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The Basics of Oxidative Biochemistry

Satomi Miwa, Florian L. Muller, and Kenneth B. Beckman

1 Chemistry of Reactive Oxygen Species

Although a thorough knowledge of oxygen chemistry is necessary to understand the biochemistry of reactive oxygen species, that topic is beyond the scope of this book (see excellent works by Sawyer [1, 2] and the outstanding textbook of Halliwell and Gutteridge [3], Chapter 1). A large number of different reactive oxygen species (ROS) and reactive nitrogen species are now known to exist in biological systems, the most relevant to the present book being superoxide (O$_2^-$/HO$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), organic (lipid, alkyl, or short chain) hydroperoxides and hydroperoxide radicals (ROOH, ROO$^-$), peroxynitrite and peroxynitrous acid (ONOO-/ONOOH), carbonate radical (CO$_3$$^{2-}$), and reactive aldehydes of varying lengths. Various oxo-metal complexes, (such as those formed from heme and H$_2$O$_2$), may also be relevant. We limit our discussion to the chemical properties relevant to the biological toxicity of the best known reactive species: superoxide, hydrogen peroxide, and the hydroxyl radical. Although we focus on these three species, this is not to say that the many other known ROS (and the downstream products thereof) are not relevant to oxidative stress and aging.

1.1 Superoxide

A one-electron reduction of O$_2$ yields superoxide (HO$_2^-$/O$_2^-$; pK$^*$ = 4.88), with a redox potential O$_2$/O$_2^-$ of −160 mV at pH 7.0 under physiological conditions [1, 2, 4, 5]. Thus, a low-potential, single electron carrier is the ideal catalyst of superoxide formation (in biological systems, iron-sulfur clusters, semiquinones, and cytochromes are low-potential, single electron carriers [6, 7]). Superoxide spontaneously dismutates to H$_2$O$_2$, with a pH-dependent second order rate of ~10$^5$ M$^{-1}$ s$^{-1}$ (at pH 7.0 [4]). After the discovery of superoxide dismutase, many questioned whether superoxide dismutase (SOD) could be physiologically relevant, because superoxide is not particularly reactive [8], and it decays spontaneously at such a
fast rate: this misconception (ultimately proven wrong by the drastic phenotypes
brought about by SOD knockouts, in diverse organisms [9–17]) arises from the
fact that superoxide dismutation by SOD is first order with regard to superoxide
concentration, whereas spontaneous dismutation is second order. Indeed, it is now
known that superoxide is extremely toxic (much more so than H₂O₂) and that
intracellular concentrations in the picomolar range are lethal. The pH-dependent
equilibrium constant for superoxide dismutation to H₂O₂ is K = 10^{18} at pH 7.0, so
the reaction can be considered irreversible except in circumstances of very high
levels of SOD and very low levels of superoxide [18]. Apart from dismutation,
superoxide can act both as a reductant (e.g., superoxide reduces high-potential
ferricytochromes to their ferrous states, with rate constants of 10⁵ to 10⁶ M⁻¹ s⁻¹
[4]) and as an oxidant (especially in its protonated HO₂⁻ form [4, 5]) via the oxida-
tion of low-potential iron sulfur clusters, thereby liberating redox-active iron. The
liberation of Fe²⁺ from iron-sulfur clusters is believed by some investigators to be
the main pathway of biological superoxide toxicity [19, 20]: the availability of
redox active iron is thought to be the rate limiting step in OH⁻ formation (see
Subsection 1.3) by Fenton chemistry. Superoxide, as HO₂⁺ [21–23], can also initi-
ate lipid peroxidation. Other oxidations of potential biological relevance include
the reaction with catechols (~10⁴ M⁻¹ s⁻¹) and thiols (from 10 to 10⁴ M⁻¹ s⁻¹), the
latter of which can proceed in a peroxidation-like chain reaction, consuming sev-
eral thiols per superoxide molecule [24]. A third fate of superoxide is of interest:
being a free radical, superoxide can react with other free radicals in annihilation
reactions, which can proceed at diffusion limited (very fast) rates, because there is
no activation energy barrier [25]. The best example of this type of reaction is that
of superoxide with NO⁺, forming peroxynitrite/peroxynitrous acid [26]. Although
beyond the scope of this review, peroxynitrite can undergo subsequent chemical
rearrangements, yielding very strong oxidants (NO₂⁺, OH⁺, CO₃⁻ [27]). The pro-
duction of these strong oxidants, coupled with the great speed of peroxynitrite
formation, has led some to argue that peroxynitrite formation is the main pathway
of biological superoxide toxicity; however, this view remains controversial [28,
29]. Peroxynitrite formation is by no means the only biologically relevant radical
annihilation reaction. It is now recognized that many enzymes use stable carbon-
centered or delocalized free radicals as part of their normal catalytic mechanism
[30]. Superoxide could theoretically react very rapidly with such radicals, causing
irreversible enzymatic inactivation. One well-documented instance of such a case
is the reaction of superoxide with the tyrosyl radical of ribonucleotide reductase,
an enzyme absolutely essential for DNA synthesis, leading to tyrosine peroxide
formation and irreversible inactivation of this enzyme [31]. There are still other
pathways of superoxide toxicity. Superoxide can initiate the oxidation of short
chain sugars [32, 33], forming toxic α, β-dicarbonyls. Superoxide also can oxidize
low-potential heme proteins, such as hemoglobin and myoglobin, yielding the
oxygen-carrying incompetent met-forms [34]. Tyrosine peroxide formation may
be another pathway of superoxide toxicity [25], and we speculate that there are
others yet to be discovered.
1.2 *Hydrogen Peroxide*

