullet}$) with $^{\bullet}NO$ yields *S*-nitrosoglutathione, a molecule with signaling properties. It is assumed that N_2O_3 or peroxynitrite mediate the formation of nitrosothiols.

$${}^{\bullet}\text{NO} + \text{RS}^{\bullet} \rightarrow \text{RSNO} \quad (12.1.25)$$

Some synthetic organic nitrates such as linsidomine (SIN-1) and the nitrosothiol *S*-nitroso-*N*-acetylpenicillamine (SNAP) are import $^{\bullet}NO$ donors. These molecules are often used in experiments to study $^{\bullet}NO$ effects. In the case of SIN-1, it is now clear that peroxynitrite is also formed in the reaction media due to autoxidation of SIN-1 followed by O_2^- formation and reaction with $^{\bullet}NO$. Thus, addition of SOD to the reaction media may enhance the yield of $^{\bullet}NO$.

12.2 ANTIOXIDANT DEFENSES

All aerobic living forms have defenses against ROS. Even various obligate anaerobe bacteria, which are not usually exposed to oxygen, have endogenous defenses against oxygen toxicity. Oxygen accumulation in Earth's primitive atmosphere and bodies of water may have provided a strong selection pressure for organisms with capacities to utilize oxygen in their energy metabolism as well as organisms that were capable of handling the toxic by-products of oxygen-based metabolism. On the other hand (as proposed by Denham Harman, University of Nebraska; *Proc Natl Acad Sci USA* **78**:7124–7128, 1981), too much protection by antioxidants on the ancient Earth would not have been favorable for evolution because it would have prevented the oxidative alterations to DNA that led to mutations and eventually to more highly developed life-forms.

Currently, existing life-forms are equipped with a very complex system for the control of free radical damage to cell constituents. The key importance of antioxidant defenses and their ancient origin is emphasized by the fact that some of the enzymatic defense systems are incredibly conserved, with strong conservation of amino acid sequences across wide phylogenetic ranges, from bacteria and humans.

The antioxidant defense systems of living forms can be divided into four subclasses:

1. Primary antioxidant defenses, of enzymatic or nonenzymatic nature, that deal directly with oxygen reactive species.
2. Auxiliary defenses that support the function of the primary antioxidant system (e.g., by recycling or synthesizing substrates of antioxidant enzymes).
3. Metal-complexing proteins/enzymes (e.g., ferritin, transferrin, ceruloplasmin, metallothionein) and low-molecular-weight compounds that prevent or minimize the participation of iron or copper (and other heavy metals) in free radical generation (see Text Box 12.1).
4. Enzymatic repair systems that repair biomolecules damaged by ROS and RNS. This last group of defenses consists mostly of a large array of enzymes that repair oxidized DNA (see Section 12.5). Moreover, a few newly discovered enzymes also provide some degree of repair to oxidized proteins (see Sections 12.2.1.5 and 12.4.2).

Most nonenzymatic antioxidant defenses are not synthesized endogenously by animals but must be provided by their diet. The plant and bacterial world has provided an incredible number of different substances (of dietary relevance or not) with potential antioxidant activity *in vivo* (plant phenols and carotenoids are the main groups of compounds), even though many substances also display prooxidant activity *in vitro*. The role of endogenous/dietary antioxidants in biology and medicine will be discussed in Section 12.2.2.

Several metals and other nonmetal elements (such as selenium) can also be considered as part of the antioxidant defense system. They are constitutive cofactors of several antioxidant enzymes (see Section 12.2.1) and of numerous other metabolic enzymes that keep the organisms alive. For example, in humans, copper is essential for ceruloplasmin and for a class of superoxide dismutase (CuZn–SOD; zinc is also crucial for this enzyme). Iron and manganese are, respectively, constituents of catalase and of the mitochondrial form of SOD (Mn–SOD) whereas selenium is crucial for several antioxidant enzymes. Iron and copper, in particular, are both essential and detrimental (as catalysts of ROS formation) for life (see Text Box 12.1).

12.2.1 Enzymatic Antioxidants, Glutathione, and Thioredoxin

The main enzymatic defenses against ROS include superoxide dismutase (several SOD forms exist), catalase, and selenium-dependent glutathione peroxidase (four GPx forms exist). SOD catalyzes the dismutation of O_2^- into O_2 and H_2O_2 [reaction (12.2.1)]. Catalase has a major role in the decomposition of H_2O_2 [reaction (12.2.2)], as does selenium-dependent GPx, which catalyzes the decomposition of H_2O_2 and also of organic hydroperoxides using the tripeptide glutathione in its reduced form (GSH) as a cosubstrate [reactions (12.2.3) and (12.2.4)]. Current evidence indicates that selenium-dependent GPx and another small protein thioredoxin (Trx, Section 12.2.1.5) are able to decompose peroxynitrite ($ONOO^-$).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (12.2.1)$$

$$2H_2O_2 \rightarrow H_2O + O_2 \quad (12.2.2)$$

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad (12.2.3)$$

$$L\text{–}OOH + 2GSH \rightarrow GSSG + L\text{–}OH + H_2O \quad (12.2.4)$$

Other peroxidases have relevant actions in cellular H_2O_2 detoxification, including ascorbate peroxidase (a hemeprotein of 28 to 34 kDa from plants, particularly abundant in chloroplasts) and cytochrome c peroxidase (CcP; from bacteria, fungi, and some trematode worms) [reactions (12.2.5) and (12.2.6)]. Plants do not have GPx. Glutathione *S*-transferases (GSTs) catalyze the conjugation of GSH with xenobiotics [reaction (12.2.7)], including aldehydes produced during lipid peroxidation (see Section 12.3), and they also display selenium-independent GPx activity toward organic hydroperoxides [reaction (12.2.4)]. Alkyl peroxidases [reaction (12.2.8)] and thioredoxin peroxidases (see Section 12.2.3.5) also participate in the defense against organic peroxides.

$$H_2O_2 + \text{ascorbate} \rightarrow \text{dehydroascorbate} + 2H_2O \quad (12.2.5)$$

$$H_2O_2 + Fe^{2+}\text{–cythocrome } c \rightarrow Fe^{3+}\text{–cythocrome } c + 2H_2O \quad (12.2.6)$$

$$GSH + \text{xenobiotic} \rightarrow GS - \text{xenobiotic} \quad (12.2.7)$$

$$L\text{–}OOH + NAD(P)H + H^+ \rightarrow L\text{–}OH + H_2O + NAD(P)^+ \quad (12.2.8)$$

Several auxiliary enzymes are also involved in antioxidant defense. Glutathione reductase (a FAD-containing enzyme widely distributed in animals, plants, and microorganisms) functions to recycle glutathione, converting the oxidized form of glutathione (glutathione disulfide; GSSG) back to GSH using the reducing power of NADPH [reaction (12.2.9)]. Hexose monophosphate shunt enzymes including glucose-6-phosphate dehydrogenase [G6PDH; reaction (12.2.10)] and 6-phosphogluconate

dehydrogenase are major suppliers of the necessary NAPDH.

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (12.2.9)$$

$$NADP^+ + \text{glucose-6-P} \rightarrow NADPH + H^+ + \text{6-phosphogluconate} \quad (12.2.10)$$

Ascorbate recycling is catalyzed by NAD(P)H-dependent ascorbate free radical reductase, a flavoenzyme present in plants, algae, fungi, and animals (this enzyme is highly active in kidney and liver of many vertebrates) [reaction (12.2.11)] and by dehydroascorbate (DHA) reductase. DHA reductase is a 23 to 28-kDa monomeric plant enzyme present in both chloroplasts and cytosol that uses the reducing power of GSH [reaction (12.2.12)].

$$NAD(P)H + H^+ + \text{ascorbyl}^\bullet \rightarrow \text{ascorbate} + NAD(P)^+ \quad (12.2.11)$$

$$2GSH + DHA \rightarrow \text{ascorbate} + GSSG \quad (12.2.12)$$

In addition, other enzymes function in the biosynthesis of GSH and in the recycling of sulfhydryl protein mixed disulfides, the latter by the thioredoxin system. These auxiliary defenses will be discussed in Section 12.2.1.5.

Moreover, the isoforms of heme oxygenase, HO-1, has been recently demonstrated to be of key relevance for antioxidant protection in cells. Heme oxygenase is the rate-limiting enzyme in heme catabolism leading to the generation of biliverdin, carbon monoxide, and free iron. HO-1 is a stress-responsive protein induced by various oxidant agents. An increase in HO-1 activity in cells generally occurs together with an increase in ferritin levels (an iron storage protein, see Text Box 12.1), which is stimulated by iron release from heme degradation.

The enzymatic antioxidant system works in a concerted way (Fig. 12.2). If, for example, there is an acute increase in H_2O_2 formation in a mammalian cell, GPx and/or catalase will decompose the peroxide to lower H_2O_2 to the previous steady-state concentration. However, if overformation of H_2O_2 is a chronic condition, where a higher steady-state concentration of H_2O_2 is established, then cells will increase the biosynthesis of H_2O_2-decomposing enzymes (GPx and/or catalase) to increase the capacity for H_2O_2 decomposition. Cells can also increase the biosynthesis of GSH, which can act as an antioxidant on its own in addition to its role as a cosubstrate of GPx and GST. These defense actions prevent the accumulation of oxidative damage to cell components, the consequences of which are highly variable in different cell types. For example, increased H_2O_2 formation can mediate $^\bullet OH$ formation (by metal-catalyzed reactions), which may

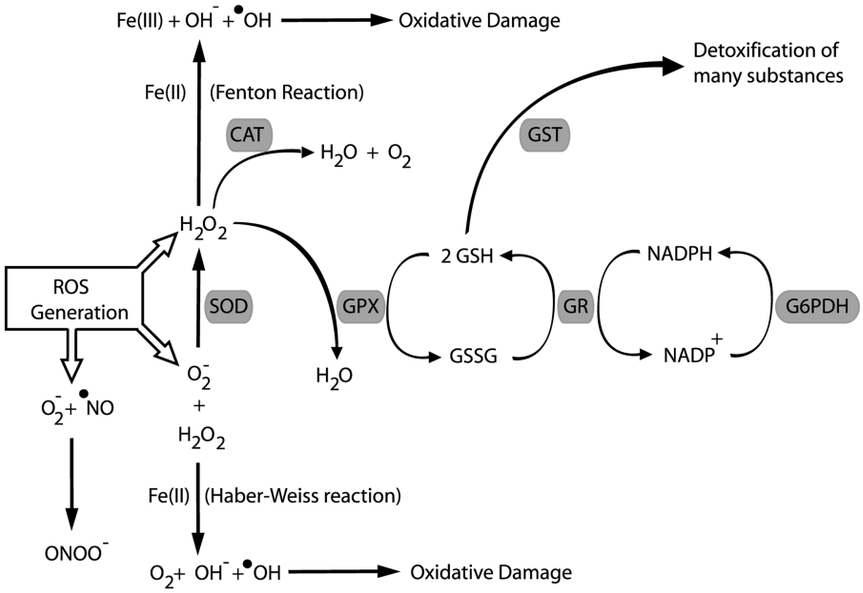

Figure 12.2 Enzymatic antioxidant defenses work in concert to protect cells against reactive oxygen species. The abbreviations SOD, CAT, GST, GR, GPX, and G6PDH represent the enzymes superoxide dismutase, catalase, glutathione *S*-transferase, glutathione reductase, glutathione peroxidase and glucose-6-phosphate dehydrogenase, respectively.

damage many kinds of biomolecules. Moreover, increased O_2^- formation (or exposure to O_2^--generating compounds) might prompt a response of the different SOD isoforms to control superoxide-mediated Fe^{3+} conversion to Fe^{2+} or peroxynitrite formation (upon reaction of O_2^- and $^\bullet NO$; Section 12.1) [see reactions (12.2.13)]:

$$O_2^- \rightarrow \text{dismutation to } H_2O_2 \qquad (12.2.13a)$$

$$O_2^- \rightarrow \text{reaction with } Fe^{3+} \text{ or } {}^\bullet NO \qquad (12.2.13b)$$

Furthermore, formation of O_2^- and H_2O_2 (and also certain lipid hydroperoxides) can occur as a consequence of signal transduction pathways (see Section 12.6). In this case, enzymatic antioxidants are necessary to ensure that the redox signal is not maintained indefinitely.

In the following subsections we will focus on some key antioxidant enzymes (SOD, catalase, GPx, GST), as well as the glutathione and thioredoxin systems.

12.2.1.1 Superoxide Dismutase The superoxide dismutase family contains four different enzymes with important structural and distribution differences. The most striking difference among the family members is the metal bound to them: There are two forms of copper–zinc SOD, a manganese SOD and an iron SOD. A nickel-containing SOD was recently identified in the bacterium *Streptomyces coelicolor*, but description of this enzyme is outside of the scope of this text.

CuZn–SOD is a 32-kDa dimeric protein that was initially described as being restricted to the cytoplasm of eukaryotes (see Text Box 12.2). However, recently it has also been detected in lysosomes, peroxisomes, nuclei, and the mitochondrial intermembrane space. Virtually all multicellular organisms contain CuZn–SOD in all their tissues; the enzyme is also present in various species of bacteria, located in the periplasmic space.

CuZn–SOD accelerates spontaneous O_2^- dismutation at pH 7.0 by several orders of magnitude at neutral pH (from $4.5 \times 10^5\ M^{-1}\ s^{-1}$ without an enzymatic catalyst to $1.6 \times 10^9\ M^{-1}\ s^{-1}$ with the enzyme), allowing the organisms to effectively control the intracellular steady-state levels of O_2^-. The copper ions in CuZn–SOD work in the dismutation reaction by means of alternate oxidation and reduction [reactions (12.2.14a) and (12.2.14b)]. Each subunit contains one copper ion, and the metal interacts with the imidazole rings of four histidine residues. The zinc ion is important for the maintenance of the structure of the enzyme and is located close to the active site. Most of the enzyme surface is negatively charged except for the active site and a nearby "track" that is positively charged, attracting O_2^-. Inhibitors of CuZn–SOD activity include cyanide and diethyldithiocarbamate, a compound

TEXT BOX 12.2 DISCOVERY OF SOD AND ITS FUNCTIONS

CuZn–SOD was first isolated from bovine erythrocytes in the late 1930s by T. Mann and D. Keilin. The crystalline copper-containing protein with molecular weight of 34 kDa was called *hemocuprein*. However, no enzymatic activity could be detected. Two decades later it was purified from human erythrocytes and baptized *erythrocuprein*. Copper proteins of similar size and copper content were isolated from other tissues, including human brain and bovine liver but, still, no apparent enzymatic function was observed. It was suggested that these copper proteins served as metal stores.

In the same year that men landed on the moon, Prof. Irving Fridovich and his predoctoral trainee Joe McCord, from Duke University (North Carolina), published a paper in *The Journal of Biological Chemistry* (**244**:6049–6055, 1969) that was the starting point for a revolution in biology and medicine. The authors reported that "erythrocuprein" (renamed superoxide dismutase) was able to catalyze the conversion of O_2^- into H_2O_2 and O_2 [see reaction (TB12.1)]. Activity of SOD was also observed in several other organs, including heart, brain, and skeletal muscle.

In those days, the only known biological source of O_2^- was the xanthine oxidase reaction, but there was literature showing that O_2^- could be generated in oxygenated aqueous solutions under X-ray or γ-ray irradiation. The study of O_2^- was still relegated to "exotic" radiation chemists—with very little biological connection. The interesting thing was that it was well-known in the years following the Manhattan Project that certain ionizing radiation sources (including those of natural origin) could have tremendous damaging effects on the organisms but only a few scientists had connected irradiation with oxygen-mediated toxicity! (See Section 12.5.1.)

In Fridovich's words (from p. 6055 of the 1969 paper), the "abundance of superoxide dismutase activity in the variety of animal tissues assayed suggests that the enzyme might play a significant, even vital, role in protecting the organism against the damaging effects of the superoxide radical." This was the basis for the "superoxide theory of oxygen toxicity," proposed by Fridovich and McCord a few years later. In the 1970s it become evident that organisms could produce O_2^- by various sources, including mitochondria, activated leukocytes, and autoxidation of several biomolecules. The superoxide theory of oxygen toxicity postulated that O_2^- was the intermediate of oxygen-mediated toxicity that could oxidize several import biological targets and was linked to the etiology of certain human disorders.

In the 1980s the importance of O_2^- was downgraded to be "just" an intermediate in the process of the metal-catalyzed formation of $^{\bullet}OH$ radical when it was realized that $^{\bullet}OH$ is the species that is really responsible for free-radical-induced toxicity. Indeed, $^{\bullet}OH$ is many orders of magnitude more reactive than O_2^-. Many studies were published describing SOD as a producer of H_2O_2 for Fenton reactions [see reactions (TB12.1) and (TB12.2)], which would make SOD a prooxidant! Others have recognized that SOD is biologically important because it keeps iron in the Fe^{3+} state [by removing O_2^- from solution; see reaction (TB12.3)], thus indirectly inhibiting Fenton-induced $^{\bullet}OH$ formation.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
(SOD speeds up this reaction) (TB12.1)

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$
(Fenton reaction) (TB12.2)

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$
(SOD inhibits this reaction) (TB12.3)

Interesting scientific battles occurred in the 1980s, with Fridovich defending the fundamental role of SOD and O_2^- in living organisms. More scientific efforts in the mid-1990s proved that O_2^- plays key roles in biology, including in the formation of $ONOO^-$ (via reaction with nitric oxide, see Section 12.1.3), inactivation of relevant enzymes with [Fe–S] clusters such as aconitase (with removal of ferrous ions, that participate in $^{\bullet}OH$ formation), and participation in cellular redox-signaling mechanisms and signal transduction pathways (see Section 12.6). So, in the end, Fridovich was right, and he is currently considered the father of the oxyradical science world. For more information about Fridovich's personal life and science achievements, read his recent review entitled "With the help of giants" (*Ann Rev Biochem* **72**:1–18, 2003).

that removes copper from the enzyme.

$$SOD\text{–}Cu^{2+} + O_2^- \rightarrow O_2 + SOD\text{–}Cu^+$$
(oxidation of O_2^-) (12.2.14a)

$$SOD\text{–}Cu^+ + O_2^- \rightarrow O_2^{2-} + SOD\text{–}Cu^{2+}$$
(reduction of O_2^-) (12.2.14b)

A different type of CuZn–SOD is the extracellular SOD (EC-SOD), a tetrameric glycoprotein with a molecular mass of 135 kDa. There are several isoforms of EC-SOD, and they seem to be bound to cell surfaces, especially in the lung and blood vessels. The biological function of EC-SOD appears to be connected with the extracellular control of O_2^- interaction with $^{\bullet}NO$ (which forms $ONOO^-$).

Mn–SOD is widely distributed in bacteria, yeast, plants, and animals. It is a 40-kDa enzyme with four subunits in mammals (two or four subunits in other species) that contains a Mn^{3+} ion in the catalytic site. Mn–SOD is insensitive to cyanide and is located in the mitochondria. The activity of Mn–SOD in mammals is about 1 to 10% of the total SOD activity (CuZn–SOD plus Mn–SOD). The enzyme plays a pivotal role in safeguarding mitochondria from oxidative stress as well as in regulating intramitochondrial O_2^- concentration (and consequently H_2O_2 concentration). Indeed, animals lacking Mn–SOD ("Mn–SOD knockouts") are incapable of surviving without antioxidant supplementation (see Text Box 12.3).

TEXT BOX 12.3 DETERMINING SOD ACTIVITY AND UNDERSTANDING ITS *IN VIVO* ROLE

There are many assays for the determination of SOD activity, and it is often difficult to compare activities between studies when different techniques are used. The basis of most SOD assays is the determination of the capacity of the sample to inhibit by 50% the reaction of O_2^- (generated in a variety of ways) with a detector molecule. The most commonly used SOD assay is based on O_2^- formation by xanthine plus xanthine oxidase and detection by determining the rate of reduction of Fe^{3+}–cytochrome *c* (Cc).

Other generators of O_2^- include Mn–EDTA/mercaptoethanol and oxygen, illuminated flavins plus oxygen, direct addition of KO_2 powder (the rate of KO_2 disappearance is determined spectrophotometrically), and autoxidation of adrenalin and pyrogallol (quantified colorimetrically). Other detectors of O_2^- include luminol (measurement of light emission), nitroblue tetrazolium (NBT), adrenalin, and hydroxylamine (quantified colorimetrically or by O_2 uptake). Nondenaturating gel electrophoresis has also been used to detect SOD activity in the presence of riboflavin–NBT. This causes formation of blue formazan in the gel, except in the areas corresponding to the SOD bands. The distinction between Mn–SOD and CuZn–SOD in tissue homogenates (or in column fractions during purification) is usually based on the inhibition of CuZn–SOD (but not Mn–SOD) by cyanide or H_2O_2 in parallel assays.

Overexpression of Mn–SOD or CuZn–SOD in transgenic mice (insertion of the human Mn– or CuZn–SOD gene in mice is the strategy of choice) and SOD-defective animals are good models to evaluate the function of SOD in biology. Transgenic mice with elevated levels of CuZn–SOD are more resistant to O_2 toxicity (and free-radical-generating compounds) than controls. Transgenic mice with increased lung Mn–SOD activity are more resistant to lung damage caused by 95% oxygen than control mice.

Knockout CuZn–SOD mice strains develop normally and show no overt motor deficits by 6 months in age. However, mice deficient in CuZn–SOD exhibit marked vulnerability to motor neuron loss after oxidative axonal injury. At the other extreme, homozygous mutant mice lacking Mn–SOD die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipids in liver and skeletal muscle, metabolic acidosis, and loss of activity of mitochondrial enzymes in several organs. Thus, the lack of control over O_2^- concentration in the mitochondrial matrix by Mn–SOD has drastic consequences. These observations suggest that the components of the antioxidant system might reorganize themselves to cope with the loss of CuZn–SOD, but not with the loss of Mn–SOD, when no exogenous stress is applied to the animals. Under oxidative stress conditions the lack of CuZn–SOD may become a problem for the organism.

The fourth form of SOD is an iron-containing enzyme (Fe–SOD) that was first described in the cell matrix of *Escherichia coli* (as was Mn–SOD). This bacterial enzyme has two or four subunits, with an amino acid sequence similar to Mn–SOD. Some bacterial species contain both Mn–SOD and Fe–SOD, and others contain just one form. Interestingly, recent reports have detected Fe–SOD in chloroplasts of higher plants. However, Fe–SOD has never been observed in the animal kingdom.

The activities of Mn–SOD and CuZn–SOD (see Text Box 12.3 for methods of SOD quantification) are highly regulated by the redox state of cells. Under oxidative stress conditions, it is common to detect increased cellular activities of both enzymes (see Section 12.6 for redox regulation of SODs). However, there are several genetic/pathological conditions that cause a decrease in SOD activities, resulting in disrupted redox balance and causing oxidative stress. One example is found in progeria, a premature aging disease. Human skin fibroblasts from affected individuals have decreased levels of Mn–SOD (but unchanged CuZn–SOD activity) as well as reduced catalase and GPx activities. The diminished capacity for H_2O_2 and O_2^- removal that ensues decreased ability of progeria cells to minimize oxidative damage may be a key factor in the disease. Indeed progeria cells have increased levels of carbonyl proteins, which are products of oxidative damage to proteins (see Section 12.4.3).

12.2.1.2 Catalase The earliest observations of catalase action date to the early nineteenth century, when the French chemist Thénard observed bubbling of H_2O_2 solutions (i.e., release of O_2) when these were added to animal tissue slices. The isolation of catalases (from tobacco, yeast, and blood) was first accomplished at the beginning of the twentieth century. It was initially imagined that H_2O_2 decomposition was a universal property of enzymes and, hence, the enzyme was named catalase because of its ability to *catalyze* H_2O_2 decomposition. In the 1930s the heme prosthetic group of liver catalase was identified.