Dismutation and oxidation reactions of superoxide yield hydrogen peroxide. Hydrogen peroxide, although more oxidizing than superoxide, is biologically less toxic: picomolar intracellular levels of superoxide are lethal, whereas micromolar levels of \( \text{H}_2\text{O}_2 \) can be tolerated. \( \text{H}_2\text{O}_2 \) is a potent oxidizer (although not always a fast oxidizer), and is much more diffusible than superoxide, because it is less reactive and is membrane permeable: \( \text{O}_2^- \) is generally considered membrane impermeable [35, 36], except in its \( \text{HO}_2^- \) form, which is in low abundance at physiological pH. \( \text{H}_2\text{O}_2 \) is usually a slow two-electron oxidizer [24], and is rather stable. However, in the presence of a metal catalyst or heme, it can act as a very rapid and indiscriminate oxidant; some biological molecules are direct oxidation targets of \( \text{H}_2\text{O}_2 \), specifically those with low-potential cysteines. \( \text{H}_2\text{O}_2 \)-induced thiol oxidation may be damaging as in the case of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [37–39], but it also may be relevant in the redox regulation of certain enzymes, a well-documented case being the PTP1B phosphatase [40, 41].

1.3 *Hydroxyl Radical*

The full oxidizing strength of \( \text{H}_2\text{O}_2 \) can be harnessed if it is single-electron reduced to \( \text{OH}^+ \), which is one of the most potent oxidizing agents known to chemistry, with redox potential +2.40 V [3]. This full strength can be achieved by Haber-Weiss chemistry, i.e., superoxide can react with \( \text{H}_2\text{O}_2 \), but the rate constants for this reaction are considered too low for biological significance [42]. Alternatively, \( \text{H}_2\text{O}_2 \) can react with reduced metal ions (most notably, \( \text{Cu}^+ \) and \( \text{Fe}^{2+} \) [43, 44]) to generate \( \text{OH}^+ \) in a reaction termed *Fenton chemistry* [20, 42, 44, 45]. \( \text{OH}^+ \) reacts at diffusion-limited rates with almost everything found in the cell (p 58 in [3]): as such, its toxicity is nonselective and its diffusion distance is very short. Because \( \text{OH}^+ \) reacts rapidly and indiscriminately, there is little that antioxidants can do, unless present in prohibitively high amounts. Thus, unlike the cases of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \), there is no enzyme that specifically detoxifies \( \text{HO}^+ \), and it seems that biological systems tightly regulate the availability of Fenton chemistry-capable metal ions to minimize \( \text{OH}^+ \) formation.

2 *Antioxidant System*

The eukaryotic antioxidant system has grown from the three classical enzymes—SOD, catalase, and glutathione peroxidase—to include a diverse and still expanding, enzymatic and nonenzymatic group of players. Furthermore, there are several enzymes that mitigate the effect of ROS by repairing oxidative damage, especially
with regard to DNA oxidative damage (see Chapter 12). Although it is beyond the scope of this review to detail each of these enzymes, we provided a brief introduction to the $O_2^-$ and $H_2O_2$ detoxifying branches of the antioxidant network, because these enzymes are discussed extensively throughout this book, and most chapters assume some familiarity with them. At the same time, we alert readers that the understanding of the antioxidant network is still incomplete, with new members still being discovered.

2.1 SOD Accelerates Dismutation of Superoxide Radical

The enzyme SOD has special significance for the free radical theory of aging. Its discovery strongly suggested that superoxide (and by extension, oxygen free radicals) was produced in vivo. SOD is the among the best known antioxidant enzymes, and knockout studies in *Escherichia coli*, yeast, flies, and mice all testify to its importance (for review, see [46, 47]). The discovery by Fridovich and McCord that erythrocyte catalyzes the dismutation of the superoxide anion led to an explosion of interest in oxygen free radicals from medical professionals and chemists alike. Indeed, SOD may be one the most well-studied enzymes. In mammalian systems, there are three different SOD isoforms encoded by three different genes [9, 48–51]. *Sod1* encodes copper-zinc SOD, which is found both in the cytoplasm and in the mitochondrial intermembrane space [52, 53]. *Sod2* encodes manganese superoxide dismutase, which is exclusively located in the mitochondrial matrix [50, 54]. *Sod3* is a copper-zinc SOD that is secreted and is extracellular in localization [49].

The general mechanism of superoxide dismutases can be summarized as follows, where $M$ stands for metal ($Cu^{2+}$ or $Mn^{3+}$), the superscripted minus sign ($\sim$) stands for negative electric charge, and the dot represents an unpaired electron. The rate constants do not vary in the pH range from 5.5 to 9.0.

$$
O_2^- + SOD-M \rightarrow O_2 + SOD-M^\sim (k = 10^9 \text{ M}^{-1} \text{ s}^{-1})
$$

$$
O_2^- + 2H^+ + SOD-M^\sim \rightarrow H_2O_2 + SOD-M (k = 10^9 \text{ M}^{-1} \text{ s}^{-1})
$$

The spontaneous decomposition of superoxide is pH dependent, and it can be written as follows (net reaction):

$$
O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 (k = 10^5 \text{ M}^{-1} \text{ s}^{-1} \text{ at pH 7.0})
$$

Thus, SOD accelerates the destruction of superoxide by increasing the rate constant for spontaneous dismutation by >1,000-fold, and also by making the rate of superoxide decay a first order rather than second order process with respect to superoxide concentration. This means that SOD is more efficient at accelerating the decomposition of superoxide, compared with spontaneous dismutation, at low superoxide concentration. However, because an enzyme cannot change the position of equilibrium, but only the rate at which it is achieved,
there is a lower limit of superoxide concentration below which the enzyme cannot reduce it.

In addition to catalyzing the (reversible) dismutation of $\text{O}_2^{-}$, SOD also can undergo a variety of radical-producing side reactions [55–58], most notably generating highly oxidizing $\text{OH}^\cdot$ and $\text{CO}_3^\cdot$ in an $\text{H}_2\text{O}_2^{-}\text{O}_2$-dependent manner termed peroxidase reaction (best documented in CuZn-SOD [58]). In addition, it also can decompose peroxynitrite to the highly reactive $\text{NO}_2^\cdot$ radical [56]. SOD can catalyze the $\text{CO}_2$ and $\text{H}_2\text{O}_2^{-}\text{O}_2$-dependent oxidation of a variety of molecules, including the dye 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein [55]. Finally, CuZn-SOD can catalyze the oxygen-dependent oxidation of thiols, in cysteine and glutathione [57]. These deleterious pro-oxidative side reactions may or may not be relevant in a non-pathological state, but they cannot be ignored when SOD is overexpressed at high levels in transgenic mice or flies.

### 2.2 Peroxiredoxins are Major Scavengers of Endogenously Produced $\text{H}_2\text{O}_2$

Although the superoxide-detoxifying system is compact with only three SOD enzymes, and the relative contribution of each enzyme is more or less understood, this is not the case for the cellular $\text{H}_2\text{O}_2$ detoxification system. There are multiple enzymes involved, including glutathione peroxidases, catalase, and peroxiredoxins. Catalase acts as a $\text{H}_2\text{O}_2$ “dismutase,” turning two $\text{H}_2\text{O}_2$ molecules into $\text{O}_2$ and $\text{H}_2\text{O}$. Peroxidases perform two-electron reduction of $\text{H}_2\text{O}_2^{-}\text{O}_2$ with its reducing equivalents ultimately originating from NAPDH.

It is traditionally held that glutathione peroxidase and catalase are the main scavengers of cellular $\text{H}_2\text{O}_2$. Based on genetic studies in yeast and mice, this view is no longer tenable. In the absence of catalase, oxidative stress and deleterious phenotypic consequences are minimal in yeast, mice, or even humans (patients with acatalasemia have no major pathology [13, 59, 60]). Nonetheless, lack of catalase does render cells susceptible to, and is the major kinetic sink for, exogenously added $\text{H}_2\text{O}_2$ [59, 60]. Although glutathione peroxidase 1 (Gpx1) is an abundant enzyme, and it also reacts rapidly with $\text{H}_2\text{O}_2^{-}\text{O}_2$, genetic ablation does not result in oxidative stress or deleterious phenotypes in either yeast or mice [61–63]; it is worth mentioning that Gpx1 is missing in *Drosophila* and even in certain mammals, such as the naked mole rat [64]. Gpx1-lacking mice are highly sensitive to bolus exogenous ROS generators such as paraquat and diquat [61–63], although they exhibit little or no elevation in basal levels of oxidative damage, and do not show any compensatory antioxidant up-regulations (Han, Muller, Perez, Van Remmen, Richardson, unpublished data). No deleterious phenotypes are evident in yeast lacking Gpx1 or catalase, even in environmental situations where excess ROS production is expected to occur, such as hyperoxia or the postdiauxic phase of yeast growth [13, 65]. It does not seem to be a question of redundancy either, because yeast strains lacking all three Gpx homologs are viable and they do not show any obvious phenotypic defects [66]. The reason why knocking out Cat or
Gpx1 does not affect phenotype or increase endogenous oxidative stress is, in our assessment, because endogenously produced H$_2$O$_2$ is detoxified by another enzyme system: the peroxiredoxins.

Peroxiredoxin was first discovered in yeast (a.k.a. thiol-specific antioxidant, tsa1 [67, 68]), and homologs are found in all kingdoms of life [68, 69]. In contrast to the lack of phenotype resulting from glutathione peroxidase or catalase yeast knockouts, knocking out tsa1 has deleterious consequences: increased oxidative damage, thermosensitivity, decreased growth under aerobic conditions, and reduced viability during the stationary phase; most intriguingly however, the rate of mutagenesis and genomic instability is dramatically (10- to 20-fold) increased [67, 70–74]. In contrast to the lack of phenotype in mice lacking catalase or Gpx1, knocking out peroxiredoxin 1 in mice causes increased oxidative damage, increased cancer incidence and shortened life span [75]. Knockout of peroxiredoxin 1 or 2 results in hemolytic anemia [75, 76].

Although Gpx1, catalase, and peroxiredoxins all react with H$_2$O$_2$, the chemical basis of their relative importance is still not fully understood. Catalase is the most economical way of removing H$_2$O$_2$, because no reducing equivalents are consumed. As such, it is very efficient at removing large quantities of H$_2$O$_2$, but at low concentrations of H$_2$O$_2$, it exhibits a nonspecific peroxidase activity, i.e., it can catalyze the H$_2$O$_2$-dependent oxidation of a variety of molecules [6]. Gpx1 consumes a two-electron reducing equivalent- for every H$_2$O$_2$ it reduces to water. What is unique about peroxiredoxins is that they are inactivated by high levels of H$_2$O$_2$, because their active site thiol is oxidized (“overoxidized”) to sulfenic acid [37, 68, 77]. As such, peroxiredoxins are ineffective at removing high levels of H$_2$O$_2$. Conversely, they seem to be the preferred target of low levels of H$_2$O$_2$, likely because of their high abundance, low redox potential, and rapid rate constant of reaction with H$_2$O$_2$ [37, 68, 77, 78].

To summarize this section, genetic evidence and recent biochemical work indicate that glutathione peroxidase, catalase, and peroxiredoxins play nonoverlapping roles in scavenging H$_2$O$_2$. High H$_2$O$_2$ levels are handled by glutathione peroxidase and catalase, whereas the lower levels of H$_2$O$_2$ produced by normal endogenous metabolism are scavenged by peroxiredoxins. This makes peroxiredoxins of great interest to the free radical theory of aging.