Mammalian catalase is a homotetrameric protein of about 240 kDa that contains one heme group and one NADPH molecule per subunit. The enzyme is localized primarily in peroxisomes, but smaller amounts are found in mammalian heart mitochondria; both of these organelles are responsible for intense H_2O_2 production. Catalase is also present in most organisms, including vertebrates, invertebrates (see Table 12.4 for examples), plants, fungi, and bacteria. In vertebrates, catalase is present in all tissues with particularly high activity in erythrocytes, liver, kidney, and adipose tissue (200 to 1500 U/mg protein in humans); by contrast, catalase activity in brain is very low (5–20 U/mg protein in humans). Catalase is exceptionally active in insects where its activity may compensate for the absence of selenium-dependent GPx activity in many species. Some parasitic trematode worms, such as the liver fluke, have no catalase and it is believed that cytochrome *c* peroxidase (CcP, found in the intermembrane space of mitochondria) replaces its function in this case.

Bacteria have two types of catalase or hydroperoxidases, HPI and HPII, that differ in structure and kinetic properties. HPI from *E. coli* has four 80-kDa subunits, contains two molecules of protoheme IX, and displays catalase activity ($2H_2O_2 \rightarrow O_2 + 2H_2O$) and a broad peroxidase activity ($AH_2 + H_2O_2 \rightarrow A + 2H_2O$; AH_2 is any substrate that is oxidized). HPII is a homohexameric protein that contains one prosthetic heme *d* group per 93-kDa subunit and displays only catalase activity. HPI is believed to be more important than HPII for H_2O_2 resistance. Moreover, studies in the mid-1980s of the adaptive responses of *E. coli* toward H_2O_2 exposure via the induction of HPI (but not SOD) opened the field to studies of the genetic regulation of the expression of antioxidant enzymes (this is further explored on Section 12.6.1).

The assay of catalase activity is usually done spectrophotometrically by following the rate of disappearance of millimolar amounts of H_2O_2 at 240 nm. Other assays are

TABLE 12.4 Hepatic Activities of Antioxidant Enzymes and Levels of Glutathione (GSH + GSSG) in Several Animals Species

	SOD (U/mg)	Catalase (U/mg)	GST (U/mg)	GR (mU/mg)	cGPx (mU/mg)	GSH-eq (μmol/gww)
Rat	50–90	350–400	0.4–0.5	25–35	600–1500	7–9
Human	40–90[b]	1200–1500	NA	30–50	20–50	4.0–5.0
Turtles[c] and snakes[d]	10–50	70–200	0.6[d] and 2.0[c]	10–30	150–300	1.0–1.2[d] and 3.0[c]
Toads[e] and frogs[f]	15–50	100–600[f] and 1300[e]	0.7–1.5	5–20	40–150	0.6–1.7
Goldfish[g]	40–50	100–150	0.5–0.6	25–30	300–600	2.5–3.0
L. littorea[h] and land snails[i]	25[h] and 50–130[i]	20[h] and 180–210[i]	0.4–1.2	10–40	5–15	0.3[h]and 1.6–2.8[i]
Insect larvae[j]	60–150	40–150	0.05–0.40	0.2–0.4	0[k]	0.9–1.1

[a]This table shows the approximate range of enzyme activities (expressed per milligram protein) and GSH levels [per gram wet weight (gww)], taken from published values for mean ± SEM. Total SOD activity was reported, using the Mn–EDTA/mercaptoethanol/NADH assay [see Paoletti et al. (1986). *Anal Biochem* **154**:536–541].
[b]We converted an average activity (from the literature) of 107,000 units/gww determined by the KO_2 assay in SOD units (per mg/protein) of Paoletti's assay.
[c]Freshwater red-eared slider *Trachemys scripta elegans*.
[d]Garter snake *Thamnophis sirtalis*.
[e]Spadefoot toad *Scaphiopus couchii*.
[f]Frogs *Rana pipiens* and *R. sylvatica*.
[g]Goldfish *Carassius auratus*.
[h]Marine gastropod *Littorina littorea*.
[i]Land snails *Helix aspersa* and *Otala lactea* [see Ramos-Vasconcelos and Hermes-Lima (2003) *J Exp Biol* **206**:675–685].
[j]Larvae of the fly *Eurosta solidaginis* and larvae of the moth *Epiblema scudderiana* [see Joanisse and Storey (1998). *Insect Biochem Mol Biol* **28**:23–30].
[k]Selenium-dependent GPX (cGPx) activity was not detectable, however, selenium-independent GPX activity (GST-Px) was 2.0 to 5.0 mU/mg protein.
NA, Information not available.
Source: Most data were adapted from Marcelo Hermes-Lima et al. (2001). Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. In *Cell and Molecular Responses to Stress*, Vol. 2, K. B. Storey and J. M. Storey (eds.). Elsevier Science, Amsterdam, pp. 263–287.

based on the quantification of O_2 evolution from H_2O_2 decomposition. The catalytic cycle of mammalian catalase involves the reaction of Fe^{3+}-catalase with one H_2O_2 molecule, forming compound I, which contains iron in a formal valency state of Fe^{5+} (it is probably Fe^{4+} bound to a porphyrin π-cation radical). Then, compound I receives two electrons from another H_2O_2 molecule, yielding H_2O and O_2. Interestingly, compound I was the first enzyme–substrate (ES) complex to be detected spectroscopically, confirming the validity of the [ES] hypothesis of Michaelis and Menten (see Chapter 2).

Catalase can be inhibited by aminotriazole, which works by "locking in" compound I, thus arresting the catalytic cycle of the enzyme. Administered to animals or plants, this inhibitor has been a very useful tool for understanding the biological role of catalase. Catalase is also sensitive to cyanide and azide. Most studies suggest that catalase is most effective in dealing with oxidative stress when the intracellular concentrations of H_2O_2 are highly elevated. Small increases in H_2O_2 seem to be better controlled by selenium-dependent GPx (see Section 12.2.1.3).

A lack of catalase can, in most cases, be overcome by a reorganization of other components of the antioxidant apparatus, as long as no further oxidative challenge occurs. For example, we observed in our laboratory in Brazil that administration of aminotriazole (at 1 g/kg) to goldfish causes a transient 80 to 90% reduction in liver catalase activity after 12 h (which lasts for several hours before the fish starts to synthesize more catalase) without increases in the levels of products of lipid peroxidation or protein oxidation in liver. Bacteria and yeast with deleted catalase genes have near-normal growth but are more sensitive to H_2O_2 exposure. Humans with acatalasemia (or Takahara's disease), a very rare condition caused by a mutation of the gene encoding catalase, have normal lives and normal life spans, except for increased incidence of mouth ulceration. This ulceration is caused by H_2O_2-producing *Streptococcus* bacteria normally found in the gums. Since the cells of acatalasemic subjects lack catalase activity, they are more sensitive to the effects of H_2O_2 or H_2O_2-generating compounds.

12.2.1.3 Glutathione Peroxidase Glutathione peroxidases (GPx) are four different selenoenzymes that have similar functions. These enzymes are typical of the animal kingdom, and some of them have been found in the last few years in plants and yeast. More recently, a

gene with homology to GPx was shown to contribute to the antioxidant defense on Streptococcus.

12.2.1.3.1 Classical GPx The "classical" form of GPx (cGPx) was first identified in erythrocytes in 1957 and has the capacity to prevent oxidative breakdown of hemoglobin. In 1973 the German group of L. Flohé identified cGPx as a selenium-dependent enzyme; this was the first selenoprotein identified in mammals. cGPx is a homotetramer, with one selenium atom per subunit and ranges in size from 76 to 105 kDa depending on the animal species. Selenium participates in the catalytic cycle of cGPx and is present as a selenocysteine residue at the active site. Interestingly, selenocysteine is coded by the codon UGA, which is usually a stop codon.

Classical GPx catalyses the decomposition of H_2O_2 and some organic hydroperoxides, such as fatty acid hydroperoxide (LOOH) and the artificial substrates cumene hydroperoxide and *tert*-butyl hydroperoxide, using GSH as a cosubstrate [see reactions (12.2.3) and (12.2.4)]. The enzyme cannot reduce membrane-bound lipid hydroperoxides unless they are removed from the phospholipids by phospholipase A2. Very recent evidence has shown that cGPx is able to decompose $ONOO^-$ to nitrite (NO_2) at high rates using the reducing power of GSH [reaction (12.2.15)]. This is a highly important enzymatic activity for minimizing the cellular toxicity of $ONOO^-$:

$$ONOO^- + 2GSH \rightarrow GSSG + H_2O + NO_2 \qquad (12.2.15)$$

Organs with high metabolic rate, such as liver, lung, and kidney, produce more H_2O_2 by their mitochondria (see Section 12.1) and have higher activities of cGPx than do other tissues. Brain, on the other hand (which also has high metabolic rate), has exceptionally low levels of cGPx. cGPx activity has been observed in all vertebrates (fish and rodents have specially high cGPx activities) and in many invertebrates including earthworms, land snails, and crustaceans (see Table 12.4 for hepatic cGPx activity in several animal species). Many insects do not have cGPx, but instead they display selenium-independent GPx activity, which is due to GST isoforms with peroxidase activity (see Section 12.2.1.4).

Depriving animals of dietary selenium causes a severe reduction in cGPx activity (other GPxs are also affected by selenium deprivation, however, with different time-dependent responses) and is associated with a series of muscular and/or cardiac problems, depending on the animal species. Severe selenium deficiency in humans causes degenerative cardiac disorders, typical symptoms of Keshan disease. This disease was first reported in the 1930s in a Chinese area with selenium-poor soil, causing selenium deficiency (blood selenium below 0.3 to 0.4 μM, as compared to 1 to 3 μM for controls). Keshan disease is treated with small doses of sodium selenite (Na_2SeO_3; high quantities of Na_2SeO_3 present in many "orthomolecular antioxidant formulas" can be very toxic!).

Several studies have suggested that cGPx is not merely complementary to catalase but has a critical role in the inhibition/prevention of cellular oxidative challenge. A classical study of the mid-1980s by Pamela E. Starke and John L. Farber (from Hahnemann University School of Medicine, Philadelphia, Pennsylvania) demonstrated that cell cultures where catalase was inhibited (by aminotriazole) were much less affected by oxidants (e.g., paraquat or H_2O_2) than cells with impaired *in situ* function of cGPx (achieved via inhibition of glutathione reductase activity by 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNU), which impairs the recycling of GSH). At low doses of H_2O_2 or paraquat (a generator of O_2^- and, consequently, of H_2O_2), the cells that were incubated with 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNC) showed higher mortality than those incubated with aminotriazole. On the other hand, at high doses of oxidants the cell mortality was similar in either BCNU- or aminotriazole-treated cells (see Starke and Farber, 1985).

The *in vivo* role of cGPx in defense against oxidative stress was better understood when researchers from Ithaca, New York, studied cGPx knockout mice strains—that is, mice completely lacking cGPx activity [Cheng et al. (1998) *J Nutr* **128**:1070–1076]. The animals had normal development up to 15 months [and normal phospholipid hydroperoxide GPx (PHGPx) activity, another form of GPx that decomposes phospholipid hydroperoxides; see Section 12.2.1.3.2] and then were injected with paraquat. The knockout mice were highly sensitive to paraquat administration (at 50 mg/kg), showing mortality rates 10 times higher than controls and increased (4-fold) lipid peroxidation in liver. Moreover, selenium-deprived mice (which causes severe reduction in cGPx activity but has no effect on PHGPx) were also highly affected by paraquat, with mortality rates similar to knockout mice with normal selenium contents. In addition, mice overexpressing cGPx (1.6 and 2.6 times higher activities in kidney and lung, respectively) had mortality rates only one-tenth those of controls when administered paraquat at 125 mg/kg. Moreover, other studies showed that neuronal cells from cGPx knockout mice are more sensitive to H_2O_2 than control cells. These results indicated that under nonstress conditions, other antioxidant defenses can substitute for a lack of cGPx. However, when there is an overproduction/exposure to H_2O_2, cGPx is of fundamental relevance for safeguarding cell homeostasis.

12.2.1.3.2 Other Glutathione Peroxidases and Their Biological Functions In 1982, a form of GPx capable of reducing phospholipid peroxides in pig liver was identified in Fulvio Ursine's laboratory in Italy and was named phospho-

lipid hydroperoxide glutathione peroxidase (PHGPx) [Ursini et al. (1982) *Biochim Biophys Acta* **710**:197–211]. This monomeric enzyme also reduces hydroperoxide groups of cholesterol esters (Ch-OOH; formed upon oxidation of cholesterol; see Section 13.4), thymine (formed upon oxidation of DNA; see Section 12.5), and lipoproteins. The small size and hydrophobic surface of PHGPx has been implicated in its ability to react with complex membrane lipids. PHGPx is present in most tissues from mammals, but its activity is much lower than cGPx (except in testis). Although PHGPx has very low activity compared to cGPx (4 to 20 mU/mg vs. 600 to 2000 mU/mg protein in rat liver, respectively), several studies have demonstrated that it is much more efficient in reducing membrane lipid hydroperoxides than cGPx plus phospholipase A2.

In the late 1980s American and Japanese scientists purified and identified a plasmatic tetrameric form of selenium-GPx (plasma GPx or pGPx). This is a small glycoprotein (about 23 kDa) that is kinetically and structurally different from the cGPx that is present in erythrocytes. pGPx is also capable of reducing phospholipid hydroperoxides. The discovery of pGPx occurred because of its very different ability to respond to selenium reintroduction (following severe selenium deficiency) than cGPx. Plasma GPx activity returned to normal 2 to 4 weeks after selenium reintroduction whereas 3 to 4 months was required before the erythrocyte enzyme activity returned to normal. This is because circulating red cells do not synthesize new proteins, and thus recovery depends on the turnover of red blood cells (which live for about 120 days; see Chapter 18). Once selenium is again available, red blood cell precursors will synthesize cGPx, and activity will be restored to the circulating pool of erythrocytes. Interestingly, pGPx seems to use either thioredoxin or glutaredoxin (see Section 12.2.1.5) as its physiological thiol substrates. The main source of pGPx synthesis is the kidney, which exports the enzyme into the plasma. Other organs also exhibit mRNA for pGPx, including liver, pancreas, muscle, and placenta (that releases pGPx into the amniotic fluid).

The fourth form of GPx is the gastrointestinal-GPx (GI-GPx) isoform. This enzyme was characterized in 1993, and it is a tetrameric selenoprotein that functions to degrade soluble hydroperoxides. It is restricted to the epithelial lining of the gastrointestinal track (in rats, these cells have no cGPx), possibly being the first line of defense against ingested hydroperoxides. Indeed, rats fed with hydroperoxide-rich diets have higher GI-GPx activity than controls. Moreover, mouse strains that are more sensitive to 1,2-dimethylhydrazine (DMH)-induced colon cancer have less GI-GPx activity. This suggests that GI-GPx may defend the organism against the carcinogenic effects of hydroperoxides in the gastrointestinal track.

The function of GPx enzymes as merely antioxidant defenses (a very important function) has recently being challenged. For example, pGPx has been linked with the regulation of extracellular hydroperoxide concentration in signal transduction pathways (see Section 12.6). PHGPx has been also implicated in modulating the function of lipid hydroperoxides as activators of lipoxygenase and cyclooxygenase, mediators in inflammation, and as signal molecules in apoptosis and receptor-mediated signal transduction.

The activity of GPx is usually quantified using a coupled assay with glutathione reductase (1 U/mL), NADPH (0.2 mM), GSH (5 mM), and H_2O_2 (added last, usually at 0.5 mM), with oxidation of NADPH followed at 340 nm. This assay gives the activity of the "selenium-dependent GPx," or simply Se–GPx (which is mostly cGPx). Several researchers use cumene hydroperoxide to measure "GPx activity" in their studies. However, they are in fact measuring the sum of Se–GPx and selenium-independent GPx activity (or GST–Px, an activity of GST), which cannot decompose H_2O_2. The percentage of GST–Px activity in relation to the total GPx in mammalian livers ranges from 30 to 85%; in insects it can even be 100%.

12.2.1.4 Glutathione* S-*transferases Glutathione *S*-transferases constitute a large family of multifunctional enzymes involved in GSH conjugation to xenobiotics and aldehydic products of lipid peroxidation (such as 4-hydroxyalkenals; see Section 12.3.3.2). Conjugates formed in hepatic cells are often excreted into bile using ATP-dependent pumps; they can also be degraded and acetylated to form *N*-acetylcysteine conjugates (mercapturic acids), which are excreted in the urine. Excretion of a mercapturic acid was first isolated in 1879 in the urine of dogs that had been fed bromobenzene. Only in the late 1950s was it discovered that mercapturates derive from GSH conjugation. GSTs are also very important for the biogenesis of prostaglandins and leukotrienes. GSTs are widely distributed in all life-forms including bacteria, fungi, plants, and animals. All animals investigated to date have GST homo- or heterodimers that are active in the cytosol or nucleus. Mammalian liver is particularly rich in GST activity; 5 to 10% of its cytosolic protein is GST!

Structural and functional studies on vertebrate GSTs have divided the several isoenzymes in four major classes: alpha (α), mu (μ), pi (π), and theta (θ) (two minor classes are sigma and kappa). These classes are based upon N-terminal amino acid sequences, substrate specificities, sensitivity to inhibitors, isoelectric point, and immunological analysis. Eric Boyland and co-workers from England discovered the existence of multiple forms of GST in the 1960s. Most GSTs have molecular masses of 40 to 55 kDa. Measurement of "total GST activity" is based on the reaction of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), forming *S*-(2,4-dinitrophenyl)-glutathione, which is quantified at 340 nm. This assay, however, does

not measure the θ class of GSTs because of their lack of reactivity toward CDNB.

Glutathione *S*-transferases also display selenium-independent GPx activity, designated as GST–Px, which is measured using the cumene hydroperoxide assay (see Section 12.2.1.3). Endogenous substrates of GST–Px include fatty acid hydroperoxides, Ch-OOH, and thymine hydroperoxide (formed upon oxidation of thymine), but not H_2O_2. The GST–Px activity is differentially distributed among the GST classes, being highest for GSTθ, moderate for GSTα, and low for the other isoforms.

Elegant studies in the late 1970s from Helmut Sies' laboratory in Germany showed that GST–Px activity is relevant in the detoxification of organic hydroperoxides in mammals. They studied GSSG efflux from rat liver under oxidative stress. When GSSG accumulates in the liver (due to cGPx-mediated oxidation of GSH to GSSG), it is pumped out into the circulation. The authors infused H_2O_2 into isolated livers from rats that had been fed a selenium-deficient diet (which severely depletes cGPx activity) and detected no GSSG efflux. However, significant GSSG efflux was observed from these livers when they infused *tert*-butylhydroperoxide (*t*-BOOH), which can be metabolized by GST–Px [reaction (12.2.16)].

$$t\text{-BOOH} + 2\text{GSH} \rightarrow t\text{-BOH} + \text{H}_2\text{O} + \text{GSSG} \quad (12.2.16)$$

Glutathione *S*-transferases are very important in cancer resistance because many endogenously produced aldehydes and many P450-activated xenobiotic drugs can damage DNA (see Section 12.5). For example, a GSTα found in mice catalyzes the detoxification of *exo*-8,9-epoxide (formed by P450 activation of aflatoxin B_1, a potent hepatocarcinogen found in badly stored peanuts), but rats do not express an enzyme with corresponding activity. This probably accounts for the much greater sensitivity to aflatoxin B_1-induced hepatocarcinogenesis of rats compared with mice. Induction of GSTs has also been linked with resistance of cancer cells toward chemotherapy because many anticancer drugs (or their P450-activated forms) are GST substrates. Tremendous efforts have been made to develop rationally designed GST inhibitors for adjuvant treatment during chemotherapy. One candidate for clinical use is the diuretic drug, ethacrynic acid, a good inhibitor of most GST isoforms.

The study of GST isoforms of insects is also relevant for crop sciences. This is because many crop-plague insects have potent enzymatic activities that can deactivate insecticides via GSH conjugation. Herbivorous insects have very high natural GST activities due to the presence of toxic compounds in the plants they feed on (notably, these toxic compounds are the plant's defensive insecticides).

Moreover, it has been recently demonstrated in Germany that manipulation of the expression of a single GST can modulate the response to oxidative stress in *Caenorhabditis elegans*. This study was the first to show a direct protective effect of GST *in vivo* (Leiers et al. *Free Radic Biol Med* **34**:1405–1415, 2003).

12.2.1.5 Glutathione and Thioredoxin Systems Glutathione is widely distributed in plants, animals, fungi, and bacteria in intracellular concentrations ranging from 0.1 to 10 mM. It also occurs in extracellular fluids and plasma at much lower levels. This antioxidant tripeptide is present primarily in its reduced from, GSH (γ-GluCysGly; see Fig. 12.3), with the ratio between GSH concentration and the disulfide oxidized form (GSSG) varying from 5 to 150, in a species- and tissue-specific manner. The GSH/GSSG ratio also changes during aging, oxidative stress conditions, and disease and is considered a relevant marker of oxidative stress (e.g., a diminution in the GSH/GSSG ratio is correlated with increased production of hydroperoxides). Cells are able to export either GSH (liver is a major exporter; see below) or GSSG into the plasma. Under oxidative stress conditions, the export of GSSG (see Section 12.2.1.4) helps cells to maintain a negative reduction potential.

Glutathione plays several roles in biology. It is classically viewed as a substrate for GPx- and GST-catalyzed reactions. GSH has also nonenzymatic antioxidant activity and can react with various carbon-centered radicals, singlet oxygen, $^{\bullet}$OH, and reactive nitrogen species to yield the GSH–thiyl radical (GS$^{\bullet}$) and other products. One product of the reaction of GSH with nitric oxide is *S*-nitrosoglutathione (GSNO), which may help to prevent $ONOO^-$ formation by neutralizing nitric oxide. Glutathione is also involved in the cellular uptake of amino acids (see below), the metabolism of ascorbic acid (see introduction to Section 12.2), leukotriene synthesis, glutaredoxin synthesis (see below), regulation of the activities of many enzymes with critical thiol groups, and in the mechanisms of protein folding and protection against oxidation of protein –SH groups. GSH is also involved in the mechanism of redox signaling (see Section 12.6).

Reduced glutathione (GSH) is synthesized in vertebrate cells by a two-step process involving γ-glutamylcysteine (γ-GluCys) synthetase and GSH synthetase [reactions (12.2.17) and (12.2.18), respectively]. GSH is a competitive inhibitor of γ-GluCys synthetase, providing feedback control of GSH formation. Intracellular levels of GSH are also controlled by secretion into plasma, as well as by enzymatic oxidation to GSSG, or conjugation with GST substrates (that are pumped out of the cells). γ-GluCys synthetase can be inhibited by buthionine sulfoximine

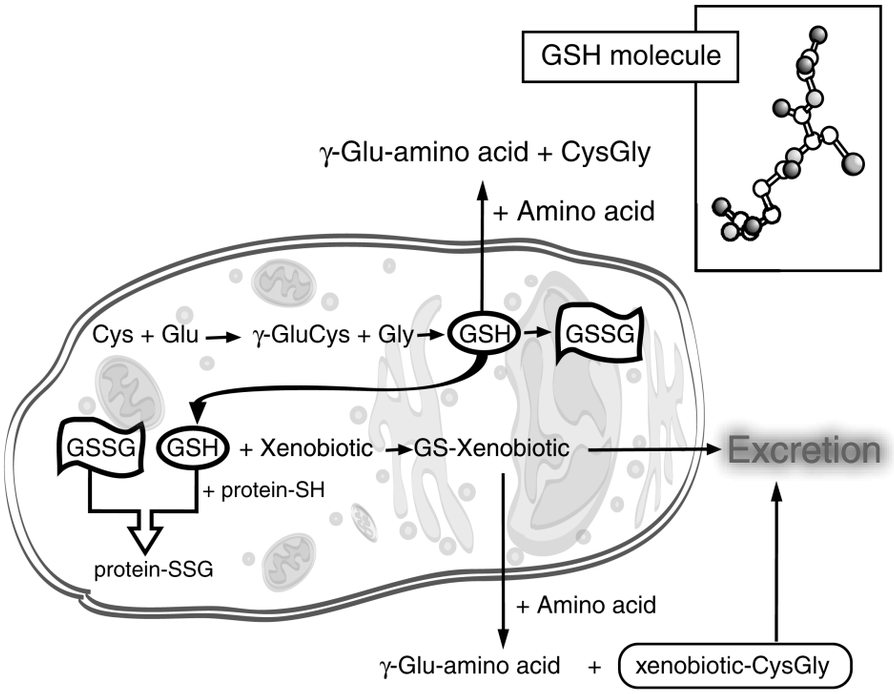

Figure 12.3 Glutathione metabolism in liver cells. The figure shows the biosynthesis of GSH and the fates of GSH and GSSG.