### 2.3 How Many Unknown Antioxidant Genes are out There?

Keep in mind that although the major players have probably been identified, new members of the antioxidant network are still being discovered. This is especially important when considering the effect of antioxidant knockout and overexpression on life span. Below, we highlight three relatively new antioxidant systems that we believe to be of great physiological significance, and that will receive increased attention in the near future.
2.3.1 Biliverdin/Bilirubin and Biliverdin Reductase

Biliverdin is a degradation product of heme that is reduced by biliverdin reductase to bilirubin. Ames’s group reported that bilirubin could act as a peroxidation chain-breaking antioxidant in a defined chemical system [79, 80]. Snyder’s group reported that biliverdin/bilirubin and biliverdin reductase form a network that catalytically detoxifies H$_2$O$_2$ [81], ablation of which results in apoptotic cell death. Although more work needs to be done (e.g., what are the phenotypical consequences of biliverdin reductase knockout?), it is probable that bilirubin will turn out to be a key player in the antioxidant network.

2.3.2 Apolipoprotein D (ApoD)

During an overexpression screen of genes for resistance to hyperoxia (100% O$_2$) in Drosophila species, Walker et al. [82] identified Glial Lazarillo, homolog of ApoD. Overexpression of ApoD thus conferred extended life span under hyperoxia. In further support of this finding, an independent group of investigators has reported that knocking out ApoD leads to a 20% reduction in median life span and increased oxidative damage [83]. ApoD belongs to the lipocalin family, which bind and transport small hydrophobic molecules [84]; although the exact biological function of ApoD is unknown, it evidently plays an important role in protection from oxidative stress, by yet unknown mechanisms.

2.3.3 Sulfiredoxin and Sestrin

Lower oxidation states of cysteine (disulfides) are readily reversible, while higher oxidation states, such as sulfinic acid, were once considered irreversible, biologically speaking. This view changed with the discovery of sulfiredoxin, an enzyme that can reduce sulfinic acid back to thiol, in an ATP-dependent manner [85]. Additional work suggests that it plays a role in resolving mixed disulfides bonds [86]. Initially discovered in yeast, sulfiredoxin is conserved in all eukaryotes, including mammals. In a perfect example of how multiple gene names can confuse the field, sulfiredoxin (Srxn1) was already known as a gene of unknown function, cloned by differential display of an in vitro model of tumorigenesis, and termed neoplastic progression 3 (Npn3), although nothing about its actual function was reported [87]. As a result, in most mouse microarray studies, sulfiredoxin is termed neoplastic progression 3, and typically classified as “cancer related” or “other” rather than as “antioxidant” [88, 89].

$Npn3/Srxn1$ is up-regulated by an exceptionally large fold-magnitude in microarray studies of oxidative stress. $Npn3/Srxn1$ is induced up to 32-fold by D3T (liver [88]), 12-fold by CdCl$_2$ (liver [89]), 4- to 10-fold by paracetamol (liver [90],...
supplemental materials), and 3.3-fold by paraquat (heart [91], supplemental materials). A survey of the Gene Expression Omnibus database also indicates that a large induction of Npn3/Srxn1 is observed in injury to the lung by hyperoxia (data set GDS247, ID 102780_at) or phosgene (GDS1244, 1451680_at). Our own microarray data indicate Npn3/Srxn1 is also strongly up-regulated in the liver of CuZn-SOD knockout mice (Han, Muller, Perez, Van Remmen, Richardson, unpublished data). That Npn3 and Sxrn1 are synonyms for the same gene has not been pointed out in any of the 15 papers written on Srxn1 since its discovery. Furthermore, this example highlights the problems associated with multiple names of genes and the need for uniform gene nomenclature in the postgenomic age.

Because it was discovered so recently, the function of sulfiredoxin is not yet fully known, and because no knockout of sulfiredoxin in mice is yet available, its true physiological importance remains to be established.

A similar catalytic activity to sulfiredoxin (reducing sulfenic acid back to sulfhydryl) was recently ascribed for the p53 target gene P26/sestrin1 and sestrin 2 [92]. This finding is intriguing, considering the increasing attention p53 is receiving as a modulator of ROS in vivo [93]. Although no knockout of sestrin 1 or 2 is yet available, knocking out p53 in mice results in increased oxidative damage, shortened life span, and cancer, which can be significantly attenuated by feeding of the antioxidant N-acetyl cysteine [93].

2.4 ROS Sources

Although it is now recognized that reactive oxygen species can be formed under many different conditions and in many different cellular compartments, the number of enzymes that have been documented to generate $O_2^-$ (either deleteriously or purposefully) is relatively small. Superoxide can be produced by NADPH oxidases [94] (first thought to be unique to phagocytes but now known to be located on the plasma membrane of most cell types [95]), xanthine oxidase [96], aldehyde oxidase [97], cytochromes P450 [98], and the mitochondrial electron transport chain [99, 100]. Many more enzyme systems (most oxygenases, e.g., monoamine oxidase) are known to generate $H_2O_2$. A particularly active source of $H_2O_2$ includes enzymes involved in the oxidation of fatty acids in the peroxisomes [6].

Despite the variety of ROS sources, most interest from a gerontological perspective has centered on the mitochondrial electron transport chain. Although it was recognized early on, through in vitro biochemical work, that the mitochondrial electron transport chain is an exceptionally strong source of superoxide, definitive proof (and its underlying biological importance) came from the finding that mice lacking mitochondrial matrix Mn-SOD (Sod2) die several days after birth [9, 10]. Mice knockouts for cytoplasmic (and intermembrane space) CuZn-SOD (Sod1) and extracellular CuZn-SOD (Sod3), although certainly not normal [51,101–108], do not exhibit as dramatic a phenotype as Sod2–/– mice, thus indicating that the mitochondrial matrix is indeed the most important site of superoxide toxicity. This
also seems to be true in *Drosophila melanogaster*, although the Sod1–/– phenotype is more severe in flies than in mice, only Sod2–/– results in true postnatal lethality [16, 109, 110]. Removing xanthine oxidase worsens, rather than ameliorates, the phenotype of Sod1 knockout flies [111]. Thus, for the present review, the mitochondrial electron transport chain remains the focus for oxidative stress and aging; however, no one would seriously argue that other nonmitochondrial sites of superoxide generation are not relevant to aging (e.g., the plasma membrane oxidoreductase [112]).

The mitochondrial electron transport chain is a series of one-electron shuttles of progressively stronger oxidants, which couple the energy released during oxidation to the pumping of protons across the inner mitochondrial membrane, and to the eventual generation of ATP [113]. There are several low-potential carriers in this system, which can potentially donate electrons to superoxide (for review, see [7]). Mitochondrial superoxide generation was first discovered as H2O2 released from mitochondria treated with the respiratory inhibitors antimycin A or rotenone [114, 115]. Not until Loschen et al. discovered that superoxide radicals are generated by submitochondrial particles, under the same conditions as H2O2 formation, was it realized that mitochondrial H2O2 originates as a dismutation product of superoxide [99]. For convenience, mitochondrial superoxide production is still largely assayed indirectly by measuring H2O2 [115, 116]. Most investigators now believe that complex I and complex III are the main sources of superoxide [7, 113] in mammalian mitochondria; it is also known that mutations can turn complex II into a superoxide generator [117, 118]. It has also been suggested that α-ketoglutarate dehydrogenase generates superoxide and H2O2 [119, 120]. Whereas it may very well produce H2O2, inhibition of the respiratory chain with KCN, which would be predicted (based on dependence on high NADH-to-NAD+ ratio) to increase superoxide production dramatically, actually rescues rather than kills a Mn-SOD lacking yeast ([12] and see Chapter 5). In mitochondria from *D. melanogaster*, glycerol 3-phosphate dehydrogenase is a very potent source of superoxide [121]. Complex I releases superoxide exclusively toward the mitochondrial matrix, whereas complex III and glycerol 3-phosphate dehydrogenase release superoxide toward both the matrix and the cytoplasm [121–123]. Considerable debate surrounds the importance of these sites, in terms of how much superoxide mitochondria truly produce in vivo. In vitro, a very large rate of superoxide production is observed when mitochondria respire on succinate and undergo reverse-electron transfer through complex I [124–126]. The often-quoted figure that “1-2% of electrons going through the respiratory chain are diverted to superoxide” comes from these studies. Although it is taken as self-evident by some investigators [127], whether or not reverse-electron transfer occurs in vivo has not yet been established. Under normal forward-electron transfer, by using the rate of H2O2 release as an indicator of superoxide production, that number is closer to 0.1% of electrons [47, 124]. Because oxygen tension is correlated with the rate of superoxide production [127, 128], even the 0.1% figure may be an overestimate, because oxygen tension in vivo is ~3% compared with 21% used in the above experiments. Some investigators have thus claimed that no H2O2 is released (and by extension, no superoxide is produced) from “normal” mitochondria in the absence of respiratory inhibitors [129, 130].
This statement is difficult to take seriously, considering that mice and flies lacking mitochondrial Sod2 die shortly after birth [9, 16] amid massive oxidative stress. Thus, although the rate of mitochondrial superoxide production is likely low in vivo (perhaps as low as 1 electron in 40,000 diverted toward superoxide formation), this is still high enough to be incompatible with life, in the absence of SOD.

One of the best ways to test the free radical theory of aging would be to modify the rate of mitochondrial superoxide production [7, 131]. However, the molecular details of this process are poorly understood. Studies with respiratory inhibitors (e.g., antimycin A, rotenone) have shown that almost anything that interrupts the “smooth” electron transfer through the respiratory chain results in a dramatic stimulation of ROS production (for review, see [7, 124, 128, 132, 133]). One can speculate that the availability of both a reduced reactive intermediate (e.g., semiquinone, iron-sulfur cluster) and O₂⁻, and the ability of the latter to reach the former, should determine the rate of O₂⁻ production. Oxygen tension, as mentioned, has already been demonstrated experimentally to modulate superoxide production [14, 127, 128, 134], and the absence of oxygen (anaerobiosis or anoxia) is the only condition known to prevent superoxide formation. Not surprisingly, heat, which denatures proteins and exposes reactive intermediates to O₂, also dramatically stimulates superoxide formation [135] (in model organisms, anaerobiosis dramatically increases theromotolerance [136, 137]). Thus, heat shock proteins may minimize superoxide production by maintaining the electron transfer complexes in the properly folded state. Finally, it is known that the higher the mitochondrial membrane potential (ΔΨ), the higher the rate of superoxide production [6, 128, 132, 138]. Superoxide production by succinate-driven reverse-electron transfer is essentially eliminated by a drop of >10 mV in ΔΨ [124, 139], which is so small that it is essentially undetectable using routine ΔΨ probes such as safranin O [139]. Even forward-electron transfer with glutamate/malate is inhibited by uncouplers (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), but the drop in membrane potential required is substantially greater [140]. The dependence of superoxide production on ΔΨ is generally explained by a higher ΔΨ causing accumulation of electrons on reactive intermediates (such as semiquinones, flavosemiquinones, and low-potential iron-sulfur clusters), which are required for proton pumping [132]. Thus, “mild” uncoupling has been proposed as a method to reduce superoxide production therapeutically [132]. It seems that nature has espoused this strategy and that it has devised special proteins, dubbed uncoupling proteins, to maintain the membrane potential at safe low levels [141].