(BSO), which is a widely used tool for the study of the effects of GSH depletion.

$$\text{Glu} + \text{Cys} + \text{ATP} \rightarrow \gamma\text{-GluCys} + \text{ADP} + \text{P}_\text{i} \quad \text{(catalyzed by } \gamma\text{-GluCys synthetase)} \tag{12.2.17}$$

$$\gamma\text{-GluCys} + \text{Gly} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{P}_\text{i} \quad \text{(catalyzed by GSH synthetase)} \tag{12.2.18}$$

Once GSH is pumped out of the cells, the ectoenzyme γ-glutamyl-transpeptidase (γGT, with the catalytic site facing the extracellular milieu) can catalyze the extracellular reaction of GSH with amino acids, yielding γ-glutamyl-amino acids and cysteinyl-glycine (CysGly) [see reaction (12.2.19) and Fig. 12.3]. γGT also catalyzes the reaction of amino acids with GSH conjugates (e.g., GSH-xenobiotic adducts produced by GST and then pumped out), yielding γ-glutamyl-amino acids and a CysGly conjugate (xenobiotic-CysGly) (Fig. 12.3). Adducts of xenobiotics with either GSH or CysGly can be converted to mercapturic acids (see Section 12.2.1.4) and then excreted in the urine.

To recycle the substrates for GSH synthesis, CysGly is hydrolyzed to the respective amino acids by ecto- or endopeptidases. Moreover, γ-glutamyl-amino acids can be converted to 5-oxoproline (plus an amino acid), which is used for the ATP-dependent synthesis of glutamic acid [Glu; reactions (12.2.20)]. Figure 12.3 summaries the various fates of GSH in cells.

$$\text{GSH} + \text{amino acid} \rightarrow \gamma\text{-glutamyl-amino acid} + \text{CysGly} \tag{12.2.19}$$

$$\gamma\text{-glutamyl-amino acid} \rightarrow \text{5-oxoproline} + \text{amino acid} \rightarrow \text{Glu} \tag{12.2.20}$$

There are a large number of assay methods for GSH and GSSG. These include HPLC-based determinations with spectrofluorometric or electrochemical detection as well as spectrophotometric assays. The most commonly used assay measures the reaction of GSH with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of NAPH and glutathione reductase (GR), which produces a yellow product quantified at 412 nm. This assay actually measures total glutathione equivalents (GSH + 2GSSG; since both forms are present in biological samples) because GSSG is converted to GSH by GR plus NAPH. GSSG content alone is then determined in parallel assays after derivatization of GSH with 2-vinylpyridine. The content of "GSH itself" is then determined by subtraction.

Plants have not only GSH but also two analogs, γ-Glu-CysAla (homoglutathione) and γ-GluCysSer, which have

similar antioxidant functions. Some parasitic protozoa such as *Trypanosoma cruzi* (the agent of Chagas disease) lack GSH and use the peptide trypanothione (which is glutathione covalently bonded to the polyamine spermidine) as a scavenger of H_2O_2. This protozoa also contains thioredoxin but not catalase nor GPx.

A reasonable amount of glutathione forms mixed disulfides with proteins (see below) and, therefore, is not "free" in cells. Accumulated GSSG can react with −SH groups of many proteins (a process called protein-*S*-thiolation) and cause their inactivation [reaction (12.2.21a)]. Protein-*S*-thiolation may result from attack of $GS^{\bullet}$. Moreover, protein-mixed disulfides are more susceptible to proteolytic degradation. GSSG can also induce crosslinkage of −SH groups at the surface of enzymes and proteins [reaction (12.2.21b)]. This crosslinkage causes inactivation of some enzymes (e.g., phosphofructokinase, pyruvate kinase) but activation/stabilization of others (e.g., glucose-6-phosphatase, fructose 1,6-bisphophatase). Hence, glutathione-mediated protein thiolation is a mechanism of posttranslational regulation of metabolic enzymes. As discussed in Chapter 1, disulfide–dithiol enzyme interconversion is a particularly important mechanism of enzyme control in photosynthetic plants.

$$\text{GSSG} + \text{protein-SH} \rightarrow \text{protein-SSG} + \text{GSH} \qquad (12.2.21a)$$

$$\text{GSSG} + \text{protein-(SH)}_2 \rightarrow \text{protein-SS} + 2\text{GSH} \qquad (12.2.21b)$$

Auxiliary defenses such as thiol–disulfide oxidoreductases can remove the glutathione group bridged to proteins as well as re-reduce thiol–disulfides in proteins. These oxidoreductases include thioltransferase (or glutaredoxin, an 11-kDa polypeptide) and the 12-kDa polypeptide thioredoxin (Trx; which contains two adjacent −SH groups). Both are present in the cells of most organisms. In mammalians, the two main thioredoxins are Trx-1, the cytosolic and nuclear form, and Trx-2, a mitochondrial form. Thioredoxin in the oxidized form (Trx-SS) is recycled to the reduced form, Trx-$(SH)_2$, by a FAD-containing selenoenzyme thioredoxin reductase (TrxR), which uses NADPH as its cosubstrate [reaction (12.2.22)].

$$\text{Trx-SS} + \text{NADPH} + \text{H}^+ \rightarrow \text{Trx-(SH)}_2 + \text{NADP}^+ \quad \text{(catalyzed by TrxR)} \qquad (12.2.22)$$

Thioredoxin can also reduce oxidized thiol groups in proteins and is a physiological substrate for the plasmatic form of GPx (pGPx; see Section 12.2.1.3.2). It also supplies the reducing equivalents to ribonucleotide reductase and thioredoxin peroxidase (TPx), an enzyme that decomposes hydroperoxides using Trx as a cosubstrate (TPx used to be called thiol-specific antioxidant up to the early 1990s). TPx is particularly important for microorganisms and helmintic parasites that lack GPx and/or catalase activity. Mammals seem to have at least two TPx isoforms: One is cytosolic and one mitochondrial. An interesting example of a physiological function of TPx is found in the adrenal gland. This gland produces high levels of H_2O_2 as a result of active sterol biosynthesis and displays very high constitutive levels of mitochondrial TPx.

Thioredoxin is present in cells at much lower levels than GSH (about 1 to 15 μM in bacteria and bovine tissues) and, at these low levels, may not act itself as a physiological antioxidant. However, it is highly important as a substrate of TPx (and possibly of pGPx). The levels of Trx can increase under certain stress/pathological conditions to provide an adaptive response. For example, increased levels of Trx-1 are found in many human tumors, associated with aggressive tumor growth. High levels of plasma Trx were also observed in hepatitis C virus-infected patients with hepatocellular carcinoma or chronic hepatitis (42 to 44 ng/mL; compared with 25 to 35 ng/mL for controls).

12.2.2 Low-Molecular-Weight Antioxidants

Animals produce several nonenzymatic low-molecular-weight metabolites that have physiologically relevant antioxidant actions. These include glutathione (and its analogs, discussed in Section 12.2.1.5), ascorbate (for humans, guinea pigs, and few other vertebrates, ascorbate is a vitamin; see Section 12.2.2.3), melanin (a product of tyrosine oxidation and polymerization, protects the skin against UV radiation), melatonin, and uric acid (see Section 12.2.2.1 for the last two molecules).

Other metabolites have *in vitro* antioxidant action, but their physiological role is still under debate. This list includes pyruvate (reacts *in vitro* with H_2O_2, $ONOO^-$, and HOCl), lactate (reacts *in vitro* with O_2^- and $^{\bullet}OH$ but cannot block lipid peroxidation; lactate might be relevant as an antioxidant in muscle during exercise), estrogens (protects LDL particles and membranes against *in vitro* peroxidation at concentrations that are 10^4 to 10^6 higher than physiological levels; therapeutic use as antioxidant is questionable), lipoic acid, coenzyme Q, and bilirubin (see Section 12.2.2.1 for the last three molecules).

Dietary antioxidants include vitamin E (Section 12.2.2.3), carotenoids (such as β carotene, a precursor of vitamin A; Section 12.2.2.4), and plant polyphenols (these are discussed in Text Box 12.4). Figure 12.4 depicts several nonenzymatic antioxidants.

Methods for the *in vivo* and *in vitro* assay of low-molecular-weight antioxidants are too numerous to be dis-

TEXT BOX 12.4 PLANT POLYPHENOLS AND HUMAN HEALTH*

Phenolic compounds are ubiquitous in plants and are abundant in fruits, vegetables, seeds, bark of trees, coffee, wine, and tea. They are produced by plant secondary metabolism from glucose that is processed to produce two main intermediates, shikimate and acetate, which are then used to generate secondary metabolites. Shikimate originates from aromatic amino acids (tryptophan, phenylalanine, tyrosine), precursors of aromatic compounds. Some secondary metabolites derive from shikimate or acetate, while some are produced by a combination of these intermediates or their derivatives, for example, flavonoids, anthraquinones, and tannins. In plants, phenolics work as structural components, and have antioxidant, antimicrobial, and antiviral activity. Many flavonoids possess attractive colors and participate in the ecology of plants by making the flowers and fruits attractive to birds and bees. Some are excellent scavengers of free radicals and metal ions. More than 4000 plant phenolics have been described. The classes of plant phenolics found in the human diet are *flavanols* (including catechin, epicatechin, and epicatechin gallate; black and green teas and red wine are rich in flavanols), *flavanones* (such as hesperidin, found in citrus fruits), *flavonols* [including kaempferol, myricetin, rutin, and quercetin (see Fig. 12.4); broccoli, onion, berries, and red wine are flavonol-rich], *flavones*, *anthocyanidins* (such as cyanidin, found in berries), and *phenylpropanoids* (including caffeic and chlorogenic acids; a fresh coffee is a souce of these). Genistein and daidzein are the main representantives of *isoflavones*; soy is the main source of these phenolics.

The beneficial effects of plant phenols (including polyphenols) have been investigated extensively in cells and tissues *in vitro* and in animal models, documenting their antiradical and antioxidant properties and their participation in prevention and therapy of several diseases. Because their relevance to human health has not been fully examined by direct measurements in humans, it is important to be able to extrapolate bioactivities observed *in vitro*, into a physiological scenario *in vivo.* In this regard, some findings are of interest. For example, black tea polyphenols decrease LDL cholesterol and LDL oxidation in rats and increase HDL content. Therefore, they can be beneficial for the treatment of atherosclerotic disorders, hypertension, and type-2 diabetes. Other studies have reported enhanced protection against oxidative stress afforded to red blood cells by polyphenols; for example, plant phenolics from blueberries ameliorate the experimentally induced formation of ROS in red blood cells using an *in vitro* model following dietary consumption by 6-month-old male rats. Polyphenols may provide protection against ROS in various cell systems. They also exhibit cytostatic and cytotoxic effects in tumorigenesis, some being able to inhibit invasion by highly metastatic cells. Polyphenols also inhibit the activity of an array of enzymes including lipoxygenases, cyclooxygenases, monooxygenases, collagenases, xanthine oxidase, protein kinases, and also increase the activity of antioxidant enzymes, such as SOD. Therefore, they may ameliorate inflammatory responses, modulate cell signaling, and improve the antioxidant status of cells.

Besides all the potential beneficial effects of polyphenols such as flavonoids and catechin, it is important to emphasize that their biological and pharmacological effects may depend upon their behavior as either antioxidants or prooxidants in the cell environment. These opposite actions may be related to their redox activity and position and the number of hydroxyls on the molecule. Flavonoids can either inhibit or enhance the formation of hydroxyl radical ($^{\bullet}OH$) by Fenton-like reactions, depending upon the redox activity of the flavonoid and the nature of the metal ion participating in $^{\bullet}OH$ formation. Most polyphenols (including flavonoids) are metal chelators and can either favor or inhibit the participation of the metal (either iron or copper) in $^{\bullet}OH$ formation. For example, we have shown that polyphenol tannic acid chelates Fe^{2+} and prevents its reaction with H_2O_2 (see *Biochim Biophys Acta* **1472**:142–152, 1999); in Brazil we are currently studing the relation between antioxidant activity of caffeic acid and biphenol ellagic acid (see Fig. 12.4) and their ability to chelate iron. Moreover, many polyphenols act as potent $^{\bullet}OH$ scavengers. Studies to elucidate the activity–structure relationships of these compounds are extremely important to distinguish the beneficial versus detrimental effects of polyphenols. Collectively, the available data suggest that polyphenols can improve human health, preventing carcinogenesis and cardiovascular diseases. For more information see reviews from Bravo (*Nutr Rev* **56**:317–333, 1998) and from Lambert and Yang (*Mut Res* **523**:201–208, 2003). We also recomend you to read the interesting interview given by Professor B.N. Ames entitled "Ames agrees with mom's advice: Eat your fruits and vegetables" (*JAMA*, **273**:1077–1078, 1995).

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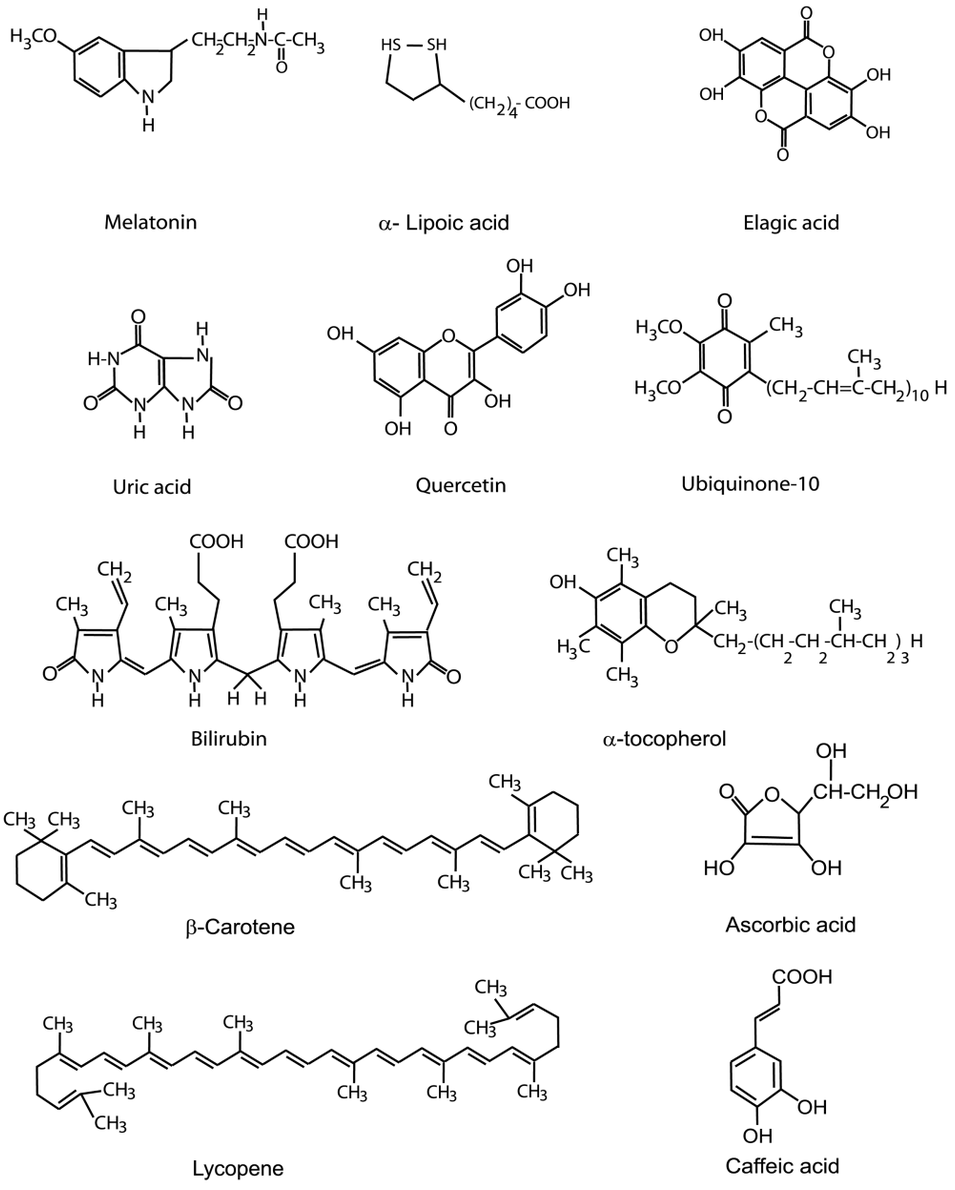


Figure 12.4 Selected nonenzymatic antioxidants of low molecular weight.

cussed in this chapter but we recommend *The Handbook of Antioxidants* (Cadenas and Packer, 1996) for this purpose. However, assessment of the antioxidant capacity of molecules is often based on two main factors. First, the standard reduction potentials (at pH 7.0; giving $E^{0\prime}$) may predict whether a redox reaction between two molecules is possible or forbidden. For example, a comparison of the $E^{0\prime}$ of half-cells (Table 12.3) indicates that dehydrolipoic acid (DHLA) could easily reduce GSSG to GSH. However, $E^{0\prime}$ values can be very different under physiological concentrations (and temperatures) and thus must be corrected by the Nernst equation to give an "effective" reduction potential. In these cases, reactions that may have appeared "impossible" become possible. One example is the autoxidation of ubisemiquinone [$CoQH^{\bullet}$, see reaction (12.2.24)], which does happen *in vivo*. Second, redox reactions that are thermodynamically possible can, in some cases, be kinetically irrelevant. In other words, they are too slow for quick biological responses. Accurate theoretical predictions of the putative actions of antioxidants or prooxidants can be subjected to so many other factors (e.g., effect of co-adjuvant metals, binding

of the reagents to membranes and/or proteins) that it is often better just to assess the reaction experimentally *in vitro* and then, as appropriate, follow up by seeking *in vivo* evidence of a particular antioxidant or prooxidant action.

12.2.2.1 Selected Endogenous Molecules with Antioxidant Activity Melatonin (Fig. 12.4) is produced from serotonin in the pineal gland and seems to regulate circadian rhythms. It is able to inhibit lipid peroxidation *in vitro* and neutralizes $^{\bullet}OH$, singlet oxygen, nitric oxide, and HOCl. However, in order to work as an endogenous antioxidant, it would have to be present in concentrations that far exceed its physiological concentrations. There are, however, new indications that melatonin has a function in redox-regulation mechanisms (e.g., stimulating increased activity of some antioxidant enzymes), immunomodulation, cellular growth, and bone differentiation. Moreover, melatonin has been used experimentally as a protective agent against a wide variety of processes and compounds that damage tissues via free radical mechanisms.

Lipoic acid is a thiol-prosthetic group in α-ketoacid dehydrogenase enzyme complexes in the mitochondria. Lipoate synthase, which catalyzes the formation of two C–S bonds from octanoic acid, is responsible for endogenous lipoic acid formation. Although the metabolic relevance of lipoic acid has been known for 40 years, it was only recently recognized as a potential antioxidant metabolite. It is able to react with $ROO^{\bullet}$, HOCl, $^{\bullet}OH$, and $ONOO^{-}$, and it is also a copper and iron chelating agent. Dehydrolipoic acid (DHLA, the reduced form of lipoic acid; see Fig. 12.4) is a powerful reducing agent (Table 12.3) and can recycle GSSG to GSH *in vitro* (see footnote *a* to Table 12.3), ascorbyl radical to ascorbate [reaction (12.2.23)], and the free radical form of vitamin E (see Section 12.2.2.3) to vitamin E. However, the endogenous levels of lipoic acid and DHLA are very low in organisms, suggesting that they may not work *in vivo* as either a free radical scavenger or as reducing agent (in the case of DHLA).

$$\text{DHLA} + \text{ascorbyl}^{\bullet} \rightarrow \text{lipoic acid} + \text{ascorbate} \quad (12.2.23)$$

On the other hand, the use of exogenous lipoic acid for treatment of a number of liver conditions linked to oxidative stress, including alcohol-induced damage, metal intoxication, and CCl_4 poisoning, has been successful in many cases. Lipoic acid effectively protects the brain against oxidative stress associated with ischemia and reperfusion and also reverses some of the effects provoked by vitamin E deficiency in mice. These observations point the way toward the clinical use of lipoic acid as an antioxidant.

Coenzyme Q (or ubiquinone; see Fig. 12.4) has been known since 1957 as a key component of the mitochondrial respiratory chain. Although most studies have focused on ubiquinone-10, several other ubiquinones occur in nature, located in most cell membranes and in lipoproteins. One-electron oxidation of ubiquinol ($CoQH_2$) yields the radical species ubisemiquinone ($CoQH^{\bullet}$), which can be sequentially oxidized to ubiquinone (CoQ) (see $E^{0\prime}$ values in Table 12.3). These reactions are fully reversible providing, for example, a continuous flow of electrons in and out of the respiratory chain. Dismutation of ubisemiquinone ($2CoQH^{\bullet} \rightarrow CoQ$ and $CoQH_2$) is also a thermodynamically favorable reaction that seems to take place *in vivo*. Moreover, ubisemiquinone is considered a highly relevant source of mitochondrial O_2^{-} formation [reaction (12.2.24) is thermodynamically favorable when calculating the "effective" $\Delta E^{0\prime}$ value, which takes in consideration the *in vivo* concentration of reactants and products; see Schafer and Buettner, *Free Radic Biol Med* **30**:1191–1212, 2001]. Ubiquinone can also be reduced to ubiquinol by the FAD-containing enzyme DT-diaphorase, which helps to maintain a low cellular $CoQ/CoQH_2$ ratio.

$$CoQH^{\bullet} + O_2 + H^{+} \rightarrow O_2^{-} + CoQ \quad (12.2.24)$$

$$CoQH_2 + LOO^{\bullet} \rightarrow CoQH^{\bullet} + LOOH \quad (12.2.25)$$

On the other hand, ubiquinol efficiently protects not only membrane phospholipids from lipid peroxidation [reaction (12.2.25)] but also mitochondrial DNA and membrane proteins from free-radical-induced oxidative damage. In LDL undergoing lipid peroxidation, ubiquinol seems to be relevant for the recycling of α-tocopherol [see reaction (12.2.31)]. The importance of ubiquinol as an *in vivo* antioxidant is still a matter of debate, even though there are indications of positive effects of coenzyme Q therapy in patients with several heart pathologies.

Uric acid (Fig. 12.4; or urate in neutral pH) is produced by the oxidation of xanthine catalyzed by xanthine oxidase or xanthine dehydrogenase. Too great a production of urate leads to gout, an inflammatory joint disease caused by the precipitation of urate crystals (most nonprimates are able to convert urate to allantoin and urea). Urate concentration in human plasma is in the range of 50 to 900 μM, being high enough to react with ozone, $^{\bullet}OH$, $ONOO^{-}$, and peroxyl radicals. Upon oxidation, urate is converted to the resonance-stabilized urate radical ($urate^{\bullet}$), which can either be recycled back to urate by ascorbate or converted (nonenzymatically) to allantoin [reactions (12.2.26) and (12.2.27)]. An indirect proof that urate may function *in vivo* as an antioxidant is the fact that allantoin is produced in patients with diseases associated with oxidative stress, such as Wilson disease (see Chapter 13) and hemochromatosis (see Text Box 12.1). Interestingly, researchers from the Federal University of Rio de Janeiro, Brazil, have proved that urate is the most important nonenzymatic

antioxidant defense in a blood-sucking insect (they produce large amounts of urate without ever developing gout!) [Souza et al. (1997) *Free Radic Biol Med* **22**:209–214].

$$\text{Urate} + {}^{\bullet}\text{OH} \rightarrow \text{urate}^{\bullet} + H_2O \qquad (12.2.26)$$

$$\text{Allantoin} \leftarrow\leftarrow \text{urate}^{\bullet} + \text{ascorbate} \rightarrow \text{urate} + \text{ascorbyl}^{\bullet} \qquad (12.2.27)$$

Bilirubin, another end product of a metabolic pathway (see Fig. 12.4), is an insoluble molecule produced by mammals from heme degradation. It has powerful antioxidant activity *in vitro* against peroxyl radicals and singlet oxygen. However, unlike uric acid, an antioxidant role for bilirubin *in vivo* is still unproven.

12.2.2.2 Vitamin C Water-soluble ascorbic acid (Fig. 12.4) is produced from glucose metabolism by plants and most animals. The enzyme-catalyzed reaction of gulono-γ-lactone (synthesized from glucose) plus oxygen yields ascorbate and H_2O_2. However, various mammalian species, such as guinea pigs and all primates, lack the ability to produce ascorbic acid, which is essential for several metabolic reactions, including noradrenalin biosynthesis, hydroxylation of proline and lysine for pro-collagen formation, and iron absorption in the gut (by reducing Fe^{3+} to Fe^{2+}). A deficiency of vitamin C intake in humans causes the "sailor's disease," scurvy, a disease associated with osteoporosis, hemorrhaging, and anemia. The Dutch doctor Ronsseus first reported the need for the consumption of oranges for scurvy prevention in sailors in the midsixteenth century. The levels of ascorbate in adult human plasma range from 30 to 100 μM, which can be maintained by daily absorption of 60 to 100 mg ascorbate. In 1970 Nobel laureate Linus Pauling popularized the use of megadoses of vitamin C, one gram per day, for preventative use against the common cold (a still unproven issue) and in the 1990s as an antioxidant supplement.

Apart from the metabolic functions of ascorbate, it is a potent nonenzymatic antioxidant defense, able to recycle vitamin E (at least *in vitro*; see Section 12.3.4) and to scavenge peroxynitrite, ozone, singlet oxygen, O_2^{-}, $HOO^{\bullet}$, and ${}^{\bullet}OH$. Considerable evidence shows that ascorbate inhibits lipid peroxidation and oxyradical-mediated damage to proteins and DNA *in vitro*. On the other hand, ascorbate can also be considered a prooxidant due to its ability to recycle iron from the ferric to the ferrous state, thus providing a Fenton reagent for ${}^{\bullet}OH$ formation. Indeed, several *in vitro* experiments designed for metal-catalyzed ${}^{\bullet}OH$ formation are initiated by addition of ascorbate (0.5 to 3 mM) to the reaction medium.

One-electron oxidation of ascorbic yields the ascorbyl radical, which is converted by another one-electron oxidation to dehydroascorbate (DHA); nonenzymatic degradation of DHA yields oxalate and other products [reactions (12.2.28)]. Recycling of ascorbate in animals and plants is enzyme-catalyzed [see reactions (12.2.11) and (12.2.12)]. It is noteworthy that the ascorbyl radical has low reactivity toward biomolecules. Ascorbyl radical can be easily detected in human plasma samples by ESR techniques (see Section 12.1 and Fig. 12.1). There is evidence that plasma of iron-overload patients (see Text Box 12.1) have decreased concentrations of ascorbate, whereas the ESR signal for ascorbyl radical is increased.

$$\underset{}{\text{Ascorbate}} \xrightarrow[(-e^-)]{} \underset{}{\text{ascorbyl}^{\bullet}} \xrightarrow[(-e^-)]{} \underset{}{\text{DHA}} \rightarrow \overset{\text{oxalate and other products}}{} \qquad (12.2.28)$$

According to the British researchers Barry Halliwell and John M.C. Gutteridge (1999; see pages 203 to 205), evidence that points to an unequivocal antioxidant role for ascorbate in mammals is still missing. For example, mutant rat strains that do not synthesize vitamin C (ODS rats) have higher levels of lipid peroxidation products, but, interestingly, ascorbate supplements did not reduce lipid peroxidation in these rats. Moreover, even though the consumption of fruits and vegetables (main sources of dietary vitamin C) can be associated with decreased risk of cardiovascular diseases and of several types of cancer—specially of the gastrointestinal tract—the actual beneficial effect of vitamin C itself is still uncertain. The results from many epidemiological studies or double-blind placebo-controlled studies show either beneficial effects or no effects, and they depend on the specific disease, genetic factors, and age and lifestyle (exercise, smoking habits, use of antioxidant or vitamin supplements) of the subjects. Furthermore, data using biomarkers of oxidative damage to DNA bases (cancer is associated with DNA oxidative damage; see Section 12.5) have given no clear evidence that vitamin C supplements can decrease the levels of oxidative DNA damage *in vivo*, except perhaps in subjects with very low vitamin C intakes.

12.2.2.3 Vitamin E Vitamin E is composed of eight naturally occurring lipid-soluble compounds, α-, β-, γ-, and δ-tocopherols (see Fig. 12.4) and α-, β-, γ-, and δ-tocotrienols. All of these function as antioxidants in membranes to prevent the propagation of lipid peroxidation (see Section 12.3.4 for details). In animals, the most active component of the "vitamin E family" is α-tocopherol, which is the form present in highest concentrations in cell membranes and in the plasma as part of lipoproteins. The levels of total tocopherol in human plasma range between 18 and 28 μM, with important variations among different populations.

Vitamin E was first detected as a fat-soluble factor essential for rodent reproduction in the early 1920s and was isolated from wheat germ oil in 1936. It was later shown to be essential in the diet of all animals (which classifies it as a vitamin) and as capable of preventing hemolytic anemia in premature babies. Sources of vitamin E in the human diet include vegetable oils, nuts, margarines, wheat germ, and green leafy vegetables.

The reaction of α-tocopherol (α-TocH) with peroxyl PUFAs [$LOO^{\bullet}$, or other radicals, $R^{\bullet}$; see reaction (12.2.29)] yields α-tocopheryl radical (α-$Toc^{\bullet}$) and LOOH. α-Tocopheryl radicals can be nonenzymatically recycled back to α-tocopherol by other antioxidants, such as ascorbate, coenzyme Q [reactions (12.2.30) and (12.2.31)], and carotenoids (see Section 12.2.2.4). The continuous recycling of α-tocopherol (and other tocopherols) seems to explain why the concentration vitamin E is so low in biological membranes. For example, there is one α-tocopherol molecule per 2000 molecules of mitochondrial phospholipid and 6 to 10 molecules of α-tocopherol per human LDL particle.

$$\alpha\text{-TocH} + R^{\bullet} \rightarrow \alpha\text{-Toc}^{\bullet} + RH \qquad (12.2.29)$$

$$\alpha\text{-Toc}^{\bullet} + \text{ascorbate} \rightarrow \text{ascorbyl}^{\bullet} + \alpha\text{-TocH} \qquad (12.2.30)$$

$$\alpha\text{-Toc}^{\bullet} + CoQH_2 \rightarrow CoQH^{\bullet} + \alpha - \text{TocH} \qquad (12.2.31)$$

Trolox, a soluble synthetic vitamin E analog, is also a powerful antioxidant that is effective against lipid peroxidation. Note that the $E^{0\prime}$ value for the $\text{Trolox}^{\bullet}/\text{Trolox}$ pair is almost the same as the value for α-$Toc^{\bullet}/\alpha$-TocH (Table 12.3).

Vitamin E deficiency in mammals causes higher susceptibility to lipid peroxidation when animals are exposed to oxidizing agents. α-Tocopherol may indirectly inhibit protein and DNA injury as well because (i) products of lipid peroxidation can damage protein residues (see Section 12.4) and DNA bases and (ii) peroxidation of membranes can prompt calcium-dependent oxidative stress (see Section 12.3), leading to DNA and protein oxidation.

Vitamin E contents in humans (either normal dietary contents or levels achieved with vitamin E supplements) have been correlated with the incidence of cardiovascular diseases and various types of cancer showing either beneficial effects or no effects. It is not easy to isolate the effect of vitamin E itself from the effects of various other dietary antioxidants and many other confounding factors. Possibly, only individuals severely deprived of vitamin E (and with very bad diet habits!), which are more prone to be at risk for cardiovascular diseases, have a better response to vitamin E supplementation (see also Section 13.4).

Vitamin E, mostly α-tocopherol, has other nonantioxidant functions in organisms. It participates in the structure of membranes and in signal transduction pathways. For example, α-tocopherol is an inhibitor of protein kinase C. These observations indicate that health-benefits roles of vitamin E may not be solely attributable to the antioxidant actions of vitamin E.

12.2.2.4 Carotenoids* In the 1930s it was discovered that vitamin A originates in the gut and liver from carotenoids. Although ~600 carotenoids have been described, only about 50 have provitamin A activity. The major carotenoids in human plasma are α- and β-carotene, lycopene (see Fig. 12.4), cryptoxanthin, and lutein; these are almost exclusively found associated with lipoproteins. In terms of the total body pool of carotenoids, major storage organs include the liver and adipose tissue. Excellent dietary sources are dairy products, yellow and green vegetables, fish, eggs, and organ meats; tomato is very rich in lycopene.

The antioxidant potential of carotenoid molecules was first described in the early 1930s by Monaghan and Schmitt (*J Biol Chem* **96**:387–395, 1932) while studying a lipid peroxidation process. In the late 1960s, Foote and Denny (*J Am Chem Soc* **90**:6233–6235, 1968) showed the quenching of singlet oxygen ($^1O_2$) by carotenoids. The antioxidant behavior of carotenoids is dependent on their structures and on the nature of the oxidizing species. Most information on their antioxidant potential has been obtained from *in vitro* experiments that test individual carotenoids in organic solvents with one species of ROS. The interaction of carotenoids with $^1O_2$ occurs by a transfer of the excitation energy to the carotenoid or by a chemical quenching of $^1O_2$; the latter results in the irreversible destruction of the carotenoid molecule. Carotenoids may interact with oxygen radicals by three main ways: electron transfer, hydrogen abstraction, and addition of a radical species [reactions (12.2.32) to (12.2.34) for the case of $ROO^{\bullet}$; carotenoids are abbreviated as CAR].

$$ROO^{\bullet} + CAR \rightarrow ROO^{-} + CAR^{\bullet +} \qquad (12.2.32)$$

$$ROO^{\bullet} + CAR \rightarrow ROOH + CAR^{\bullet} \qquad (12.2.33)$$

$$ROO^{\bullet} + CAR \rightarrow (\text{ROO-CAR})^{\bullet} \qquad (12.2.34)$$

In biological systems carotenoids rarely occur as free molecules but are associated with proteins or lipoproteins, and their distribution is very heterogeneous in tissues. The microenvironment where the carotenoid molecule is located may have a profound effect on its antioxidant properties. β-Carotene and lycopene, for example, lie parallel

*Section 12.2.2.4 was prepared in collaboration with Ph.D. student Sandra Arruda, from Universidade de Brasília.

to the membrane surface, within the hydrophobic core, whereas the dihydroxy carotenoids such as zeaxanthin span the membrane entirely. β-Cryptoxanthin and zeaxanthin have greater protective effects against peroxyl radicals in the aqueous phase of liposomal membranes as compared to β-carotene and lycopene, even though β-carotene and zeaxanthin have the same conjugated C=C chain length. The predicted collision rates of β-carotene with peroxyl radicals within the membrane would be very low and, in this case, these molecules are expected to be poor antioxidants. Therefore, differences in the antioxidant actions of various carotenoids can be attributed to differences in their location within the lipid bilayer.

Carotenoids can function synergistically with α-tocopherol and vitamin C to provide an effective barrier against oxidation. An integrated mechanism for the interaction of vitamins C and E with β-carotene has been proposed, where the carotenoid molecule recycles vitamin E [reaction (12.2.35)] and the resulting carotenoid radical is, in turn, "regenerated" by vitamin C [reaction (12.2.36)].

$$\text{CAR} + \alpha\text{-Toc}^{\bullet} \rightarrow \alpha\text{-TocH} + \text{CAR}^{\bullet+} \qquad (12.2.35)$$

$$\text{CAR}^{\bullet+} + \text{ascorbate} + \text{H}^{+} \rightarrow \text{CAR} + \text{ascorbyl}^{\bullet} \qquad (12.2.36)$$

The synergistic protection afforded by carotenoids and other co-antioxidants is dependent on the balance between all the components. In humans that smoke, a diminished plasma vitamin C concentration, compared with nonsmokers, leads to an accumulation of β-carotene radical due to an inefficient "regeneration" process. The antioxidant behavior of carotenoids is, in part, dependent upon the partial oxygen pressure; at low pO_2 carotenoid molecules act *in vitro* as a chain-breaking antioxidant. At a high pO_2 carotenoid radicals may react with oxygen to produce a carotenoid peroxyl radical, which may function as a prooxidant.

Epidemiological studies show good correlation between declining incidence of cardiovascular diseases, such as ischemic heart disease, and an increase in dietary intake and plasma concentration of carotenoids and vitamin A. Plasma concentrations of β-carotene above 0.4 to 0.5 μmol/L and of vitamin A in the range of 2.2 to 2.8 μmol/L have been proposed to reduce the risk of such diseases. However, those studies do not exclude some lifestyle choices (as discussed above for the case of vitamin E) that may reduce the risk of cardiovascular diseases.

Human studies with rigorous control of different variables may provide a better view of the role of carotenoids in public health. The double-blind placebo-controlled studies ATBC (Alpha-Tocopherol Beta-Carotene; see *N Engl J Med* **330**:1029–1035, 1994) and US CARET (β-carotene and retinol efficacy trial; *N Engl J Med* **334**:1150–1155, 1996) tested whether vitamin E and/or β-carotene supplementation could reduce the incidence of cancers and cardiovascular diseases in former smokers or workers exposed to asbestos. Curiously, analysis of both trials found a higher incidence of fatal coronary heart disease and lung cancer in β-carotene-supplemented groups. On the other hand, experimental studies in rats subjected to ischemia-reperfusion stress (see Section 13.5) showed a significant decrease in plasma and liver retinol, suggesting that carotenoids may act against oxidant injury in infarction.

Much more work is required to reconcile the vastly different results from intervention trials and epidemiological and experimental studies regarding the *in vivo* role of carotenoids. In any case, it is a good precaution for smokers (who will not quit this bad habit!) to avoid β-carotene supplements.

12.3 LIPID PEROXIDATION

Most of today's knowledge about the chemical aspects of lipid peroxidation in biological systems was borrowed from the oil and food industry. It has long been known that rancidity of oils and meat is caused by lipid peroxidation. The first determination of the relevance of oxygen to oil viscosity during storage was done in the early nineteenth century (see Section 12.3.2). However, it was not until the 1960s that we became aware that lipid peroxidation could be a relevant event in biology and medicine.

12.3.1 Introduction

The process of lipid peroxidation is considered to be a major cause of cell injury and death. Many pathological conditions and human diseases are connected with lipid peroxidation, as will be discussed later in this chapter. Basically, lipid peroxidation is a chain reaction, in most cases catalyzed by transition metals, where strong oxidants cause the breakdown of membrane phospholipids that contain polyunsaturated fatty acids (PUFAs). Lipid peroxidation damage to biological membranes can have several levels of severity depending on the nature and concentration of the oxidant, ranging from local reductions in membrane fluidity to full disruption of bilayer integrity. LDL particles are also major targets of lipid peroxidation.

Lipid peroxidation of endoplasmic or sarcoplasmic reticulum may cause uncontrolled Ca^{2+} flux into the cytoplasm. This can be a disastrous event for cellular metabolic processes since elevated Ca^{2+} can activate/deactivate enzymes at inappropriate times. Moreover, Ca^{2+}-dependent proteases, phospholipases, and endonucleases can undergo uncontrolled activation, causing degradation of enzymes, membranes, and DNA. Calcium also stimu-

lates nitric oxide synthase, which will prompt $ONOO^-$ formation from nitric oxide and O_2^- (see Section 12.1.3). Furthermore, peroxidation of mitochondrial membranes alters the functioning of the respiratory chain, which could lead to insufficient ATP production and exhaustion of cell energy. Peroxidative damage to mitochondria, a process that is also mediated by Ca^{2+}, causes swelling, membrane disruption, and a loss of the selective permeability of the inner membrane.

Peroxidation of the plasma membrane can affect membrane permeability (the membrane may even be ruptured) and the proper functioning of hormone receptors and membrane proteins involved in signal transduction pathways. Moreover, cell contents, from small molecules to proteins, can be released into the extracellular fluids as a consequence of membrane rupture (see Text Box 12.5 for the effect of H_2O_2 in the induction of cellular lipid peroxidation).

12.3.2 Initiation and Propagation Phases of Lipid Peroxidation

The initiation of lipid peroxidation involves abstraction of a hydrogen atom of a methylene group of a PUFA molecule (LH), caused by the oxidizing species and forming a carbon-centered lipid radical ($L^\bullet$) [reaction (12.3.1); see also Fig. 12.5]. Methylene-bridged double bonds of common PUFA species [18 : 2(ω-6) linoleic, 18 : 3(ω-3) α-linolenic, 20 : 4(ω-6) arachidonic, 20 : 5(ω-3) eicosapentaenoic, and 22 : 6(ω-3) docosahexaenoic acids] are much more easily oxidized than are monounsaturated fatty acids [such as 16 : 1(ω-7) palmitoleic and 18 : 1(ω-9) oleic acids]. This is due to the weaker nature of the bis-allylic C–H bonds (bond dissociation energy (BDE) of about 315 kJ/mol) compared to monoallylic C–H bonds (BDE of about 370 kJ/mol). Moreover, the higher the number of double bonds of a PUFA, the greater the susceptibility of the fatty acid molecule to undergo peroxidation. Thus, 18 : 2(ω-6) and 22 : 6(ω-3) have the lowest and the highest susceptibilities, respectively, for peroxidation among common PUFA species.

Thermodynamics is useful to explain why bis-allylic hydrogen is highly oxidizable (the $E^{0\prime}$ of the $PUFA^\bullet$/PUFA couple is +0.60 V; see Table 12.3) as compared with aliphatic hydrogen from saturated fatty acids ($E^{0\prime} = +1.9$ V). By thermodynamic rules, several oxidizing agents can abstract hydrogen from PUFAs. Observe in Table 12.3 that oxygen and H_2O_2 cannot react with PUFAs due to their half-cell $E^{0\prime}$ values (−0.33 V and +0.32 V for the couples O_2/O_2^- and $H_2O_2/{}^\bullet OH$, respectively). Superoxide radical cannot initiate peroxidation, even though its $E^{0\prime}$ value (+0.94 V for the couple O_2^-/H_2O_2) would allow it. This is because of the charged nature of superoxide, making it very unlikely to enter the hydrophopic interior of the membrane bilayer. However, the protonated form of superoxide ($HOO^\bullet$; $E^{0\prime}$ value of +1.06 V for the couple $HOO^\bullet/H_2O_2$) is able to abstract hydrogen from PUFAs; the rate constant of the reaction is about $2 \times 10^3\ M^{-1}\ s^{-1}$.

Hydroxyl radical is to be considered the most important species involved in the initiation of lipid peroxidation. The rate constant for the reaction of $^\bullet OH$ with artificial membranes under very controlled conditions is about $5 \times 10^8\ M^{-1}\ s^{-1}$. However, there is major controversy in the literature about the nature of the $^\bullet OH$ sources and the locations and velocities of $^\bullet OH$ formation when considering the biological/pathological relevance of $^\bullet OH$-mediated initiation of lipid peroxidation. Hydroxyl radicals also have an extremely short half-life (a few nanoseconds), and they react with many other cell targets before reaching the interior of a membrane bilayer. Several authors have considered that peroxynitrite, $^\bullet NO_2$, perferryl species (Fe^{3+}–O_2^-), ferryl species (Fe^{4+}=O), and even the radical form of vitamin E (see Section 12.3.3) are of importance for the initiation reactions of lipid peroxidation. Ozone can also react with PUFAs generating ozonides, which decomposes to cytotoxic aldehydes; thus, ozone is not an initiator of lipid peroxidation. Iron ions may also participate in the initiation step of peroxidation by directly promoting the formation of perferryl and ferryl species, as well as catalyzing the formation of $^\bullet OH$ radicals. Copper ions are also biologically active in promoting $^\bullet OH$ formation and lipid peroxidation, especially in studies of copper-mediated peroxidation of LDL particles (see discussion in Chapter 13 about atherosclerosis).

After a carbon-centered lipid radical forms, it tends to stabilize by means of electron resonance forming a conjugated diene [reaction (12.3.2); see Fig. 12.5]. Conjugated dienes can be measured at 234 nm and studies with artificial bilayer preparations, microsomes, LDL particles, or cell cultures under free radical attack have shown a marked time-dependent increase in absorbance at 234 nm. Thus, measurement of the kinetics of conjugated diene formation under different experimental protocols is an important tool in biochemistry, with clinical and biological applications. This assay allows for the measurement of the effect of agents that increase (oxidants) or decrease (antioxidants) the rate of lipid peroxidation reactions. The assay is also relevant for studies of the peroxidability of different membrane compositions. Preformed conjugated dienes in animal and plant tissues can also be quantified using a second-derivative spectrum analysis after lipid extraction in organic solvents. This technique has been subject to much criticism due to the fact that it is not possible to determine the absolute amount of conjugated diene in the samples. However, when comparing results with

TEXT BOX 12.5 CELL DAMAGE CAUSED BY H_2O_2-MEDIATED LIPID PEROXIDATION

As mentioned in Section 12.3.1, lipid peroxidation can cause major injury to cells and tissues. In the early 1990s, David Janero and co-workers from Ciba Pharmaceuticals, New Jersey, provided an excellent example of the damaging effects of H_2O_2 in cultures of neonatal-rat cardiomyocytes (see *J Cell Physiol* **149**:347–364, 1991). They observed that H_2O_2 caused dose-dependent lipid peroxidation (by measuring TBARS, conjugated dienes and endogenous vitamin E contents). The illustration (Fig. TB12.1) shows the concentrations of conjugated dienes and TBARS as a function of the period of exposure of cardiomyocytes to 250 μM H_2O_2. The peaks of concentration of conjugated dienes and TBARS occurred at 30 and 45 min, respectively; similar results were observed with 50 or 500 μM H_2O_2. The offset in peak concentration occurs because conjugated dienes are one of the first products of the "lipid peroxidation pathway" (see Fig. 12.5), whereas TBARS are termination products and therefore take longer to accumulate. Moreover, both are produced and decomposed; conjugated diene levels diminish due to the ongoing chain of peroxidation reactions whereas malondialdehyde and other aldehyde products that can react with TBA (see Text Box 12.7) are metabolized by aldehyde dehydrogenases. In addition, vitamin E concentration in cardiomyocytes decreased very rapidly upon H_2O_2 exposure, indicating that it was used as a chain-breaking antioxidant.

The lethal effect of H_2O_2 on cardiomyocytes was attested by the fall in ATP content. The lag period preceding ATP loss (see Fig. TB12.1) is indicative of a process that is induced by lipid peroxidation. Sarcolemmal rupture of the cardiomyocytes mediated by H_2O_2 was demonstrated by leakage of the enzyme lactate dehydrogenase into the medium. Addition of Trolox or iron chelators (*o*-phenanthroline and deferoxamine) to the cell cultures before addition of H_2O_2 (at 500 μM) prevented lipid peroxidation and sarcolemmal disruption. These results indicate that lipid peroxidation is mediated by iron-catalyzed reactions. H_2O_2 added to the cultures reacts with intracellular iron and produces ROS (via Fenton reactions) that initiates/propagates the peroxidation process, leading to membrane disruption and cell death.