2.5 Measuring Oxidative Damage

Because ROS have short half-lives, and they are found at low concentrations in vivo, they are exceedingly difficult to observe. The alternative that biochemists have gravitated toward is measuring the end products of ROS, i.e., oxidative damage. Measuring oxidative damage is almost as old as the free radical theory itself.
It has a history of controversy, and many techniques used at one point in time have subsequently proved inappropriate, usually due to artifactual oxidation of the sample during preparation (see discussion on pp 388 and 407 in [3]). In this section, we briefly discuss the main current techniques for the measurement of end product oxidative damage. What makes a good marker of oxidative damage? A good marker of oxidative damage must be increased when oxidative stress is present (i.e., when induced by known treatments or agents that cause oxidative damage, e.g., paraquat, diquat, ionizing radiation, hyperoxia), and it must remain unchanged when oxidative stress is absent. The marker must measure a product that is endogenously present, not produced during the isolation procedure. This latter requirement is not trivial, and it affects several assays: even if a difference between a treatment and a control is observed, if the majority of the signal is artifactually produced during isolation, it will be very difficult to conclude that the difference between the samples did not arise during the preparation procedure (rather than having been endogenously present).

2.5.1 Lipid Peroxidation

Lipid peroxidation is a chain reaction in which carbon-centered radicals at the allylic position of polyunsaturated fatty acids (PUFA) react with molecular oxygen (at near diffusion-limited rates), thereby forming a peroxyl radical (ROO'). ROO' can then abstract the allylic H-atom from nearby PUFA (becoming a lipid peroxide ROO-H), which creates another carbon-centered radical, thereby repeating the process described above (for review, see p 291 in [3]). The initial proton abstraction event (“the chain initiator”) is thought to be initiated mainly by superoxide (in its protonated form, HO₂⁺ [21, 22]). The higher the number of double bonds (unsaturation) in a fatty acid, the greater its propensity to peroxidize. In subsequent reactions, lipid peroxides can undergo a variety of reactions, yielding a myriad of end products, e.g., reactive aldehydes (malondialdehyde), alkanes, isoprostanes, and isoketals. Vitamin E (α-tocopherol) plays a critical role in minimizing lipid peroxidation; in fact, dietary induction of vitamin E deficiency results in profound oxidative stress, and if unchecked leads to death [142].

The oldest assay to measure lipid peroxidation is the thiobarbituric acid-reactive substances assay (p 407 in [3]). This assay measures thiobarbituric acid-reactive substances: the reactive end product aldehydes formed during peroxidation of polyunsaturated fatty acids. It is now understood that, although suitable for in vitro chemical systems, this assay is inappropriate to determine lipid peroxidation in vivo, because >90% of the signal actually originates from artifactual oxidation during the harsh isolation procedure.

In the last decade, many new assays to measure lipid peroxides in vivo have been developed. The most popular marker for measuring lipid peroxidation is the gas chromatography-mass spectrometry F₂-isoprostan assay developed by Roberts and Morrow [143, 144]. F₂-isoprostanes are cyclooxygenase-independent oxidation products of arachidonic acid, which are produced in every tissue where this fatty
acid is present [145, 146]. F$_2$-isoprostanes are eliminated via the bloodstream, enabling estimation of whole-organism lipid peroxidation by measuring plasma F$_2$-isoprostanes [147]. F$_2$-isoprostanes have been reported to be elevated in a variety of human pathologies [148]. Since isoprostanes are terminal end products, isolation artifacts are minimized (though not eliminated, because F$_2$-isoprostane levels increase if tissues are stored below −80 °C). It is our opinion that plasma F$_2$-isoprostanes are currently the most robust marker for measurements of oxidative damage. It has been reported that F$_2$-isoprostanes are dramatically increased in situations of oxidative stress (e.g., diquat, CCl$_4$), with the increases being much higher than that with previously used markers (e.g., 8-oxo-7,8-dihydro-2′-deoxyguanosine [8-oxo-dG]). In addition, Jackson and Morrow have extended their initial findings with isoprostanes, demonstrating that peroxidation products of docohexanoic acid, F4 neuroprostanes [149], are also useful markers of lipid peroxidation, especially in neuronal tissues.