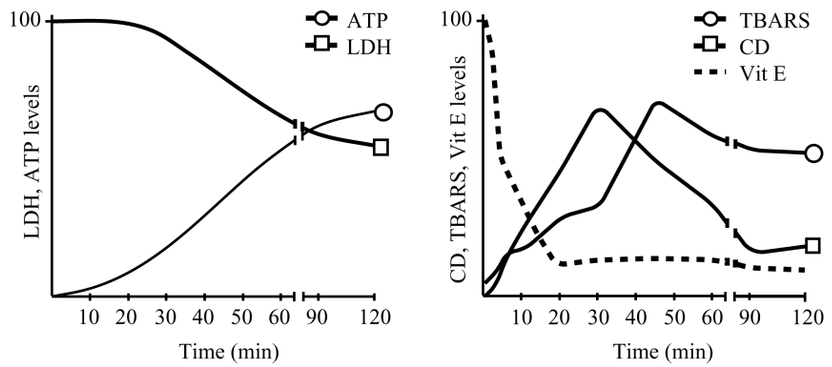


Figure TB12.1 Oxidative stress in cultured cardiomyocytes induced by H_2O_2. The figure on the left shows the decline in cellular ATP levels and the augmentation of extracellular lactate dehydrogenase (LDH) levels (caused by leakage from cells) after challenge by 0.25 mM H_2O_2. The figure on the right shows the corresponding changes in α-tocopherol (Vit E) and lipid peroxidation products: conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS). Data modified from Janero et al. (1991).

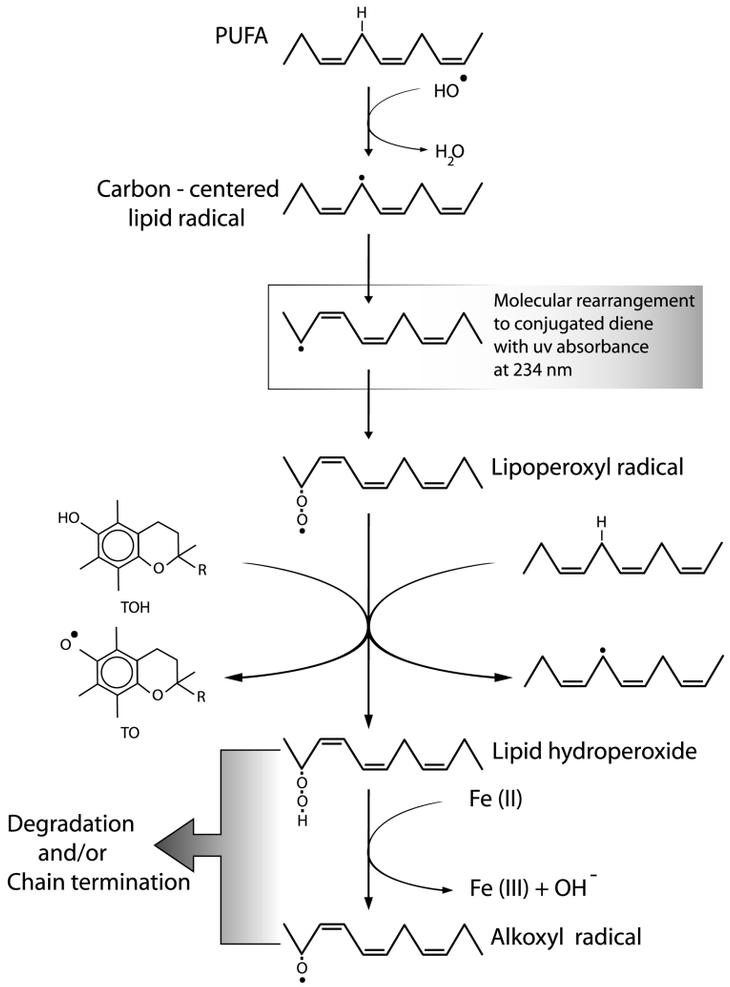


Figure 12.5 The basic scheme of lipid peroxidation reactions. A lipid molecule reacts with an oxidizing agent ($^{\bullet}$OH in this case) resulting in a carbon-centered lipid radical. Upon reaction with oxygen, it is converted in a lipoperoxyl radical, which may react (i) with another PUFA (producing a chain reaction, since a new carbon-centered lipid radical is produced) or (ii) with α-tocopherol (TOH), which is converted in tocopheryl (TO), producing in either case a lipid hydroperoxide. The later is then converted to an alkoxyl lipid radical upon reaction with iron. Lipid hydroperoxides and alkoxyl radicals undergo numerous routes for degradation and chain termination.

proper controls, relative values of conjugated dienes (percent increases or decreases) have been well-accepted in the literature.

Initiation reactions:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH \qquad (12.3.1)$$

$$L^{\bullet} \rightarrow \text{conjugated diene } L^{\bullet} \qquad (12.3.2)$$

$$O_2 + \text{conjugated diene } L^{\bullet} \rightarrow LOO^{\bullet} \qquad (12.3.3)$$

The next step in the *peroxidation pathway* is the reaction of a PUFA-conjugated diene (a carbon-centered $L^{\bullet}$ species) with molecular oxygen to yield a peroxyl radical ($LOO^{\bullet}$) [reaction (12.3.3); see Fig. 12.5] that feeds the propagation step of peroxidation. $L^{\bullet}$ can also undergo reaction with other $L^{\bullet}$ isomers, but this is only quantitatively relevant under very low O_2 concentrations. ESR methods of spin-trapping (see Section 12.1), mainly using nitroso compounds such as DMPO and PBN, have proven the formation of $LOO^{\bullet}$ in biological membranes. Interestingly, it has been calculated that, in LDL particles, a single $L^{\bullet}$ can initiate the oxidation of 30 to 50 other PUFA molecules.

Propagation reactions:

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (12.3.4)$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \quad (12.3.5)$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \quad (12.3.6)$$

Propagation of lipid peroxidation takes place by the reaction of $LOO^{\bullet}$ with a methylene group on a nonoxidized PUFA molecule to yield a lipid hydroperoxide (LOOH; see Text Box 12.6) and another PUFA carbon-centered radical ($L^{\bullet}$) [reaction (12.3.4)]. The new carbon-centered radical can react with O_2 to start a chain reaction (Fig. 12.5). The O–O bond of $LOO^{\bullet}$ or LOOH species can also undergo cyclization and/or fragmentation, leading to termination reactions (see Section 12.3.3).

The most common (indirect) method for assessing the peroxidation reaction is O_2 uptake (using a classical Clark-type electrode). Since this method is very simple [it determines reaction (12.3.3)], many studies have taken advantage of its usefulness for determining the effects of oxidants and antioxidants under *in vitro* conditions.

The oldest assessment of peroxidation reactions was done early in the nineteenth century by de Saussure who determined air absorption (using a mercury manometer) into walnut oil during long-term storage (see Halliwell and Gutteridge, 1999). Over the course of 8 months of observation very little air was absorbed, only 3 times the volume of oil (actually oxygen was being consumed and incorporated into the oil). After that, within 10 days, a fast reaction occurred and air absorption was then 60 times the oil volume; the oil viscosity also increased incredibly, producing an "evil smell" in de Saussure's words. Finally, the rate of air absorption decreased gradually over the following 3 months. What de Saussure had observed was the 3 main phases of lipid peroxidation: *initiation*, *propagation*, and *termination*. The kinetics of both conjugated diene formation and O_2 consumption in biomembrane preparations in modern experiments also display a slow phase (lag phase, or initiation) followed by a very rapid phase (log phase, or propagation) and a termination phase (Fig. 12.6).

TEXT BOX 12.6 LIPID HYDROPEROXIDE DETERMINATION

Various methods have been used to determine LOOH in biological samples. Iodometric determinations of LOOH were mostly employed in the 1970s and 1980s. These are based on the reaction of iodide (I^{-}) with LOOH, yielding L-OH (an alcohol) and iodine (I_2), the latter quantified with sodium thiosulfate. This reaction is not very useful for tissue samples because many other biological oxidizing agents can catalyze I_2 formation. Determination of LOOH by a glutathione peroxidase (cGPx) assay that measures the yield of NADPH oxidation at 340 nm ($LOOH + 2GSH + NADPH \rightarrow L\text{-}OH + H_2O + GSSG + NADP^{+}$), was developed in the 1980s. However, this assay needs highly purified cGPx (an expensive enzyme) and cannot measure LOOH from membranes unless these are first disrupted using purified phospholipase. Determination of LOOH by oxidation of a ferrous complex with xylenol orange at acid pH has been used for membrane preparations. The resulting Fe^{3+}–xylenol orange complex is measured at 580 nm (the reaction involves propagation mechanisms that are still not fully understood).

In the mid 1990s, Hermes-Lima and co-workers adapted the xylenol orange assay for use with tissue extracts to compare control versus stressed conditions in biological and clinical studies (see *Free Radic Biol Med* **19**:271–280, 1995). For example, it is well-known that ischemia/reperfusion of mammalian organs induces an increase in lipid peroxidation products, including increased levels of conjugated dienes (see Chapter 13). The ventricular levels of iron–xylenol orange-reactive LOOH increased by about 1.6-fold after 20 min of coronary occlusion, followed by 10 min of reperfusion. The corresponding increase in ventricular TBARS (a classical assay for peroxidation; see Text Box 12.7) was about 1.5-fold in these experiments.

12.3.2.1 Metals and Lipid Peroxidation Propagation Iron and copper ions play a pivotal role in the process of lipid peroxidation, mostly by means of LOOH decomposition (metals are also involved in the initiation of lipid peroxidation, by catalyzing the formation of ROS). Fe^{2+} and Fe^{3+} [bound to ligands such as ethylenediaminetetraacetic acid (EDTA), citrate, or adenosine diphosphate (ADP)] may react with LOOH, producing either peroxyl ($LOO^{\bullet}$) or alkoxyl species ($LO^{\bullet}$), which feed the propagation process [reactions (12.3.5) and (12.3.6)].

For example, Fe^{2+}-citrate or Fe^{2+}–ADP (but not Fe^{2+} alone) can induce severe damage *in vitro* to mitochondrial functions, such as loss of transmembrane electrochemical potential. The severity of the damage is correlated with the increase in the initial rate of O_2 uptake (caused by lipid peroxidation) of the mitochondrial membranes and the formation of aldehydic by-products of peroxidation (see Section 12.3.3.2).

Certain iron chelators (see Text Box 12.1) such as deferoxamine and pyridoxal isonicotinoyl hydrazone (PIH) are

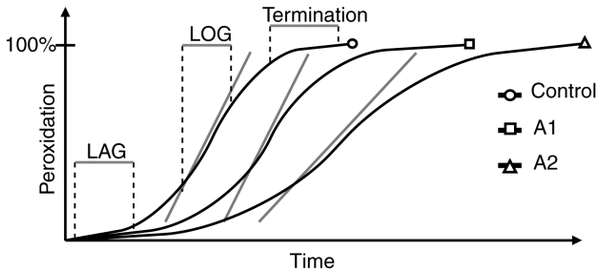


Figure 12.6 A typical time course of a lipid peroxidation reaction (the *y* axis represents product formation, such as TBARS or conjugated dienes), showing the three phases of the process: initiation (lag phase), propagation (log phase), and termination. It also shows the effect of two types of antioxidants (A1 and A2). Both augment the period of the initiation phase, but just one of them (A2) affects the log phase of the reaction.

able to inhibit lipid peroxidation in biomembranes by forming a complex with Fe^{3+} [see reaction (12.3.7) for the case of PIH]. The resulting complex is not redox active and thus cannot receive electrons from LOOH [or very low rates of reaction (12.3.8) may occur]. By functionally arresting $LOO^{\bullet}$ formation, these chelators may stop the propagation of the peroxidation process and lead to a diminished formation of by-products of lipid peroxidation, such as aldehydes.

$$\begin{aligned} Fe^{2+}\text{-citrate} + 2PIH &\rightarrow Fe^{2+}\text{–}PIH_2 + \text{citrate} \\ &\rightarrow Fe^{3+}\text{–}PIH_2 \end{aligned} \qquad (12.3.7)$$

$$\begin{aligned} Fe^{3+}\text{–}PIH_2 + LOOH &\rightarrow Fe^{2+}\text{–}PIH_2 \\ &\quad + LOO^{\bullet} + H^{+} \end{aligned} \qquad (12.3.8)$$

In the 1980s, American researcher Steven D. Aust proposed that iron-catalyzed lipid peroxidation depends on an optimum 1 : 1 ratio of Fe^{2+} to Fe^{3+} at the beginning of the process [Minotti and Aust (1987) *J Biol Chem* **262**:1098–1104]. Aust suggested that a $Fe^{2+}\text{–}Fe^{3+}\text{–}O_2$ complex is involved in the initiation of the peroxidation reaction. This view has permeated the literature for over two decades, even though the proposed oxidant was never isolated. Many authors (including myself) do not believe this complex exists.

Lipid peroxidation mediated by iron and copper ions has been associated with the etiology of several human diseases. Metal-mediated peroxidation is also considered a "worsening event" in many diseases and pathological process. One of the best-studied cases is the liver cirrhotic condition caused by hemochromatosis, a genetic disease with high prevalence in North America that is caused by a defect in the iron absorption mechanism that leads to ferritin–iron accumulation in liver and heart (see Text Box 12.1). This continuous load of hepatic iron increases the levels of redox-available "free iron," which is iron bound to low-molecular-weight molecules (such as citrate, ATP, ADP, and phosphate). Free iron may then initiate/propagate peroxidation reactions, inflicting membrane damage and causing the formation of toxic peroxidation termination products (such as aldehydes, see section below), which can react with DNA and proteins.

In addition, several neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, are connected to increased iron levels in certain brain areas, leading to lipid peroxidation and neuronal dysfunction (see Chapter 13). Copper-mediated peroxidation of LDL particles has been also associated with the etiology of plaque formation in atherosclerosis. The determination of termination products of lipid peroxidation has been very useful for the study of lipid peroxidation in human diseases.

12.3.3 Termination of Peroxidation Reaction and Its Consequences

Termination of the peroxidation chain is caused by (i) reaction between two lipid radical species [reaction (12.3.9)], (ii) decomposition of LOOH, $LO^{\bullet}$, and $LOO^{\bullet}$ species to form many by-products (including hydrocarbon gases, F_2-isoprostanes, and aldehydes) [reaction (12.3.10)], or (iii) the presence of lipid-soluble phenolic antioxidant compounds such as α-tocopherol or butylated hydroxytoluene (BHT, an artificial antioxidant used extensively in the food industry) [reaction (12.3.11)]. Antioxidant molecules (AH) react with $LOO^{\bullet}$ yielding LOOH and a phenolic radical that is resonance-stabilized ($A^{\bullet}$). For example, tocopheryl radical is formed upon reaction of α-tocopherol with $LOO^{\bullet}$ (Fig. 12.5; see Section 12.3.4 for further

discussions about chain-break antioxidants).

$$LO^{\bullet} + LO^{\bullet} \rightarrow LOOL \quad (12.3.9)$$

$$LOOH \rightarrow\rightarrow\rightarrow \text{aldehydes, alkanes, and isoprostanes (mostly metal-mediated reactions)} \quad (12.3.10)$$

$$AH + LOO^{\bullet} \rightarrow A^{\bullet} + LOOH \quad (12.3.11)$$

The fate of the termination products of lipid peroxidation is also important for their effects in the cells and for analytical biochemistry. We shall next discuss some relevant classes of peroxidation by-products.

12.3.3.1 Hydrocarbon Gases Volatile hydrocarbon gases, mainly ethane and pentane, are produced by β-scission reactions of $LO^{\bullet}$ species in metal-catalyzed reactions. Although formation of hydrocarbons from lipid peroxidation constitutes only a minor degradation pathway, they are of special interest for noninvasive studies in laboratory animals and in humans because these volatile gasses can be collected from exhaled breath for analysis by gas-liquid chromatography.

Alkane formation is dependent on the nature of the PUFA. Oxidation of omega-3, omega-4, omega-6, and omega-7 PUFAs enriches the yield of ethane, propane, pentane, and hexane, respectively. Because omega-3 and omega-6 PUFAs are the most abundant PUFAs in biological membranes, ethane and propane are routinely quantified by researchers. Ethane production as a marker of lipid peroxidation has been especially well-documented and validated in studies with rats.

Several studies have correlated increased formation of breath alkanes in humans or laboratory animals under stress conditions (e.g., hyperoxia, extenuating exercise, smoking, liver transplantation), natural aging, and in patients suffering from several pathologies [e.g., rheumatoid arthritis, cystic fibrosis, acute myocardial infarction, multiple sclerosis, schizophrenia, acquired immunodeficiency syndrome (AIDS), Alzheimer's disease]. For example, increased breath pentane has been found in patients with alcoholic hepatitis (1.7 ± 0.7 pmol/mL) and alcoholic cirrhosis (3.4 ± 0.7 pmol/mL) as compared with controls (0.9 ± 0.1 pmol/mL). Hepatic lipid peroxidation is well-known to be elevated in alcoholic cirrhosis. Determination of breath pentane in nonsmoking patients suffering from Crohn's disease, which is an acute intestinal inflammation with intense influx of neutrophils, indicated significantly higher levels than in control subjects (11.6 ± 1.7 vs. 5.8 ± 0.5 pmol/kg/min). Control smokers had breath pentane levels (9.4 ± 1.4 pmol/kg/min) that were similar with those found in patients with Crohn's disease.

12.3.3.2 Aldehydic Products Aldehydes are the most relevant class of products of lipid peroxidation in quantitative terms. The carbonyls formed during lipid peroxidation are composed of several subclasses including *n*-alkenals, 2-alkenals, 2,4-alkadienals, alkatrienals, hydroxyalkenals, hydroxylperoxy-alkenals, α-dicarbonyls, alkanes, alkenes, and (un)saturated ketones. Major carbonyls produced during peroxidation of PUFAs are malondialdehyde (MDA), hexanal, 4-hydroxy-2,3-*trans*-nonenal (4-HNE), 4-hydroxy-2,3-*trans*-hexenal, and 2-propenal (acrolein) (Fig. 12.7).

Several methods have been used for the determination of soluble carbonyls. The most popular one is the formation of thiobarbituric acid reactive substances (TBARS) (see Text Box 12.7). Others include derivatization with dinitrophenylhydrazine (DNPH) followed by separation with HPLC and gas-chromatography coupled with electrochemical determination (CG–EC). These methods have been applied to blood, tissue samples, and urine. For example, rats under treatment with classical peroxidation-inducing chemicals, such as Fe(III)-NTA (nitrilotriacetic acid) or CCl_4, show increased levels of soluble carbonyls in the urine. TBARS (and MDA determination) has also been widely used for studies in animal comparative biochemistry, plant physiology, and food science. Various stress conditions are associated with increased TBARS/MDA (either in specific tissues or determined in the urine or plasma; many of these examples are discussed further in Chapter 13) including hyperoxia, extenuating exercise, reperfusion injury in rodent organs, burn injuries, AIDS, cystic fibrosis, acute myocardial infarction, Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, and diabetes.

The aldehydic products of lipid peroxidation are of a highly toxic nature. The dialdehydes can crosslink and aggregate membrane proteins. For example, valine residues of hemoglobin are very reactive with MDA, forming a stable product with a half-life of several days. Moreover,

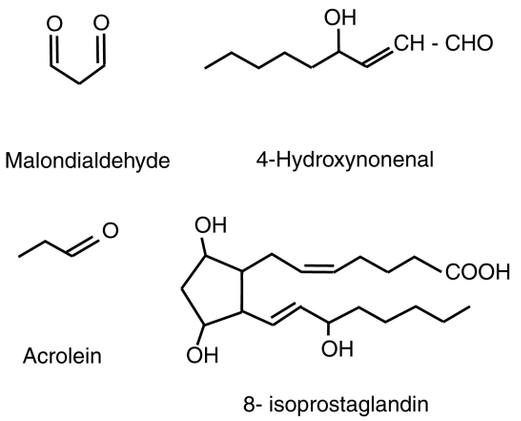


Figure 12.7 Selected end products of lipid peroxidation.

TEXT BOX 12.7 THE TBARS ASSAY

Malondialdehyde (MDA) is one of the end products of lipid peroxidation. It reacts with thiobarbituric acid (TBA) under acid pH and elevated temperature to form a pink fluorescent MDA : TBA (1 : 2 ratio) adduct. Hundreds of studies have used the TBA test (also called thiobarbituric-acid-reactive substances assay or TBARS assay) to quantify MDA formation. However, this assay is neither specific for MDA (other aldehydes formed during peroxidation are also reactive) nor is MDA the main aldehyde product of lipid peroxidation. Thus, the use of the TBARS assay for determination of MDA content in biological samples (using calibration curves with pure MDA) gives an overestimation of the true concentration of this molecule. Moreover, the yield of MDA in peroxidation reactions is dependent on the membrane composition and the nature of the oxidant insult. Since MDA is just one of many products of lipid peroxidation, determination of MDA by HPLC (or by methods of chromatography coupled with mass spectrometry) may underestimate the true extent of the lipid peroxidation process under study. In addition, MDA is not a metabolically inert product of peroxidation; several aldehyde dehydrogenases are capable of converting MDA (and other aldehyde products of peroxidation) to their corresponding alcohols.

Even though measurements of TBARS have been under severe attack from many researchers (a ballot was proposed in the 1995 International Free Radical Meeting for a ban of the TBARS assay!), it is still considered a relevant biomarker of lipid peroxidation in tissue sample preparations, especially for comparative purposes. However, to make conclusions about a specific sample, other methods of measuring lipid peroxidation should be used concomitantly.

accumulation of aldehyde-modified soluble enzymes and membrane proteins compromises normal metabolic functions and may cause cell dysfunction and death. This seems to be a relevant factor in neuronal dysfunction in Alzheimer's disease (see Section 13.2).

Aldehydes also react with DNA, mainly with guanine bases, forming stable adducts. Levels of the fluorescent products (excitation at 315 to 390 nm, emission at 420 to 460 nm) of the interaction of DNA with aldehydes correlate positively with the disruption of DNA physical-chemical properties and the loss of template activity for RNA polymerase. Recently, gas chromatography–mass spectrometry (GC–MS) has been used to identify adducts of aldehydes with DNA. For example, DNA from disease-free human liver was found to contain 5400 MDA–deoxyguanosine adducts per cell. Several studies have shown that DNA modifications caused by lipid peroxidation products have cytotoxic, mutagenic, and carcinogenic consequences (see Section 12.5).

12.3.3.3 Chemiluminescence The reaction of two alkoxyl PUFA radicals yields an alcohol (L-OH) in the ground state and an excited-state ketone [L═O*; see reaction (12.3.12)], which can emit light as it decays to the ground state. Peroxyl radicals can undergo self-reactions yielding singlet oxygen ($^1O_2$) and a carbonyl product that may or not be in the excited state (triplet carbonyl). The determination of light emission from these Russell-type reactions has been an important tool for lipid peroxidation research. Recent studies that have followed chemiluminescence in isolated LDL particles under peroxidation stress have shown good correlation with more traditional methods for quantifying lipid peroxidation, such as measurement of conjugated dienes. Chemiluminescence can also be useful for probing the oxidation resistance of membrane preparations (e.g., caused by lipid-soluble antioxidants under evaluation) without the need of time-consuming extraction procedures.

$$LO^{\bullet} + LO^{\bullet} \rightarrow L{=}O^{*} + L\text{-}OH \qquad (12.3.12)$$

Low-level chemiluminescence can be detected in cells, tissue samples, and isolated organs with the use of highly sensitive photodetectors. The use of chemical enhancers of light emission, such as lucigenin and luminol, can also be helpful. The technique has the advantage of being a noninvasive method for continuous determination of lipid peroxidation. For example, chemiluminescence by using lucigenin was applied for studies of oxidative stress associated with ischemia and reperfusion in isolated rat heart. Lucigenin (at 10^{-5} M) was used in the perfusates to enhance the luminescence signal. Chemiluminescence decreased from 219 ± 11 counts per second (cps) at baseline to 149 ± 9 cps during a 12-min ischemia but markedly increased to a peak of 476 ± 36 cps during 3 to 5 min of reperfusion before decreasing again to near control values over the next 15 to 20 min. Addition of SOD to the perfusate (2000 U/min) caused a significant reduction in chemiluminescence during reperfusion, suggesting that O_2^- radicals participate is the complex mechanism of lipid peroxidation. The studies from the early 1990s demonstrated that free radical generation and lipid peroxidation occurs during the reperfusion phase of the ischemia/reperfusion cardiac stress (see Section 13.5).

12.3.3.4 Isoprostanes A group of prostaglandin-like compounds, known as F_2-isoprostanes, are produced by a free-radical-catalyzed mechanism during peroxidation of arachidonic acid. Their generation involves the formation of peroxyl radical isomers of arachidonic acid, which undergo endocyclization to form prostaglandin-like compounds with subsequent reduction to F_2-isoprostane-like molecules. F_2-isoprostanes are released in the free form from esterified arachidonic acid undergoing peroxidation possibly by the action of phospholipases. The F_2-isoprostane, 8-isoprostaglandin (8-EPI-PGF_{2a}; see Fig. 12.7) was identified some years ago as a product of *in vivo* peroxidation in rats induced by CCl_4.

Determination of F_2-isoprostanes requires sophisticated and expensive methods such as GC–MS. Simpler immunodetection assays for 8-EPI-PGF_{2a} in tissue samples, plasma, and urine have been used since the early 1990s, but there is still controversy over the reliability of this assay for quantification of absolute levels of 8-EPI-PGF_{2a}. In addition, recent studies have shown that isoprostanes are not merely markers of lipid peroxidation. They can also invoke biological and pathological responses in several cell types, especially in lung cells.

As an example of F_2-isoprostane determination in a stress condition, it has been observed increased plasma concentrations of isoprostanes associated with cigarette smoking. Plasma levels of free and esterified F2-isoprostanes were significantly higher in smokers (0.24 ± 0.15 and 0.57 ± 0.22 nM, respectively) than in nonsmokers (0.10 ± 0.02 and 0.35 ± 0.06 nM). Smoking had no short-term effects on the circulating levels of F2-isoprostanes. However, the levels of free and esterified F2-isoprostanes fell significantly (by 38 and 25%, respectively) after 2 weeks of abstinence from smoking. In another study, 8-EPI-PGF_{2a} (measured in the esterified form by a GC–MS technique) was found to be increased in the plasma of non-insulin-dependent diabetes mellitus (NIDDM) patients (0.93 ± 0.07 nM, $n = 39$) when compared to control subjects (0.28 ± 0.04 nM, $n = 15$).

Interestingly, both cigarette smoking and NIDDM have been linked with oxidative stress when biomarkers of DNA oxidative damage (see Section 12.5), protein oxidation (see Section 12.4), and other markers of lipid peroxidation have been assessed.

12.3.4 Antioxidants That Function Against Lipid Peroxidation

Many lipid- and water-soluble molecules provide defenses against lipid peroxidation. These include vitamin E, carotenoids, plant polyphenols, bilirubin, lipoic acid, coenzyme Q, melatonin, uric acid, and various synthetic antioxidants, such as probucol (an antiatherogenic drug; see Section 13.4), BHT, and Trolox (a water-soluble analog of α-tocopherol). Ascorbate can be either a prooxidant by recycling metals to the reduced state, or a quencher of free radicals that could initiate lipid peroxidation. In the following discussions we will focus on α-tocopherol.

As discussed earlier in this chapter (Section 12.2.3), all components of vitamin E are able to function as antiperoxidative agents. In the case of α-tocopherol, it works as an antioxidant of peroxidation reactions by donating hydrogen to lipid hydroperoxyl radicals ($LOO^\bullet$), resulting in tocopheryl radical and LOOH [reaction (12.3.13), which has a rate constant 10,000-fold higher than the reaction of $LOO^\bullet$ with lipids]. Other antioxidants also react with $LOO^\bullet$, including other vitamin E components, coenzyme Q, uric acid, bilirubin, BHT, and probucol. The hydrophobic tail of α-tocopherol anchors the molecule in the membrane, positioning the phenolic –OH groups for reaction with $R-OO^\bullet$ groups (from oxidized phospholipids) at the hydrophilic surface of the membrane. The propagation of *in vitro* peroxidation reactions of biomembranes will only occur when α-tocopherol is depleted; thus, it is called a chain-breaking antioxidant. The increase in α-tocopherol concentration in membranes under pro-peroxidative conditions will further elongate the "lag phase" of lipid peroxidation, but usually not changing the "log phase" (see Fig. 12.6).

$$\alpha\text{-TocH} + LOO^\bullet \rightarrow LOOH + \alpha\text{-Toc}^\bullet \quad (\text{rate constant} = 10^6\ M^{-1}\ s^{-1}) \tag{12.3.13}$$

$$\alpha\text{-Toc}^\bullet + AH(\text{antioxidant}) \rightarrow A^\bullet + \alpha\text{-TocH} \tag{12.3.14}$$

$$\alpha\text{-Toc}^\bullet + LH \rightarrow L^\bullet + \alpha\text{-TocH} \tag{12.3.15}$$

Tocopheryl radical (α-$Toc^\bullet$) can be stabilized by resonance; it can be "recycled" to α-tocopherol by water-soluble ascorbate or lipid-soluble coenzyme Q [reaction (12.3.14)]. Accumulation of α-$Toc^\bullet$ may lead to initiation of lipid peroxidation [reaction (12.3.15)], which explains the prooxidant effect of α-tocopherol. Tocopherol-mediated lipid peroxidation (TMP) is currently a subject of many studies and conflicting conclusions. One can argue that the low rate constants of TMP reactions (about $0.1\ M^{-1}\ s^{-1}$) make them insignificant under *in vivo* conditions, especially considering that the steady-state levels of α-$Toc^\bullet$ are very low. Moreover, several antioxidants may reduce very quickly α-$Toc^\bullet$ to α-tocopherol (rate constants ranging from 10^4 to $10^6\ M^{-1}\ s^{-1}$).

12.4 PROTEIN OXIDATION

Awareness by the scientific community of the clinical and biological relevance of protein oxidation started relatively

later in the short history of free radical research. The boom of studies on this subject started only in the early 1990s. Most investigations have focused on oxidative damage to specific purified enzymes (or structural proteins) or on studies in cells/organelles and tissue samples, where overall protein oxidation is determined and linked to general processes of oxidative stress. As in the case of lipid peroxidation, many diseases and pathological (or even physiological) processes have been connected with protein oxidation (see below).

Unlike the situation of DNA damage, there are few cellular mechanisms involved in providing protection against protein oxidation or acting to repair damaged proteins (see Section 12.4.2). Instead, damaged proteins are targeted for degradation (often via ATP-dependent ubiquitination) by endogenous proteases, including cathepsin c, calpain, trypsin, and the proteasomes. The recycling of oxidized proteins by means of proteolysis is the way that cells prevent the accumulation of malfunctioning proteins. Non-oxidized amino acids are released to be reused for biosynthesis of new proteins. It is interesting that protease biosynthesis is up-regulated under oxidative stress conditions.

Proteins can be damaged by various free-radical-mediated mechanisms including irradiation, products of lipid peroxidation and sugar oxidation (mostly aldehydes, forming carbonyl adducts with proteins), nitric-oxide-derived metabolites, and metal-catalyzed oxidation systems. Other nonradical mechanisms are also of great importance such as cold and heat denaturation, pressure-induced denaturation, and nonenzymatic glycation of proteins (by Amadori chemistry). Glycated proteins are also subjected to oxidation that leads to advanced glycation end products (AGE), which are of major importance in aging and Alzheimer's disease. Glycation of many relevant proteins including the lens proteins (causing cataract) and hemoglobin is also associated with diabetes.

Superoxide radicals inactivate selected enzymes including aconitase (by reducing Fe^{3+} in the enzyme molecule and causing the release of ferrous ions) and other [Fe–S] cluster-containing dehydratases. Inactivation of aconitase is of tremendous biological significance because it is a key enzyme for the normal functioning of the Krebs cycle. Alkyl peroxides (mostly LOOH) and H_2O_2, in general, are not capable of inflicting relevant protein damage. Various reports of H_2O_2-induced *in vitro* enzyme inactivation must be carefully examined because the presence of contaminating transition metals in the solutions can promote $^{\bullet}OH$ formation via Fenton-like reactions.

$$O_2^- + Fe^{3+}\text{–ligand} \rightarrow Fe^{2+} + \text{ligand} + O_2 \quad (12.4.1)$$

$$Fe^{2+} + \text{protein} \rightarrow Fe^{2+}\text{–protein} \quad (12.4.2)$$

$$Fe^{2+}\text{–protein} + H_2O_2 \rightarrow Fe^{3+}\text{–protein} + OH^- + {}^{\bullet}OH\,(\text{site-specific formation}) \quad (12.4.3)$$

$$Fe^{3+}\text{–protein} + {}^{\bullet}OH \rightarrow \text{oxidized protein} + Fe^{3+} \quad (12.4.4)$$

Hydroxyl radicals formed by various types of irradiation can oxidize virtually all amino acid residues of proteins. Irradiation studies with purified proteins have led to the identification of most of the products of oxidation of amino acid residues (see Table 12.5). Moreover, peptide bond cleavage can also result from attack by ROS on glutamyl, aspartyl, and prolyl residues. The most important source of protein oxidation seems to be meditated by the binding of low amounts of iron or copper to the protein structures. This prompts the formation of radical species, upon reaction with H_2O_2 or alkyl peroxides, and induction of site-specific protein damage [reactions (12.4.1) to (12.4.4)]. Metal-catalyzed oxidation is involved in the damage to most enzymes, membrane receptors, and structural proteins.

TABLE 12.5 Oxidation of Amino Acid Residues and Some of Their Products

Phenylalanine → 3-nitrophenylanaline,[a] *ortho*-, *para*-, and *meta*-tyrosine
Tyrosine → 3-nitrotyrosine,[a] dityrosine, dihydroxyphenylalanine (DOPA)
Histidine → 2-oxo-histidine
Tryptophan → 6-nitrotryptophan,[a] hydroxytryptophan, kynurenine, formylkynurenine
Methionine → methionine sulfoxide, methionine sulfone
Cysteine → disulfite derivatives
Leucine and valine → hydroxy derivatives
Threonine → 2-amino-3-ketobutyric acid
Lysine and arginine → carbonyl derivatives
Proline → carbonyl derivatives, 2-pyrrolidone

[a]Products of reactive nitrogen species (RNS) attack.
Source: Data adapted from Stadtman and Levine (2000).

12.4.1 *In Vitro* Studies on Protein Oxidation

Many studies were conducted through the 1990s on oxidative damage to purified proteins. Typically, the focus was on the relationship between enzyme activity and the concentration of the oxidizing agent, whereas enzyme structural changes were also assessed by fluorescence studies, electrophoresis, and immunoblotting. Levels of oxidized products of amino acid residues, such as carbonyls, oxidized –SH groups, and dityrosine were also quantified.

An interesting example of the study of protein inactivation by oxidation was conducted several years ago in Earl Stadtman's laboratory (National Institutes of Health, Maryland). It assessed the effect of micromolar concentrations of Fe^{2+} and H_2O_2 on the loss of activity by glucose-6-phosphate dehydrogenase (G6PDH) purified from *Leuconostoc mesenteroides* (Szweda and Stadtman, *J Biol Chem* **267**:3096–3100, 1992). Enzyme inactivation was correlated with the formation of one carbonyl group (see Section 12.4.3) per enzyme subunit, showing that inactivation is the result of site-specific oxidative modification. The results suggested that Fe^{2+} binds to the glucose-6-phosphate binding site and that interaction of the enzyme-bound Fe^{2+} with H_2O_2 leads to the oxidative modification of amino acid residues that are essential for enzyme activity. H_2O_2 alone was unable to trigger a loss of enzyme activity. This example is of special relevance because G6PDH is key to NADPH production for antioxidant defense (see Section 12.2).

Another interesting study determined the inactivation of purified RNase and lysozyme by means of irradiation or copper-catalyzed oxidation. Using fluorometric methods, increased formation of *ortho*-tyrosine and dityrosine in both enzymes under oxidative attack was observed.

TEXT BOX 12.8 DETERMINATION OF PROTEIN THIOLS

Reduced thiols from soluble or membrane proteins are widely measured by the Ellman procedure, which was developed in the 1950s. This reaction with 5,5′,dithiobis-2(2-nitrobenzoic acid) (DTNB) yields *p*-nitrothiophenol anions that absorb at 412 nm (extinction coefficient = 13.6 $mM^{-1}\,cm^{-1}$). A note of caution for experimental design is the fact that the *p*-nitrothiophenol anion is oxidized/decomposed by ROS. Thus, addition of catalase to the assays before inclusion of DTNB can help to prevent severe underestimation of protein –SH groups in studies with purified enzymes or cell extracts under oxidative stress. For example, ROS induce the loss of 30 to 45% of –SH groups within 30 min in purified rabbit liver metallothionein, a protein that has cysteine as one-third of its amino acid content. These determinations would be jeopardized if catalase was not used in the analytical procedure (Suzuki et al., *Free Radic Biol Med* **9**:479–484, 1990).

12.4.2 Oxidation of Sulfur-Containing Residues and Tyrosine Residues

Cysteine and methionine residues are highly susceptible to oxidation by ROS. Cysteine residues are converted to disulfides and methionine residues are converted to methionine sulfoxide (MeSOX) residues. Virtually all biological systems contain thiol–disulfide oxidoreductases (see Section 12.2.1.5) and MeSOX reductases, which can reconvert the oxidized residues into the original forms. These are the only oxidative modifications to proteins that can be repaired. Moreover, it has been proposed that the cycle of oxidation/re-reduction of methionine residues functions as a "sink" for free radical attack on proteins (notably, the oxidation of exposed methionine residues generally has little effect on enzyme activities). Thus, methionine residues can be considered oxyradical scavengers. It is of relevance that the activity of MeSOX reductase is decreased in brains of Alzheimer's patients, and this may be connected with the increased damage to the methionine-containing amyloid β-peptide (Aβ) in this disease (see Section 13.2).

Assessment of the loss of protein –SH groups (by means of oxidation) is also a relevant indicator of oxidative stress affecting proteins. Several reactive species such as *t*-butyl hydroperoxide, menadione, $ONOO^-$ and $^{\bullet}OH$ are capable of oxidizing protein –SH groups. The oxidative loss of activity by many enzymes (in studies with purified enzymes) correlates with the oxidation of sulfhydryl groups. Protein thiols are also easily measurable in tissue samples (see Text Box 12.8) and constitute a relevant marker of oxidative stress.

Reactive nitrogen species (RNS) are also capable of reacting with several amino acid residues, including tyrosine, cysteine, tryptophan, and methionine. Particularly interesting is the reaction of nitrogen dioxide ($^{\bullet}NO_2$) and peroxynitrite with tyrosine residues, yielding 3-nitrotyrosine and dityrosine (also formed from $^{\bullet}OH$ attack), which can alter enzyme activities. Determination of dityrosine is done by fluorescence detection (excitation 284 nm, emission 410 nm) in purified enzymes after acid hydrolysis. Formation of 3-nitrotyrosine can be measured at 428 nm (extinction coefficient = 4.2 $mM^{-1}\,cm^{-1}$) from purified enzymes such as bovine serum albumin. Moreover, detection of 3-nitrotyrosine in tissue samples (e.g., in brain of Alzheimer's patients) by means of GC–MS or Western blotting is considered a relevant biomarker for *in vivo* damage induced by RNS.

12.4.3 Carbonyl Protein

The carbonyl derivatives of proteins are the most relevant products of free radical attack on proteins. Protein carbonyl can also be formed from the reaction of aldehydes (formed from lipid peroxidation and sugar oxidation) with proteins. The presence of carbonyl proteins in cell and tissue samples has become a widely accepted biomarker of oxidative

stress. This has led to the development of several highly sensitive methods for their determination. The quantification of protein carbonyl groups is done by derivatization with 2,4-dinitrophenyl-hydrazine (DNPH), acid precipitation, and measurement of absorbance at 350 to 380 nm (extinction coefficient = 22 $mM^{-1}\,cm^{-1}$). Other assays are based on immunohistochemical detection of carbonyl proteins and HPLC separation of carbonyl protein adducts with DNPH.

Protein carbonyl in control samples from many different sources averages about 2 nmol/mg protein, which is approximately 0.1 mol of carbonyl per mole of protein. If this is not proven by future research to be an overestimation, it means that about 10% of cellular proteins have a carbonyl group in their structures. This may represent the steady-state between damaged protein formation and damaged protein degradation. A two- to threefold increase in carbonyl protein content under oxidizing conditions could create a high percentage of dysfunctional proteins that may be a major contributor to cell damage and death due to oxidative stress.

Increased levels of carbonyl protein are present (in tissue samples) in many stress conditions and pathologies associated with oxidative stress including smoking, extenuating exercise, hyperoxia (in laboratory animals), ischemia/reperfusion of mammalian organs, rheumatoid arthritis, cystic fibrosis, Alzheimer's disease, Parkinson's disease, alcoholic liver disease, cataractogenesis, and diabetes (several of these conditions/diseases are further discussed in Chapter 13). Aging is also related to carbonyl protein accumulation (see Text Box 12.9). However, not all cases have clear-cut "radical explanations." For example, an interesting study was done in the 1990s with postmortem brain tissue from patients with Parkinson's disease (see Chapter 13) and age-matched controls. In brain areas associated with Parkinson's disease, such as the substantia nigra and caudate nucleus, there were significant increases in protein carbonyl levels. These results are of apparent clinical relevance since increased lipid peroxidation and increased levels of the DNA oxidation product 8-OH-dGua (see Section 12.5) have been previously observed in the substantia nigra of Parkinson's disease patients. However, increased carbonyl levels were also found in areas of the brain that may not be affected in Parkinson's disease. This may suggest that oxidative stress is related to the therapeutic treatment of Parkinson's disease with L-DOPA (3,4-dihydroxyphenylalanine), which can exert prooxidant properties *in vitro* (see Chapter 13).

12.5 DNA OXIDATIVE DAMAGE

Damage to DNA by ionizing radiation and oxygen radicals (as well as DNA repair) has been a field of intense investigation for the last 40 years. It is an impossible task to cover all the chemical, analytical, biological, and clinical aspects of it in a single textbook chapter. Thus, an overview of several aspects of cellular oxidative stress connected to DNA damage is presented below.

12.5.1 DNA Damage: Introductory Remarks

Determinations of DNA damage were part of the first studies of the biological effects of free radicals. These studies began in the post–World War II atomic era, when researchers became interested in understanding the biochemical basis of the toxicity of ionizing radiation. Rebeca Gerschman from Argentina was the first to theorize, in a 1953 lecture at the University of Rochester, that ionizing radiation damage and oxygen toxicity were due to free radical formation. The proposal was published one year latter in the journal, *Science*, in collaboration with Daniel L. Gilbert (a medical student at that time but currently at the National Institutes of Health in the United States) and co-workers. Later they reported that high oxygen pressures change the viscosity of DNA solutions. In 1958, they also correlated their *in vitro* data with studies of mouse mortality induced by high oxygen tensions. The overall idea was that the univalent reduction of oxygen, caused by ionizing radiation or metabolic processes (see Scheme 12.1), would cause the formation of reactive intermediates that could damage cell components such as DNA. They were right. But in those days oxygen was still considered only a "good boy," essential for aerobic life. Only with the discovery of SOD (see Section 12.1 and Text Box 12.2) and the establishment of the role of ROS in biology in the early 1970s was more attention given to the link between DNA damage by ionizing radiation and oxygen.

In the early 1980s, studies of DNA damage by ionizing radiation were "replaced" by the studies of the effects of H_2O_2, since H_2O_2 is formed from the radiolysis of water. Furthermore, H_2O_2 is simpler and safer to work with.

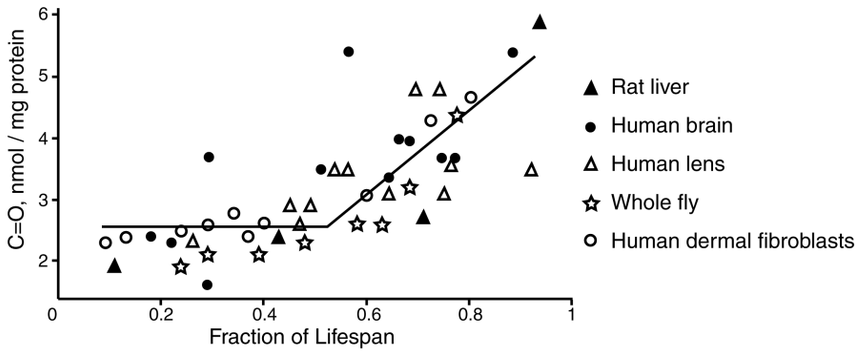
$$O_2 + e- \rightarrow O_2^- + e- \rightarrow O_2^{=} + e- \rightarrow O^{=} + O^-$$
$$O^- + e- \rightarrow O^{=}$$

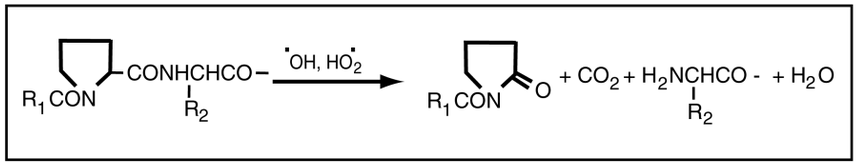
Scheme 12.1 Scheme of univalent reduction of oxygen exactly as proposed by Gerschman and co-workers [Oxygen poisoning and X-irradiation: A mechanism in common. *Science* **119**:623–626 (1954)], showing the formation of hydrogen peroxide (shown as $O_2^{=}$), superoxide radical (O_2^-), and hydroxyl radical (shown as O^-); OH^- was represented as $O^{=}$. Compare this 1950s scheme with the currently accepted one [reactions (12.1.8)]. The poor acceptance of women in medical sciences in the 1950s contributed to the low impact of Gerschman's radically different ideas (see Gilbert, *Ann NY Acad Sci* **899**: 1–14, 2000).

TEXT BOX 12.9 CARBONYL PROTEIN AND AGING

Natural aging is associated with a number of changes in biochemical parameters, including an increase in carbonyl protein levels in many animals species. The carbonyl content of protein in cultured human fibroblasts increases exponentially as a function of the age of the fibroblast donor. There is also a clear relationship between the carbonyl content of different tissues (human, rat, fly) and the fraction of the life span of the species (Fig. TB12.2). Moreover, dietary caloric restriction increases the average life expectancy (but not the maximum life span) of several species, and this correlates with reduced amounts of carbonyl protein, compared with age-matched controls.

Why would carbonyl protein increase with aging? There are several factors. (1) With aging, there is a decrease in the levels of several low-molecular-weight antioxidants that can quench free radicals and prevent protein oxidation. (2) Proteolytic activity decreases during aging, contributing to accumulation of damaged proteins. (3) Some damaged proteins with intra- or intermolecular crosslinks between a carbonyl group and a lysine residue are resistant to proteolysis and may also inhibit the activity of the 20s proteasome. That would further contribute to accumulation of damaged proteins, being particularly important in long-lived nondividing neuronal cells. (4) Aging may also increase the rate of mitochondrial generation of O_2^-, which could set up a situation for increased protein oxidation. These ideas are consistent with the observations of increased carbonyl protein levels in the frontal and occipital poles of human brain, eye lens proteins, and skeletal muscle as a function of age.





An example of a carbonyl product formation (2-pyrrolidone).

Figure TB12.2 Carbonyl protein versus aging. The figure was obtained (with permission by Dr. Stadtman) from Stadtman and Levine (2000). As an example of carbonyl formation, it shows the oxidation of proline residues leading to 2-pyrrolidone formation and peptide bond cleavage.

Even researchers from radiation chemistry/biochemistry laboratories started to study the toxic effects of H_2O_2 on cells and DNA, and they made major advances in elucidating the chemistry/biochemistry of DNA fragmentation, modification, and repair.