2.5.2 DNA Oxidative Damage

Much work has gone into exploring the hypothesis that oxidative damage to DNA causes mutations and cancer. At least 100 different types of oxidative DNA lesions have been reported, including base modifications (e.g., 8-oxo-dG, thymidine glycol, and 8-hydroxycytosine), single- and double-strand breaks and interstrand cross-links [150, 151]. Measuring DNA oxidative damage has a tortuous history that is still not fully settled [152]. Although DNA oxidation yields many products, only a few have been rigorously quantified in vivo [153, 154]; of these, the most popular is 8-oxo-dG. The levels of 8-oxo-dG are measured by high-performance liquid chromatography (HPLC), typically using an electrochemical (EC) detector. The applicability of this assay to estimating the [low] endogenous levels of DNA oxidative damage in vivo has been questioned [155], because large artifactual increases occur during DNA extraction. For example, the values of 8-oxo-dG for the same tissue from the same species have ranged over 3 orders of magnitude [152]. This assay is typically carried out on whole fresh tissue or frozen cells. The sample is first homogenized, and digested with proteinase K at 56 °C; DNA is extracted with phenol and subsequently hydrolyzed into nucleotides (using nuclease P1), then converted to nucleosides by using alkaline phosphatase. The nucleosides are then injected into the HPLC, and 8-oxo-dG is detected electrochemically. Considering the harshness of these treatments, the low redox potential of guanosine, and the large excess of deoxyguanosine (dG) vs. 8-oxo-dG (even a 0.01% artifactual oxidation of dG would translate into a 10-fold artifactual increase of 8-oxo-dG [156]), it is easy to see how artifactual oxidations could inflate the measured amount of 8-oxo-dG. Several factors causing artifactual oxidation have been identified [157], including light from fluorescent lamps [158]. The use of phenol during the DNA extraction procedure also seems problematic [159]; this can be avoided by substituting NaI in the DNA extraction protocol [157, 159]. Other strategies to minimize oxidation involve the addition of desferrioxamine (to prevent Fenton chemistry) or antioxidant enzymes such as catalase [157, 160]. Using these optimizations, the level of 8-oxo-dG has
been measured to be ∼0.5 per 10^6 dG in human lymphocytes and ∼2 per 10^6 dG in rat liver DNA [157, 160]. Even if artifactual oxidation during DNA extraction were reduced to zero, this would not address the possibility of artifactual DNA oxidation during tissue homogenization: this is relevant because homolytic bond cleavage and free radical formation have been demonstrated under those conditions. Experiments with H_2^{18}O under anaerobic conditions may resolve this issue, because any 8-oxo-dG produced artfactually would have been distinguishable by mass spectroscopy [160]. Significantly, even the lowest values obtained using the HPLC-EC method are still an order of magnitude higher than those obtained with the formamidopyrimidine-DNA glycosylase (Fpg-glycosylase) comet assay [152, 161]. The comet assay does not require tissue homogenization and DNA hydrolysis, but can only be used on cells, not whole tissues [162, 163]. It quantifies the number of strand breaks after treatment with Fpg-glycosylase, which converts 8-oxo-dG into single-strand breaks. The ∼10-fold discrepancy between these two assays has not yet been resolved, and it cannot be attributed to differences in endogenous endonuclease activity, since the difference persists even in Ogg1 knockout mice, which lack base excision repair [164, 165].

2.5.3 Protein Oxidation

Numerous oxidative modifications have been documented in proteins. Proteins vary in their susceptibilities to different types of oxidants, and in the sites and degree of oxidation. Such differences are generally influenced by types of accessible amino acid residues in the proteins. The best-known types of protein oxidation are the nitration of tyrosine, the sulfoxidation of methionine, and the carbonylation of most amine-containing amino acid residues [166–169].

Peroxynitrite (ONOO-), which can be formed by the reaction of NO with superoxide, NO + O_2^{−} → ONOO^(−) (k = 7 × 10^9 M^{-1} s^{-1}), can convert tyrosine to nitrotyrosine. The hydroxyl group of certain tyrosine residues is critical in some enzymes and cell signalling molecules (e.g., tyrosine phosphorylation). It was shown that for glutamine synthetase in E. coli, nitration of either one of two different tyrosine residues inhibited the enzymatic function [170]. There is also evidence indicating that nitrotyrosilation of Mn-SOD decreases its activity [171, 172].

Sulfur-containing amino acids (methionine and cysteine) can be oxidized by hydrogen peroxide, superoxide, peroxynitrite, and perhaps by molecular oxygen itself. However, these are the only oxidative modifications of proteins that can be repaired. ROS-mediated oxidation of methionine (Met) residues leads to methionine sulfoxide (MetO), consisting of a mixture of the S- and R-epimers of MetO. Methionine sulfoxide reductases (MsrA) catalyze the thioredoxin-dependent reduction of MetO back to Met. The S-epimer of MetO is reduced back to Met by MsrA, and the R-epimer by MsrB. Importantly however, the oxidized form of thioredoxin produced during the reduction of MetO can be converted back to reduced form by the enzyme thioredoxin reductase, in an NADPH-dependent reaction. This cyclic oxidation and reduction of methionine has been proposed to play an antioxidant role [173]. Reversible oxidation of cysteine (thiol) residues in turn mediates the
antioxidant function of glutathione, thioredoxin, metallothioneins, glutaredoxin, and peroxiredoxins. Oxidation of thiols (sulfhydryl) yields disulfides, and further oxidation yields sulfenic and sulfinic acids, and eventually sulfones [174]. Certain enzymes, such as GAPDH, have active site cysteines that can become irreversibly oxidized [37–39, 175]. A newly discovered antioxidant gene, termed sulfiredoxin, can reduce sulfenic acid back to sulfhydryl (thiol) [85, 176]. The sulfones are still considered irreversible.

Carbonylation of proteins can occur by several different oxidative pathways, including metal-catalyzed oxidation of specific amino acid side chains (histidine, arginine, proline, lysine, and threonine) and adduction of carbonyl-containing oxidized lipids (e.g., 4-hydroxynonenal, malondialdehyde) and sugars [177]. Carbonyl content of samples has been popularly used as a global indicator of protein oxidation levels, perhaps due in part to the simplicity of the assay (carbonyl groups can be detected spectrophotometrically after their reactions with 2,4-dinitrophenylhydrazine), although this assay is also not free from controversy [178]. Specific proteins, such as adenine nucleotide translocase [179] and aconitase [180], seem be preferentially carbonylated with age in flies. In mice, carbonic anhydrase 3 and CuZn-SOD are preferentially carbonylated during aging [178, 181]. Oxidative modification of bacterial glutamine synthetase results in its inactivation and degradation [182, 183]. Protein carbonylation also can be quantified by gas chromatography-mass spectrometry [184, 185]. For example, glutamic and aminoadipic semialdehyde are the markers of direct protein oxidation by ROS, and they are found to be the major constituent of the total protein carbonyl value [184], whereas \( \text{N}^\epsilon-(\text{malondialdehyde})\text{lysine} \) and \( \text{N}^\epsilon-(\text{carboxymethyl})\text{lysine} \) (CML) arise from lipid peroxidation products [186]. \( \text{N}^\epsilon-(\text{carboxyethyl})\text{lysine} \) and CML also can be formed through glycoxidative damage from sugars [186, 187].