12.5.2 DNA as Target of Reactive Oxygen Species

Damage to DNA has been observed in many cell types, from invertebrates to mammals, and in response to multiple different forms of oxidative stress. Oxidative damage to DNA participates in many stress conditions and diseases, especially in carcinogenesis and aging (see Section 12.5.5). Moreover, DNA fragmentation is related to apoptosis, which is a crucial anticancer physiological mechanism of organisms. Exposure to ozone, ionizing radiation, chemical/metal-catalyzed generation of $^{\bullet}OH$ radicals, hyperbaric oxygen, metabolic by-products of xenobiotic compounds (e.g., aldehydes, H_2O_2), iron-overload, and nitrogen reactive species all induce DNA strand breaks and/or base modifications (see Section 12.5.3). It is also interesting to note that the quantification of oxidative damage to DNA has been widely developed as a probe to test the potentially damaging (and potentially mutagenic) effects of various chemicals, natural compounds, and metals. These studies use different DNA sources including plasmid DNA, purified mammalian nuclear DNA, isolated mitochondria (for studies of mtDNA), and cells under conditions of oxidative stress.

Superoxide radicals and H_2O_2 are not directly involved in DNA damage. However, their interaction with transition metals promotes DNA strand breaks and base modifications. Almost two decades ago Rogerio Meneghini's group at the University of São Paulo, Brazil, proposed that H_2O_2 induces DNA damage (measured as DNA fragmentation by alkaline digestion techniques; see Section 12.5.4) in cultured cells indirectly, by means of its interaction with intracellular transition metals, mainly iron (MelloFilho et al. *Biochem J* **218**:273–275, 1984). They also proposed that iron ions bound to the DNA structure would react with H_2O_2 (which crosses biological membranes easily) causing Fenton-type reactions, site-specific $^{\bullet}OH$ generation, and thus DNA damage. This mechanism was actually much like the one proposed years later for metal-catalyzed protein oxidation (see Section 12.4).

It is known from *in vitro* studies that iron and copper bind tightly to DNA at neutral pH values. Copper appears to bind preferentially to GC-rich residues in DNA. However, there are still doubts about whether iron or copper binds to DNA *in vivo.* Indirect evidence points to this in the case of iron, including the presence of a P-ATPase that transports Fe^{3+} into the nucleus, as observed by Meneghini's research group. New evidence suggests that superoxide mediates the removal of iron (as Fe^{2+}) from protein-bound iron–sulfur clusters (and many of these clusters are in the nuclei). This Fe^{2+} may bind to DNA, react with H_2O_2, and lead to $^{\bullet}OH$-mediated site-specific DNA damage (see Fig. 12.8).

Until the mid-1990s, an alternative proposal made by S. Orrenius in Sweden (see *Trends Pharmacol Sci* **10**:281–285, 1989) for the mechanism of DNA damage was well accepted by the scientific community. Orrenius's ideas were that oxidative stress would cause DNA damage (as fragmentation, but not base oxidative modification) indirectly. Free radicals would induce lipid peroxidation and rupture of the endoplasmic reticulum, which could release Ca^{2+} into the cytoplasm and activate Ca^{2+}-dependent endonucleases. This idea began to lose favor when studies in the 1990s showed that membrane-permeable iron chelators (such as 1,10-phenanthroline, which prevents Fe^{2+} reaction with H_2O_2) protected DNA from oxidative damage and cell killing under conditions of oxidative stress. On the other hand, the role of calcium in the processes of cellular redox-regulation and oxidative stress cannot be underestimated. Moreover, Ca^{2+} activation of endonucleases, caused by loss of cellular calcium homeostasis, has been recognized recently as a relevant component of the apoptotic processes.

It is also relevant to mention the literature on polyphenols and phenolic acids (see Text Box 12.4) that are present in red wines, vegetable oils, and many dietary vegetables which include compounds such as quercetin, caffeic acid, murin, tannic acid, elagic acid, and kaempferol. These may act as either antioxidants or prooxidants, but studies have shown that their action is mostly related to the capacity to chelate transition metals and/or to intercalate in the DNA structure.

12.5.3 Oxidative Damage (and Repair) to DNA Bases

Hydroxyl radicals can damage all DNA bases. They can react with thymine and cytosine yielding many products including cytosine glycol and thymine glycols, as shown in Table 12.6 (see also Fig. 12.9). Thymine–hydroperoxide is also detected after free radical attack on DNA. Reaction of $^{\bullet}OH$ with guanine and adenine residues at C4, C5, and C8 positions leads to hydroxylated and deaminated products, such as 8-hydroxyguanine (8-OH-Gua), 8-hydroxyadenine (8-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and other modified bases with an open ring. Pyrimidine dimers are also relevant products of UV damage to DNA (mediated by $^{\bullet}OH$ formation), resulting from crosslinking of adjacent pyrimidines. Singlet oxygen also damages DNA bases, generating mostly guanine-derived products, including 8-OH-Gua and FapyGua.

Reactive nitrogen species (RNS) also produce base modifications, resulting in 8-nitroguanine (this is not

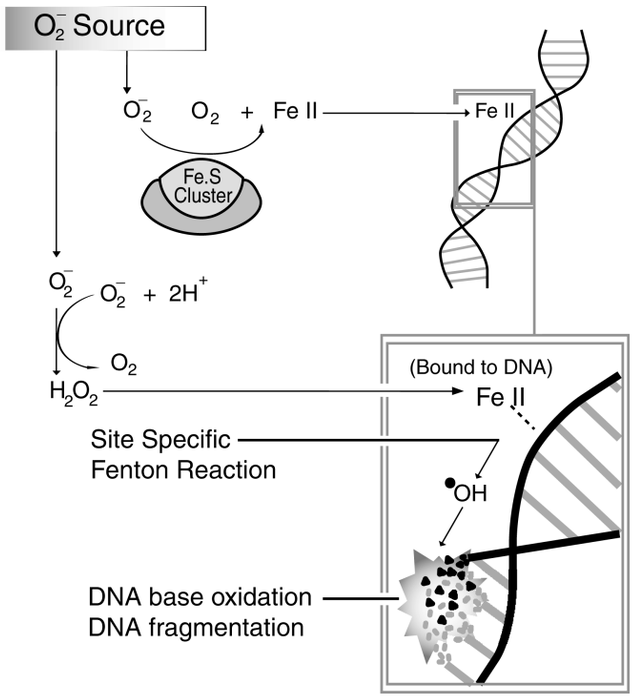


Figure 12.8 The role of iron in DNA oxidative damage. Superoxide radicals formed from cellular sources (such as mitochondria and endoplasmic reticulum) undergo (i) dismutation into H_2O_2 and (ii) cause Fe(II) release from [Fe-S]-containing proteins. When Fe(II) bound to DNA reacts with H_2O_2, it produces site-specific Fenton reaction (and $^{\bullet}OH$ formation), leading to DNA oxidative damage.

TABLE 12.6 Selected Products of DNA Base Oxidation/ Modification by ROS and RNS

Base	Product
Guanine	8-Hydroxyguanine (8-OH-Gua)
	2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FapyGua)
	8-Nitroguanine[a]
	Xanthine[a]
Adenine	8-Hydroxyadenine (8-OH-Ade)
	4,6-Diamino-5-formamidopyrimidine (FapyAde)
	Hypoxanthine[a]
Thymine	Thymine glycol (*cis*- and *trans*-)
	5-Hydroxymethyluracil
	5-Hydroxy-5-methylhydantoin
	5,6-Dihydrothymine
	5-Hydroxy-6-hydrothymine
Cytosine	Cytosine glycol
	5-Hydroxyhydantoin
	5-Hydroxycytosine
	5-Hydroxyuracil
	5,6-Dihydroxyuracil
	Uracil[a]

[a]Products of RNS attack.

formed by $^{\bullet}OH$ attack), 8-OH-Gua, FapyGua, thymine glycol, and 5-hydroxy-methyluracil. Formation of 8-OH-Gua (and other "typical" $^{\bullet}OH$-oxidation products such as FapyGua) as products of the direct action of $ONOO^-$ on DNA has been a matter of debate because they might also be formed as a result of DNA attack by decomposition products of ONOOH, such as $^{\bullet}OH$.

In general, mispaired, oxidized, and deaminated bases (or the nucleotides with the damaged bases) are removed from DNA by repair enzymes. Nonspecific endonucleases can remove a stretch of DNA containing an oxidized base, whereas DNA glycosylases remove one specific oxidized base. Methods that quantify these excised bases and nucleotides in cell extracts, intercellular fluids, blood, and urine are widely used to assess oxidative damage to DNA. For example, measurement of 8-OH-Gua in systems under oxidative stress provides an evaluation of the balance between the rates of DNA damage and repair. If there is an increase in 8-OH-Gua in blood or urine samples from animals under a certain stress/pathological condition, this could reflect an increase in DNA damage and/or a decrease in repair. Other methods for evaluating oxidative damage to DNA involve purification and digestion of the DNA (enzyme-catalyzed or by acid hydrolysis),

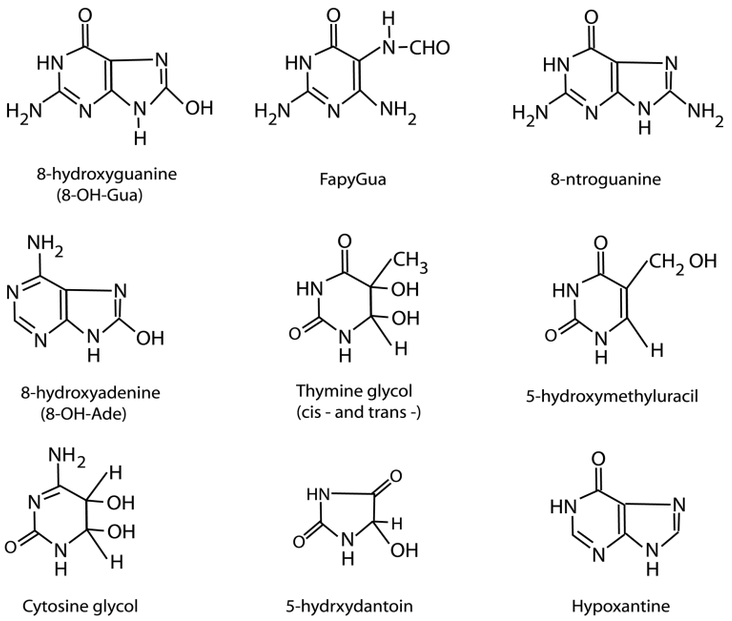


Figure 12.9 Selected oxidized/modified DNA bases.

so that the actual amounts of oxidized nucleosides or bases can be quantified.

8-Hydroxydeoxyguanosine (8-OH-dGua) and its free base 8-OH-Gua (also called 8-oxo-Gua) are the products most frequently measured as indicators of oxidative DNA damage. This product constitutes about 5% of all DNA adducts. Quantification is typically by one of two methods: (1) HPLC separation coupled with electrochemical detection (HPLC-EC) after bases are removed from DNA by enzymatic hydrolysis, or (2) gas chromatography–mass spectrometry (GC–MS) analysis after DNA is hydrolyzed in weak acid under heating.

The validity of the two methods is highly debated because both over- and underestimation of results can occur. HLPC analysis usually gives concentrations of 8-OH-dGua that are several-fold higher than results obtained from GC–MS. The GC–MS method for 8-OH-Gua quantification is also subject to overestimation due to 8-OH-Gua generation as a result of the chemical hydrolysis of 8-OH-dGua during the assay or from RNA free radical oxidation. Thus, RNA-free samples are needed for determinations of DNA oxidation products. Baseline levels of 8-OH-dGua in rodent livers (rat, mouse, and hamster, measured by HPLC-EC) were described in the mid-1990s as being in the range of 1.5 to 50 molecules of 8-OH-dGua per 10^6 DNA bases, which is equivalent to a range of 0.6 to 20 mol of 8-OH-dGua per 10^5 guanines. The current lowest baseline estimates of the ratio 8-OH-dGua/guanine in rat hepatocytes and HeLa (human transformed epithelial) cells are around 0.5×10^{-5}. Even though the battle for the correct measurement of baseline levels of 8-OH-dGua (and other modified bases) continues, comparative measurements are still of high relevance for clinical and biological studies. For more detail on the measurement of DNA oxidation see the recent trial published at *Free Radic Biol Med* **34**:1098–1099, 2003).

Levels of 8-OH-dGua in urine and tissue samples are significantly increased in humans and laboratory animals under conditions of oxidative stress (see Text Box 12.10). Examples include the presence of augmented 8-OH-dGua levels in patients suffering from systemic lupus erythematosus (in lymphocytes) and amyotrophic lateral sclerosis (in the spinal cord), cystic fibrosis, rheumatoid arthritis, and diabetes (in urine samples), as well as in postmortem brain analyses of patients with Parkinson's and Alzheimer's diseases. Currently, 8-OH-dGua is the most popular biomarker of free radical damage to DNA.

12.5.4 Other Types of DNA Alterations, Including Strand Breaks and Fragmentation

Oxygen and nitrogen reactive species also cause various other alterations to DNA structure. Crosslinks between DNA bases and amino acid residues of proteins are one example. Loss of purine bases, leaving apurinic sites, is another consequence of free radical attack on DNA. Cyto-

TEXT BOX 12.10 DETERMINATION OF 8-OH-dGua IN SELECTED STUDIES

Japanese scientists reported in 1990 that injecting rats with ferric nitrilotriacetate (Fe–NTA), a known renal carcinogen, induces a significant increase in the levels of 8-OH-dGua and lipid peroxides in kidney. The dose-dependent effect of Fe–NTA in 8-OH-dGua formation was parallel with the appearance of nephrotoxic responses in terms of serum biochemical and histopathological changes. The researchers also determined the levels of 8-OH-dGua in several organs of aging rodents (comparing 6- to 30-month-old rats). They observed increased 8-OH-dGua levels in liver and kidney of aged animals.

Studies from a research group in Singapore revealed that the levels of 8-OH-dGua in the sperm DNA of smokers are significantly higher than in the sperm DNA of nonsmokers (6.2 ± 1.7 vs. 3.9 ± 1.3 mol of 8-OH-dGua per 10^5 mol of dGua). Moreover, it has been found that infertile men have about twice the amount of 8-OH-dGua in sperm DNA as do fertile men (the results were also correlated with conventional seminal parameters). Thus, oxidative damage to sperm DNA might be important in the etiology of male infertility.

Genomic damage was also correlated with several autoimmune diseases (associated with inflammation; see Chapter 13) by researchers from England. Levels of 8-OH-dGua, determined by HPLC, were 6.8 ± 0.8 mol of 8-OH-dGua per 10^5 mol of dGua in blood lymphocytes from healthy subjects. However, the levels of 8-OH-dGua were significantly increased in lymphocytes of patients suffering from rheumatoid arthritis (9.8 ± 1.6), systemic lupus erythematosus (13.7 ± 2.8), vasculitis (10 ± 3.2), and Behcet's disease (9.2 ± 1.9). However, 8-OH-dGua levels did not correlate with disease duration, disease severity, or age.

Systemic DNA lesion was studied in non-insulin-dependent diabetes mellitus (NIDDM) by assessments of urinary 8-OH-dGua. Researchers from Finland collected urine samples (24-h collection) from 81 NIDDM patients 9 years after the initial diagnosis and of 100 control subjects matched for age and gender. The total urinary excretion of 8-OH-dGua was markedly higher in NIDDM patients than in the controls (68.2 ± 39.4 vs. 49.6 ± 37.7 μg). Moreover, glycosylated hemoglobin (the usual clinical test for diabetes) at high levels was associated with elevated concentrations of urinary 8-OH-dGua. Interestingly, NIDDM patients also have increased plasma levels of biomarkers of lipid peroxidation, such as 8-EPI-PGF_{2a} (see Section 12.3.2.4).

sine can be deaminated and produce uracil (this can have mutagenic consequences, see Section 12.5.5). In addition, deoxyribose (the sugar of the DNA structure) is fragmented by $^{\bullet}OH$, yielding an array of products, including peroxyl radicals and carbonyls (MDA is one product of DNA damage). Damage to deoxyribose may cause DNA strand breaks.

Studies on double-strand breaks to DNA are of special relevance for understanding oxidative stress processes. Many analytical methods have been employed for this purpose. However, some of them are problematic because they actually increase the number of nicks due to manipulations and, thereby, overestimate DNA fragmentation. Thus, DNA fragmentation assays are mainly used to quantify relative levels of damage in control versus experimental samples.

Many assays use gel electrophoresis to produce a "footprint" of the nuclear DNA fragmentation pattern. The fluorescent dye ethidium bromide is used to stain the DNA. The anticancer drug bleomycin is well-known for its effects on DNA fragmentation. It forms a complex with the DNA structure and, in the presence of transition metals, causes oxygen-dependent single- and double-stranded lesions to DNA. Different oxidizing agents can produce unique DNA footprints; the pattern may depend on the base composition of the DNA. For example, Japanese researchers observed in the late 1990s that cupric nitriloacetate, a potent carcinogen, causes severe nuclear DNA fragmentation to human promyelocytic HL-60 cells in culture, as observed by gel electrophoresis. They also observed increased 8-OH-dGua formation caused by cupric nitriloacetate. The effect was dose-dependent and was associated with apoptosis of the HL-60 cells under stress.

The DNA plasmids are often used for simple model studies of the effects of oxidants and antioxidants. Intact plasmids are supercoiled (SC) whereas plasmids containing single-strand breaks will form open-circular (OP) forms; double strands will cause fragmentation. SC and OP forms migrate differently on agarose gel electrophoresis and damage can be quantified by measuring the loss of the SC band and/or the increase in the OP band intensity. Metal-catalyzed $^{\bullet}OH$ formation, singlet O_2, and $ONOO^-$ are able to cause plasmid DNA fragmentation and damage; antioxidants, such as catalase, and certain metal chelators can prevent DNA strand-break formation.

12.5.4.1 Alkaline Digestion or Unwinding of DNA for Determination of DNA Fragmentation Other classical assays to determine DNA fragmentation are based on alkaline digestion of the DNA followed by enzymatic incorporation of radioactively labeled nucleotides. Enzyme-catalyzed incorporation of tritiated thymidine is often used. The fragments are then separated (by HPLC or regular chromatographic columns) and the radioactivity

quantified. The use of cesium salt gradients to separate fragmented DNA labeled with H^3-thymidine (without alkaline digestion) is another method to analyze DNA damage. This class of assays (which can be used for cells or tissue samples) highly overestimates the actual number of DNA nicks, but, in relative terms, oxidatively damaged DNA will have more fragments than controls.

It was observed two decades ago that incubation of isolated rat liver with ferrous ions causes a dose-dependent fragmentation of DNA, measured by alkaline digestion, as well as lipid peroxidation. Interestingly, in this study DNA damage was inhibited by chain-break antioxidants (BHT and vitamin E; see Section 12.3). This study by Thomas Shires (*Biochem J* **205**:321–329, 1982) was one of the first to show that products of lipid peroxidation contribute to DNA fragmentation.

Another method for analyzing DNA damage uses alkaline exposure of DNA at low temperature to produce unwinding of intact or nicked double helix; unwinding is faster in DNA with strand breaks. A fluorescent probe that intercalates with DNA (e.g., ethidium bromide) is then introduced and used to determine the extent of DNA damage. Using this assay (fluorometric analysis of DNA unwinding, FADU assay), it has been observed that 100 μM $ONOO^-$ causes DNA strand breaks in isolated rat thymocytes (a process inhibited by Trolox) and oxidation of cellular thiols.

12.5.4.2 TUNEL and Comet Assays to Quantify DNA Damage The enzymatic labeling techniques used to detect and quantify DNA fragmentation are based on the addition of fluorescent bases (or modified bases, detected using antibodies) to free OH groups created by strand breaks. The currently popular TUNEL assay (TUNEL stands for TdT-mediated X-dUTP nick end-labeling; TdT = terminal deoxynucleotidyl transferase) is an example of this kind of methodology. These techniques can be applied to cell cultures provided that the cells are made permeable for the entry of the enzymes and substrates.

Another popular technique of the late 1990s is the comet assay. This is based on cell electrophoresis; after exposure to an oxidizing agent (e.g., H_2O_2 or hyperbaric oxygen), membranes are removed by treatment with a detergent, followed by staining of the DNA. Fragmented DNA migrates more quickly to the anode than does intact DNA and so a "tail" is produced, very similar to that of a comet in the sky; the bigger the comet tail the greater the DNA fragmentation. The presence of oxidized DNA bases can also be detected by exposing cell lysates to endonuclease III, which cuts the DNA at sites of oxidized pyrimidines.

12.5.5 Biological Effects of DNA Damage

The most studied consequences of oxidative DNA damage are the mutagenic and carcinogenic effects (Fig. 12.10). Aging, cigarette smoking, and several human diseases have been also connected to DNA damage (see end of Section 12.5.3 and Text Box 12.10). Damage to mitochondrial DNA (mtDNA) is also a key factor in several human degenerative pathologies.

Organisms have a finite capacity to deal with continuous insults to DNA bases (e.g., oxidation, nitration, deamination) using their endogenous repair mechanisms. Excessive oxyradical generation (caused by many cellular processes and by a huge number of substances) can overwhelm the capacity of repair enzymes to correct DNA structure. Misreading of altered bases can then lead to mutations during DNA replication. For example, *C:G → T:A transversion mutations* (see Fig. 12.11) may arise from misreading of 8-OH-Gua during DNA replication because 8-OH-Gua can also pair with adenine. Likewise, 8-OH-Ade can

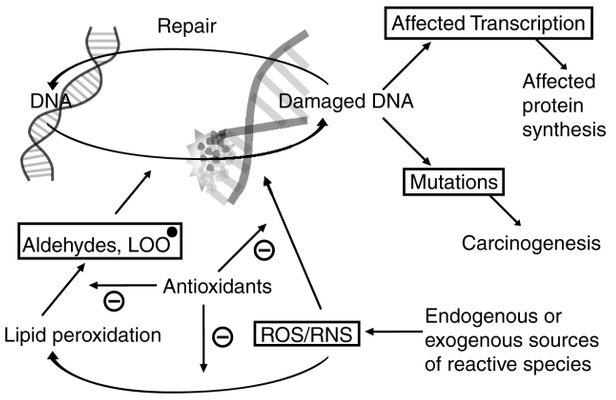


Figure 12.10 Connection between DNA oxidative damage and DNA repair with basic biological effects. The (−) symbol indicates inhibitory actions of antioxidants.

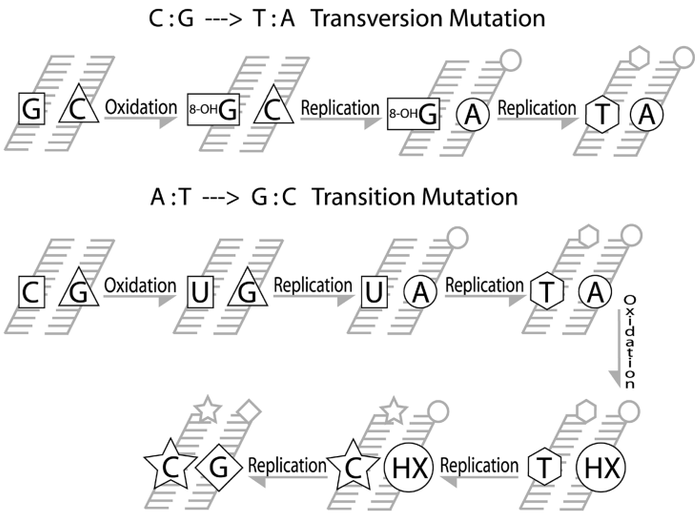


Figure 12.11 Two types of DNA mutations. After every DNA replication, the figure shows a new DNA strand marked with a symbol. U and HX represent uracil and hypoxanthine, which are oxidative deamination products of cytosine and adenine, respectively, upon RNS attack.

mispair with guanine, causing another transversion mutation. Moreover, oxidative deamination of cytosine to uracil (which pairs with adenine) may induce *A:T → G:C transition mutations* because oxidative deamination of adenine to hypoxanthine (caused by $ONOO^-$ attack on adenine) will form a hypoxanthine–cytosine pair during new replication (Fig. 12.11).