2.6 How Does Oxidative Damage Kill or Compromise the Function of the Cell?

In the preceding sections, we outlined the chemistry of some of the best-described ROS, and the most easily measureable types of damage that ROS can inflict on macromolecules. Here, we summarize how these oxidative modifications can compromise cellular function or lead to cell death.

2.6.1 Lipid Peroxidation

Lipid peroxidation of membranes has the immediate effect of decreasing membrane fluidity and increasing ionic permeability [188]. High enough levels of lipid peroxidation can directly lead to loss of membrane barrier function, cell lysis, and cell death. Because ionic gradients play a critical role in several physiological processes, including energy metabolism (mitochondrial inner membrane) and neuronal
conductance (plasma membrane), even moderately increased lipid peroxidation has the potential to be highly disruptive to overall tissue and organism function. Deficiency of the chain-breaking antioxidant, vitamin E, results in many deleterious phenotypes, including fertility loss, neurological phenotypes [189], myopathy [142], lipofuscin accumulation [190], and erythrocyte lysis; if severe enough, vitamin E deficiency results in death [142]. Absence of the lipid hydroperoxide-scavenging enzyme glutathione peroxidase 4 results in early embryonic lethality in mice [191], testifying to the great toxicity of lipid peroxidation.

2.6.2 DNA Oxidative Damage

Damage to DNA by reactive oxygen species falls into two main categories: damage to nucleotide bases and strand breaks (which, biologically, predominantly result in mutagenesis and genomic instability, respectively). Mutations, in turn, contribute to carcinogenesis, whereas genomic instability contributes both to carcinogenesis and, more critically, to cell death. There is an increasing body of evidence in model organisms (e.g., yeast and E. coli) that oxidative stress is a strong contributor to both mutagenesis and genomic instability. For example, genetic ablation of CuZn-SOD and peroxiredoxin 1 (tsa1) cause a 5- and 10-fold increase, respectively, in mutagenesis in yeast, as measured by the canavanine method [15, 72, 192–194]. This increased mutagenesis is entirely prevented by growth under anaerobic conditions [15, 73] (complete absence of oxygen implies no formation of superoxide and H2O2). In fact, spontaneous mutagenesis also is decreased ∼3-fold by anaerobic conditions in wild-type yeast, indicating that oxidative stress is an important driver of mutagenesis even under antioxidant enzyme sufficient conditions [73]. Similar results also have been obtained in E. coli [195]. Oxidative stress brought about by antioxidant enzyme ablation also increases genomic instability in yeast. Knockout of peroxiredoxin 1 (tsa1) resulted in a 10-fold increase in gross chromosomal rearrangements [73, 74]. Again, anaerobic conditions dramatically reduce the gross chromosomal rearrangement rate, not only in the antioxidant knockouts, but also in wild-type control yeast [73].

Finally, although knockout of neither sod1 nor tsal is lethal by itself in yeast grown under optimal conditions (rich media), it was recently reported that combining sod1+/− or tsal+/− with a wide assortment of knockouts of DNA repair genes resulted in synthetic lethality [196]. For example, a double knockout of either sod1 or tsal with rad51 (involved in double-strand break recombination repair) was synthetically lethal [73, 196]. In the case of tsal+/−rad 51+ double knockout, the synthetic lethality could be rescued by growth under anaerobic conditions [73].

These data in single-celled model systems complement basic chemical work, and they establish that ROS are a significant cause of DNA damage in vivo, driving both mutagenesis and genomic instability. However, it is worth remembering that yeast and E. coli grow at 21% O2 (normal atmospheric oxygen tension), whereas most mammalian cells are exposed to ~10 times less oxygen in situ; the relative importance of DNA oxidative damage may therefore be less in higher organisms.
2.6.3 Oxidative Damage to Proteins

Oxidative damage to proteins can be physiologically detrimental by several pathways. We briefly discuss two such pathways here: inactivation of enzymatic function and stimulation of protein aggregation.

There are several well-described examples of direct, selective inactivation of enzymatic function by different ROS: aconitase (superoxide [197], reversible), ribonucleotide reductase (superoxide [31], irreversible), GAPDH (H$_2$O$_2$, irreversible [37–39, 175]), carbonic anhydrase [181], and glutamine synthetase [182]. In certain cases, the ROS-dependent inactivation has clearly demonstrable phenotypical and metabolic consequences, e.g., the lysine and methionine auxotrophies of sod1$^{-/-}$ yeast can directly be ascribed to the superoxide-dependent inactivation of iron sulfur-containing enzymes [198–200].

It is increasingly appreciated that protein aggregation plays a critical role in many (most prominently neurodegenerative) diseases [201]. The notion that ROS can contribute to protein aggregation has been long known, especially considering the role of ROS in formation of lipofuscin, the age pigment composed of aggregated, oxidized proteins and lipids [202, 203]. Increasing evidence indicates that, at least in vitro, oxidative modification increases thermodynamic instability and facilitates protein aggregation, e.g., α-synuclein (Parkinson’s disease) [204] and CuZn-SOD (amyotrophic lateral sclerosis). It has been suggested that this is a key pathway by which oxidative damage contributes to aging [205].

3 Conclusions

Chemical studies indicate that ROS can damage a number of different cellular macromolecules, and physiological studies in model organisms indicate that ROS can compromise cell function and viability in many ways. However, an efficient and complex network of enzymatic and nonenzymatic players does exist; how much oxidative damage “escapes” the effects of this network under normal conditions is still not fully resolved. Finally, it is important to recall that oxygen tension in animals is typically 10 times lower than that to which the above-mentioned model organisms are exposed.

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