Other effects not directly related to mutations include the fact that ring-fragmented bases can block DNA replication and that DNA–protein crosslinks interfere with chromatin unfolding, DNA repair, replication, and transcription. In addition, DNA lesions (induced by ROS or RNS) may cause activation of the chromatin-bound enzyme poly(ADP-ribose) polymerase (PARP), which is responsible for ADP-ribosylation of proteins involved in DNA repair. Excessive activation of PARP may deplete the cellular NAD^+ pool, challenge the normal regulation/function of metabolism, and interfere severely with ATP synthesis.

Damage to mtDNA also has severe consequences to cells. Mitochondria have DNA repair mechanisms that are much less efficient than the nuclear DNA repair systems. The physical proximity of mtDNA to the intramitochondrial sites of O_2^- and H_2O_2 formation, as well as the absence of histones on mtDNA, make it a highly susceptible target for oxidative damage. Moreover, the intramitochondrial Fe^{2+} pool, which can be released from aconitase by O_2^-, could prompt Fenton reactions and $^{\bullet}OH$-mediated mtDNA oxidative injury. Damage to mtDNA may compromise cellular bioenergetics since several proteins (or protein subunits) involved in ATP synthesis are encoded by mtDNA. For example, it has been observed that mtDNA from rat hepatoma cells are susceptible to iron-mediated strand breaks, in contrast to mtDNA from normal hepatocytes. The much higher levels of Mn–SOD (the mitochondrial form of SOD; see Section 12.1.1) and coenzyme Q in hepatocytes, in comparison with hepatoma cells, may explain the absence of mtDNA strand breaks in normal cells. Indeed, Mn–SOD is an unique form of cell defense against oxidative stress; the absence of this enzyme in knockout mice strains has devastating effects and causes very high mortality (see Text Box 12.3).

12.6 FREE RADICALS AND SIGNAL TRANSDUCTION

A few more key points about reactive oxygen/nitrogen species remain to be discussed. First, conditions of increased free radical formation (that do not cause immediate cell death) stimulate cells to raise the activities of antioxidant enzymes and the synthesis of low-molecular-weight antioxidants. This has been well-described in multiple tissues and species under many different conditions of oxidative stress. However, the signaling mechanisms involved in these antioxidant responses were a mystery until the late 1980s, when Bruce Demple's team from Harvard University started to reveal some secrets of these mechanisms in bacteria [see paper by Greenberg and Demple (*EMBO J* **7**:2611–2617, 1988)

for the effects of H_2D_2]. They observed that H_2O_2 (or O_2^- and $^{\bullet}NO$, in later publications) can regulate the expression of genes that code enzymes directly or indirectly linked to bacterial survival under an ROS-enriched environment. Even though a wealth of information has been amassed on the mechanisms of redox regulation in bacteria (see Section 12.6.1), much has still to be learned about the components, regulation, and function of comparable systems in higher organisms.

Second, if ROS (and RNS, such as $^{\bullet}NO$) are able to regulate the production of antioxidant defenses, they may also be involved in the regulation of many other cellular processes, directly or indirectly linked with the redox state of cells. Currently, it is known that over 100 genes are activated in response to oxidative stress in mammalian cells. For example, changes in the cellular redox state modulate transcriptional activation of metallothionein and collagen genes, posttranscriptional control of ferritin mRNA, and activation of transcription factors including Myb and Egr-1. Several proto-oncogenes are transcriptionally activated by increased cellular oxidation. Exposure of normal or transformed cells to UV radiation or H_2O_2 stimulates the expression of *jun*-B, *jun*-D, c-*fos*, and *fos*-B. Results obtained over the last 10 years in many laboratories have proven that ROS and RNS can be considered relevant second messengers in signal transduction in many molecular cascades. However, redox-regulation of vertebrate cells, leading to cell response and adaptation to numerous stimuli, including oxidative stress, is a very complex and intricate process and its full comprehension is still in its infancy. One interesting example addressed in Section 12.6.3 is the involvement of ROS in the process of oxygen sensing, that is, in the ability of cells/organisms to respond to changes in oxygen tensions.

12.6.1 Bacterial Regulons *sox*RS and *oxy*R

Escherichia coli has independent multigene responses to two kinds of oxidative stress: elevated H_2O_2 triggers the *oxy*R regulon, and excess O_2^- (and $^{\bullet}NO$) triggers the *sox*RS regulon. Singlet oxygen has also been recently implicated in *soxRS* activation in *E. coli*. These regulons coordinate the induction of numerous promoters related to protective responses. Several similar mechanisms exist in other bacterial species, including Mycobacteria.

Gene activation by *sox*RS occurs in two stages: (1) constitutive SoxR protein is activated by a redox signal (see below for the mechanism of SoxR activation), which strongly activates the transcription of *soxS* mRNA; and (2) SoxS protein is synthesized and induces at least 15 genes [see reactions (12.6.1) to (12.6.3)]. The products of these genes include endonuclease IV (initiates repair of oxidatively damaged DNA; see Section 12.5.3), Mn–SOD (see Section 12.2.1.1), G6PDH (to supply NADPH reducing power for antioxidant enzymes; see Section 12.2.1), and superoxide-insensitive fumarase C (a metabolic enzyme). These gene products are all directly or indirectly involved in adaptive responses to O_2^--induced oxidative stress. Other gene products that are triggered by the *sox*RS regulon are related to antibiotic resistance and efflux pumps. Thus, *sox*RS contributes to clinical antibiotic resistance, in part, by counteracting compounds that induce oxidative stress:

$$O_2^- + \text{SoxR}-[2\text{Fe}-2\text{S}]^{2+}(\text{inactive}) \rightarrow O_2 + \text{SoxR}-[2\text{Fe}-2\text{S}]^{3+}(\text{active}) \quad (12.6.1)$$

$$\text{Active SoxR} \rightarrow \text{triggers production of } soxS \text{ mRNA and SoxS protein} \quad (12.6.2)$$

$$\text{SoxS protein} \rightarrow \text{activates transcription of various genes (Mn–SOD, G6PDH, etc.)} \quad (12.6.3)$$

The SoxR protein of *E. coli* is a homodimer containing two [2Fe–2S] clusters per monomer. One-electron oxidation of [2Fe–2S] clusters by O_2^- or $^{\bullet}NO$ causes the activation of the SoxR protein (by means of an allosteric/torsional event) as a transcription factor. It is interesting to note that O_2^- usually causes damage to Fe–S clusters of enzymes, but not to SoxR. The role of $^{\bullet}NO$ in the activation of bacterial SoxR has a direct relevance for bacterial defense against the destructive products of phagocytes. As pointed out by Bruce Demple, several bacteria have "evolved to sense and respond to a major cytotoxic weapon of the immune system."

In addition to the effects of $^{\bullet}NO$ and O_2^-, excess H_2O_2 causes peroxide resistance in *E. coli.* This resistance is dependent on the *oxy*R gene, which governs a series of genes that constitute the *oxy*R regulon. The OxyR protein activates the synthesis of gene products with clear involvement in antioxidant defense; these include glutathione reductase, glutaredoxin, NADPH-dependent alkyl hydroperoxidase, and catalase (HPI; see Section 12.2.1.2). OxyR also activates the synthesis of the gene products of *fur* (encoding an iron-binding repressor of iron transport) and *dps* (encoding a DNA- and iron-binding protein that protects DNA from oxidative attack). This gene product is an iron-binding repressor of iron transport, which prevents loading of iron when excess of H_2O_2 is present (this is a good strategy against Fenton-mediated $^{\bullet}OH$ formation). Activation of the transcription factor is dependent on the H_2O_2-mediated oxidation of two specific –SH groups per monomer in its tetrameric structure. Following oxidative stress, deactivation of OxyR seems to be mediated by the GSH–glutaredoxin system, reducing OxyR disulfide bonds.

12.6.2 Oxidants and Cellular Redox Signaling

Extracellular signaling molecules such as insulin, growth factors, and cytokines induce changes in cell metabolism via complex mechanisms that involve transmission of the signal from the plasma membrane to the nucleus, where gene expression is altered. The first step of a signaling cascade generally involves the activation of receptors that either have protein kinase activity or activate protein kinases (and/or phospholipases) in the cytoplasm. The signal is eventually transmitted to the nucleus, where it activates the transcription factors that regulate gene expression.

Stimulation of cells by ROS causes signal transduction via the same signaling pathways as those triggered by growth factors or cytokines (in some instances ROS bypass steps of the signaling chains). For example, oxidants activate receptor-type protein tyrosine kinases (RTK), non-receptor-type protein tyrosine kinases (PTKs), and downstream signaling components including mitogen-activated protein (MAP) kinases, protein kinase C (PKC), phospholipase C-γ, and calcium flux in cells.

12.6.2.1 ROS, Phosphorylation, and Signal Transduction As pointed out by Robert Floyd's group, from the Oklahoma Medical Research Foundation, "the common paradigm in all redox-sensitive signal transduction pathways is the presence of the intermediate protein kinases which are activated by phosphorylation of specific regulatory domains." H_2O_2 and other oxidants mediate these activations/phosphorylations (Hensley et al. *Free Radic Biol Med* **28**:1456–1462, 2000).

For example, the binding of insulin to its receptor tyrosine kinase (RTK) increases the receptor's enzymatic activity and results in the phosphorylation of both the receptor (autophosphorylation) and the insulin receptor substrate-1 (IRS-1) (see Chapter 5). It has been observed that H_2O_2 has insulin-mimetic effects, such as the stimulation of metabolism, via the activation of the insulin receptor and the phosphorylation of IRS-1. Moreover, both epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulate cell growth via activation of their respective receptors, and phosphorylation of the EGF and PDGF receptors is also induced by exogenous treatment with H_2O_2, stimulating downstream signaling systems.

It is interesting that H_2O_2 not only activates early components of signaling cascades, but H_2O_2 is also produced endogenously upon the binding of growth factors to their cellular receptors, causing activation of RTKs. The stimulation of formation of H_2O_2 then effects downstream components of signal transduction cascades. The first clear-cut evidence implicating H_2O_2 as an endogenous messenger was obtained in the mid-1990s by Toren Finkel's group, from the National Institutes of Health, Maryland (Sunderesan et al. *Science* **270**:296–299, 1995). They observed that stimulation of rat vascular smooth muscle cells by PDGF transiently increased the intracellular concentration of H_2O_2. The response of vascular smooth muscle cells to PDGF, which includes tyrosine phosphorylation, MAP kinase stimulation, DNA synthesis, and chemotaxis, was inhibited when the growth-factor-stimulated rise in H_2O_2 concentration was blocked (by increasing the intracellular concentration of catalase or by the addition of the chemical antioxidant *N*-acetylcysteine).

Sue G. Rhee and research associates, from the National Institutes of Health, obtained similar results (see Bae et al. *J Biol Chem* **272**:217–221, 1997) working with human epidermoid carcinoma cells stimulated with EGF. They observed that growth-factor-stimulated H_2O_2 production is a key event for tyrosine phosphorylation. Rhee also suggested that inhibition of protein tyrosine phosphatase (PTP) activity by H_2O_2 is required for EGF-induced protein tyrosine phosphorylation to be manifested (see Section 12.6.2.1.1). Indeed, PTPs contain a nucleophilic cysteine in the active site, which can be oxidized by H_2O_2; GSH restores the enzyme to the active form.

The enzymatic source(s) of the endogenous H_2O_2 that is produced during stimulation by growth factors (or cytokines) remained elusive until very recently. Activation of cyclooxygenases or lipoxygenases (see Table 12.2) by growth factors has been suggested as relevant sources of superoxide and H_2O_2 generation. NADPH oxidase (similar to the enzyme found in phagocytes; see Section 13.3) is currently considered the main primary source of ROS involved in signaling in mammalian cells such as smooth muscle cells, chondrocytes, and kidney epithelium.

12.6.2.1.1 Phosphatases and ROS Although oxidants stimulate many PTKs (such as Lck, Fyn, Src, Syk, and Lyn) and RTKs, it seems that oxidants do not act directly on them. Rather, it seems clear that inhibition of PTPs by oxidants is the crucial event in the activation/phosphorylation of the EGF-receptor, Lck, and Fyn. Thus, blockage of phosphatase action (by the use of inhibitors) allows maximal signal output through the protein kinase cascade. Reactivation of PTPs seems to be mediated by thioredoxin plus GSH (see Section 12.2.1.5) and leads to dephosphorylation of intermediate protein kinases and transcription factors (see below), thereby terminating the redox-sensitive signal.

On the other hand, chronic situations of oxidative stress (and ROS generation) may overwhelm the normal capacity of cells to turn-off signal transduction cascades, which may be harmful. Pharmacological agents that maintain phosphatase (PTPs) activity during an oxidative challenge would be expected to antagonize the redox signaling process. Indeed, there is a great deal of evidence showing the antagonizing

Growth factor binding to receptor → RTK autophosphorylation
→ phosphorylation of Grb-2/Sos → SHC–Grb-2–Sos complex formation (activation by H_2O_2)
→ activation of G-protein Ras
→ Raf-1 or Mekk activation (via phosphorylation) (*)
→ MKK/MEK activation (via phosphorylation) (activation by H_2O_2) (**)
→ ERK or JNK activation (via phosphorylation) (***)
→ TCF formation (and phosphorylation by JNK or ERK)
→ TCF–SRF complex formation at the serum response element (SRE) of c-*fos* → c-*fos* gene expression

Note: Looking at the MAP kinase pathway, some stages are activated by H_2O_2 and others phosphorylation: MAPKKK (*) → MAPKK (**) → MAPK (***)

Scheme 12.2 JNK/ERK pathways and ROS.

effects of exogenous antioxidants (or overexpression of antioxidant enzymes) on upstream signal transduction pathways.

12.6.2.1.2 MAP Kinases One of the most studied families of signal transduction pathways is the MAP kinase family (see Chapter 5). Activated RTKs stimulate the MAP kinase cascade, mainly via the activation of Ras, a small guanosine 5′-triphosphate (GTP) binding protein. Four MAP kinase subfamilies have been identified to date: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), p38 MAP kinases, and Big MAP kinases. All these pathways contain redox-sensitive sites and several of the MAP kinases are directly activated by H_2O_2.

In the ERK pathway, the activation of growth factor(s) receptors results in tyrosine autophosphorylation and prompts tyrosine phosphorylation of Grb-2/Sos, which binds to an adapter protein the SH-containing (SHC) protein. The resulting SHC–Grb-2–Sos complex activates the G-protein Ras (Ras-GDP is converted to Ras-GTP) (see Text Box 5.1 on page 136). The formation of the SHC–Grb-2–Sos complex is also stimulated by H_2O_2. Activated Ras stimulates the phosphorylation of Raf-1 (Raf-1 is a MAP kinase kinase) by means of PTKs and serine/threonine kinases and PKC. Once activated, Raf-1 phosphorylates MKK/MEK (MEK1, but not MEK2, is stimulated by H_2O_2 treatment), which then activates ERK by means of phosphorylation (see Scheme 12.2 and Fig. 5.5 on page 135, for other Map kinase pathways). Oxidative stress also activates protein phosphatases which can dephosphorylate MAP kinases. This might represent a feedback control of ROS activation of MAP kinase.

Members of the ERK, JNK, and p38 MAP kinase subfamilies phosphorylate the transcription factors Elk-1 and SAP-1, which then associate with other nuclear proteins to form the ternary complex factor (TCF). TCF associates with another transcription factor, the serum response factor SRF, which finally activates the expression of genes such as c-*fos* (Scheme 12.2). The c-*fos* gene is one of the best-studied early response genes, thus the study of its redox induction has been useful in elucidating the mechanisms of gene expression by ROS.

12.6.2.1.3 Protein Kinase C (PKC) and Phospholipase Cγ (PLCγ) The PKC is activated by diacylglycerol (produced from receptor-mediated hydrolysis of inositol phospholipids) or phorbol 12-myristate 13-acetate (PMA). PKC is also regulated by means of phosphorylation, lipid cofactors, and calcium binding. PKC can activate transcription factors nuclear factor κB (NF-κB) and activator protein 1 (AP-1) (see Section 12.6.2.2), which are subject to redox-regulation.

Treatment of cells with H_2O_2 and redox-cycling quinones leads to stimulation of PKC activity. Moreover, Japanese scientists recently observed that low levels of H_2O_2 induce tyrosine phosphorylation of several PKC isoforms (independent of receptor-coupled hydrolysis of inositol phospholipids), prompting enhanced activity of the phosphorylated PKC. Menadione, which generates O_2^-, also induced tyrosine phosphorylation as well as activation of PKC isoforms.

Furthermore, PKCs contain unique structural features that are susceptible to oxidative modification. The N-terminal regulatory domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by H_2O_2. When oxidized, the autoinhibitory function of the regulatory domain is compromised and, consequently, cellular PKC remains active. The C-terminal catalytic domain contains several reactive cysteines that are targets for various chemopreventive antioxidants such as selenocompounds, polyphenolic agents, and vitamin E analogs. Modification of these cysteines decreases cellular PKC activity. Thus, PKC may be subjected to a complex redox-regulation, which affects its role in tumor promotion and the control of cell growth. This makes PKC an interesting target for pharmacological manipulation.

Intracellular calcium homeostasis is also regulated by the redox state of cells. Hydrogen peroxide activates PLCγ phosphorylation, turning on the production of inosi-

tol 1,4,5-trisphosphate (Ins-1,4,5-P_3). Moreover, the Ins-1,4,5-P_3 receptor/calcium channel in the endoplasmic reticulum is activated (causing a rise in cytoplasmic calcium) not only by the binding of Ins-1,4,5-P_3 but also by the direct action of oxidants on the Ins-1,4,5-P_3 receptor. Recent evidence indicates that the redox state of cells directly modulates the activities of Ca^{2+}-ATPases and the $Ca^{2+}-Na^{+}$ exchanger.

12.6.2.2 Transcription Factors and ROS When upstream signaling pathways finally reach the nucleus, they stimulate changes in gene expression mediated by the activation of several transcription factors, such as NF-κB, AP-1, the tumor suppressor p53, and the pro-oncogene product Myb (see Chapter 5). The action of several transcription factors is redox sensitive. For example, the activity of AP-1 (which is formed by the oncogene products Jun and Fos) is inhibited by oxidation of conserved cysteine residues located in its DNA-binding site domain. Reducing agents (such as the protein Ref-1) restore the DNA-binding activity of AP-1. Moreover, cysteine residues in a reduced state are also essential for the DNA-binding activity of NF-κB. Thioredoxin and Ref-1 can sustain the transcriptional activity of NF-κB (see Scheme 12.3). For more information about redox regulation of transcription factors, see Chapter 6.

The earliest evidence that showed direct effect of oxidants on a protein involved in signal transduction appeared in the early 1990s in studies on NF-κB. NF-κB is a multiprotein complex that activates the transcription of a variety of genes involved in the early defense reactions of higher organisms. In nonstimulated cells, NF-κB resides in the cytoplasm in an inactive complex with the inhibitor IκB. Pathogenic stimuli cause the release of the IκB subunit (in the case of IκBα, it is hyperphosphorylated and then degraded by the 26S proteasome). This allows NF-κB to enter the nucleus, bind to DNA control elements and induce the synthesis of specific mRNAs (see Chapter 6). Since the mid-1980s it has been known that activation of NF-κB is triggered by a variety of agents including the cytokines interleukin-1 and tumor necrosis factor (TNF), viruses, endotoxins, phorbol esters, UV light, and ionizing radiation. In 1991 German scientists reported that low concentrations of H_2O_2 also activate NF-κB by causing the phosphorylation and release of IκB; this is prevented by various antioxidants (Schreck et al. *EMBO J* **10**:2247–2258, 1991).

Activation of NF-κB is currently considered a relevant marker of oxidative stress in several pathological situations, including HIV-1 (human immunodeficiency virus) infection, atherosclerosis, reperfusion stress, and Alzheimer's disease. NF-κB is also a factor involved in the transcriptional activation of genes encoding γ-GluCys synthetase (see Section 12.2.1.5) and Mn–SOD.

12.6.3 Oxygen Sensing and ROS

Scientists have been curious for decades about how major metabolic pathways are regulated at the gene level by changes in oxygen tension, including responses to oxygen limitation by hypoxia-sensitive species and adaptive responses to hypoxia/anoxia by anoxia-tolerant species. It has been postulated from experiments with rat carotid body preparations that a heme-containing oxidase (similar to the NADPH oxidase of phagocytes) might work as an oxygen-sensing protein. This oxidase could produce O_2^- (that is dismutated to H_2O_2) in proportion to different oxygen tensions. H_2O_2 production is PO_2-dependent, being highest under normoxia and lowest under hypoxia.

The erythropoietin (EPO) gene is one of the well-known genes whose expression is enhanced under hypoxia (see Chapter 6). It has been demonstrated in Hep G2 cells that EPO expression is under the control of PO_2-dependent H_2O_2 production. In hypoxia, when H_2O_2 production decreases, full expression of the EPO gene occurs. The role of H_2O_2 as the signaling molecule in the oxygen response has been also substantiated in studies with other genes that are induced by hypoxia including aldolase A, phosphoenolpyruvate carboxykinase (PEPCK), glucokinase (see Text Box 12.11), and tyrosine hydroxylase.

Exogenous sources of ROS and/or ligand-stimulated ROS formation
⇓
Activation of RTKs or PTKs (e.g., by ROS-mediated inhibition of PTPs)
⇓
Activation (mediated or not by ROS) of downstream pathways
(MAP kinases, PKC, PLCγ, calcium signaling, etc.)
⇓
Activation of transcription factors (NF-κB, AP-1, p53, etc.)
⇓
Expression of stress-response gene products

Scheme 12.3 ROS and signal transduction.

TEXT BOX 12.11 CARBOHYDRATE METABOLISM, OXYGEN SENSING, AND ROS

In liver, the periportal and perivenous hepatocytes are subjected to different oxygen tensions (70 and 35 mmHg, respectively), causing a hepatic *metabolic zonation*. Hepatocytes from the periportal zone have more gluconeogenic capacity than perivenous hepatocytes, which are better equipped for glycolysis. Hydrogen peroxide formation is higher in the periportal zone (maximum production of 1.2×10^{-9} M) than the perivenous zone (0.8×10^{-9} M).

When analyzing cultured rat hepatocytes, Helmut Acker and co-workers (from Germany; see *News Physiol Sci* **15**:202–208, 2000) recently observed that glucagon-dependent PEP carboxykinase (PEPCK) mRNA production is reduced under low oxygen tensions. However, addition of H_2O_2 (50 μM) to the cell cultures under low oxygen tensions restored maximal PEPCK mRNA production. On the other hand, insulin-mediated production of glucokinase mRNA transcripts is stimulated under low oxygen tensions but inhibited by H_2O_2. These results indicate that ROS (produced under different oxygen tensions) have the ability to regulate both gluconeogenic and glycolytic pathways, at least in rat liver.

The authors also provided evidence that H_2O_2 is only an intermediate in Fenton-mediated $^{\bullet}OH$ radical formation and that $^{\bullet}OH$ is the chemical species more closely linked with the regulation of PEPCK and glucokinase genes. Possibly, $^{\bullet}OH$ may oxidize –SH groups in certain candidate transcriptional factors, thus shifting the balance between reduced and oxidized states.

The main transcription factor involved in the oxygen-sensing mechanisms is the hypoxia inducible factor 1 (HIF-1) (see Chapter 6), whose activity seems to be redox-regulated. HIF-1 is a heterodimer protein complex that activates transcription through binding to specific hypoxia-responsive sequences present in genes that are activated by hypoxia, including those for glycolytic enzymes, growth factors, and vasoactive peptides. Hypoxia induces HIF-1 complex formation by stabilizing the HIF-1α subunit, which under normoxic conditions is degraded by an ubiquitin–proteasome system. The possiblity of either ROS-induced HIF-1 activation (under hypoxia) or ROS-induced HIF-1 deactivation (under normoxia) is still a matter of intense debate (see Michiels et al. *Free Radic Biol Med* **33**:1231–1242, 2002).

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