Background

Before we consider the enzymatically controlled reactions of dioxygen in living systems, it is instructive to consider the uncontrolled and deleterious reactions that must also occur in aerobic organisms. Life originally appeared on Earth at a time when the atmosphere contained only low concentrations of dioxygen, and was reducing rather than oxidizing, as it is today. With the appearance of photosynthetic organisms approximately 2.5 billion years ago, however, the conversion to an aerobic, oxidizing atmosphere exposed the existing anaerobic organisms to a gradually increasing level of oxidative stress.25,26 Modern-day anaerobic bacteria, the descendants of the original primitive anaerobic organisms, evolved in ways that enabled them to avoid contact with normal atmospheric concentrations of dioxygen. Modern-day aerobic organisms, by contrast, evolved by developing aerobic metabolism to harness the oxidizing power of dioxygen and thus to obtain usable metabolic energy. This remarkably successful adaptation enabled life to survive and flourish as the atmosphere became aerobic, and also allowed larger, multicellular organisms to evolve. An important aspect of dioxygen chemistry that enabled the development of aerobic metabolism is the relatively slow rate of dioxygen reactions in the absence of catalysts. Thus, enzymes could be used to direct and control the oxidation of substrates either for energy generation or for biosynthesis. Nevertheless, the balance achieved between constructive and destructive oxidation is a delicate one, maintained in aerobic organisms by several means, e.g.: compartmentalization of oxidative reactions in mitochondria, peroxisomes, and chloroplasts; scavenging or detoxification of toxic byproducts of dioxygen reactions; repair of some types of oxidatively damaged species; and degradation and replacement of other species.6

The classification "anaerobic" actually includes organisms with varying degrees of tolerance for dioxygen: strict anaerobes, for which even small concentrations of O2 are toxic; moderate anaerobes, which can tolerate low levels of dioxygen; and microaerophiles, which require low concentrations of O2 for growth, but cannot tolerate normal atmospheric concentrations, i.e., 21 percent O2, 1 atm pressure. Anaerobic organisms thrive in places protected from the atmosphere, for example, in rotting organic material, decaying teeth, the colon, and gangrenous wounds. Dioxygen appears to be toxic to anaerobic organisms largely because it depletes the reducing equivalents in the cell that are needed for normal biosynthetic reactions.6

Aerobic organisms can, of course, live in environments in which they are exposed to normal atmospheric concentrations of O2. Nevertheless, there is much evidence that O2 is toxic to these organisms as well. For example, plants grown in varying concentrations of O2 have been observed to grow faster in lower than normal concentrations of O2.22 E. coli grown under 5 atm of O2 ceased to grow unless the growth medium was supplemented with branched-chain amino acids or precursors. High concentrations of O2 damaged the enzyme dihydroxy acid dehydratase, an important component in the biosynthetic pathway for those amino acids.28 In mammals, elevated levels of O2 are clearly toxic, leading first to coughing and soreness of the throat, and then to convulsions when the level of 5 atm of 100 percent O2 is reached. Eventually, elevated concentrations of O2 lead to pulmonary edema and irreversible lung damage, with obvious damage to other tissues as well.6 The effects of high concentrations of O2 on humans is of some medical interest, since dioxygen is used therapeutically for patients experiencing difficulty breathing, or for those suffering from infection by anaerobic organisms.6
Biological Targets

The major biochemical targets of O₂ toxicity appear to be lipids, DNA, and proteins. The chemical reactions accounting for the damage to each type of target are probably different, not only because of the different reactivities of these three classes of molecules, but also because of the different environment for each one inside the cell. Lipids, for example, are essential components of membranes and are extremely hydrophobic. The oxidative damage that is observed is due to free-radical autoxidation (see Reactions 5.16 to 5.21), and the products observed are lipid hydroperoxides (see Reaction 5.23). The introduction of the hydroperoxide group into the interior of the lipid bilayer apparently causes that structure to be disrupted, as the configuration of the lipid rearranges in order to bring that polar group out of the hydrophobic membrane interior and up to the membrane-water interface. DNA, by contrast, is in the interior of the cell, and its exposed portions are surrounded by an aqueous medium. It is particularly vulnerable to oxidative attack at the base or at the sugar, and multiple products are formed when samples are exposed to oxidants in vitro. Since oxidation of DNA in vivo may lead to mutations, this type of damage is potentially very serious. Proteins also suffer oxidative damage, with amino-acid side chains, particularly the sulfur-containing residues cysteine and methionine, appearing to be the most vulnerable sites.

Defense and Repair Systems

The biological defense systems protecting against oxidative damage and its consequences are summarized below.

1. Nonenzymatic Oxidant Scavengers

Some examples of small-molecule antioxidants are \((\alpha\text{-tocopherol (vitamin E; 5.24, which is found dissolved in cell membranes and protects them against lipid peroxidation, and ascorbate (vitamin C; 5.25 and glutathione (5.26, which are found in the cytosol of many cells. Several others are known as well.\)
2. Detoxification Enzymes

The enzymatic antioxidants are (a) catalase and the various peroxidases, whose presence lowers the concentration of hydrogen peroxide, thereby preventing it from entering into potentially damaging reactions with various cell components (see Section VI and Reactions 5.82 and 5.83), and (b) the superoxide dismutases, whose presence provides protection against dioxygen toxicity that is believed to be mediated by the superoxide anion, $\text{O}_2^-$ (see Section VII and Reaction 5.95).

Some of the enzymatic and nonenzymatic antioxidants in the cell are illustrated in Figure 5.1.

3. Systems for Sequestration of Redox-active Metal Ions

Redox-active metal ions are present in the cell in their free, uncomplexed state only in extremely low concentrations. They are instead sequestered by metal-ion storage and transport proteins, such as ferritin and transferrin for iron (see Chapter 1) and ceruloplasmin for copper. This arrangement prevents such metal ions from catalyzing deleterious oxidative reactions, but makes them available for incorporation into metalloenzymes as they are needed.

In vitro experiments have shown quite clearly that redox-active metal ions such as $\text{Fe}^{2+/3+}$ or $\text{Cu}^{+2+}$ are extremely good catalysts for oxidation of sulfhydryl groups by $\text{O}_2$ (Reaction 5.27).\(^\text{30}\)

$$4\text{RSH} + \text{O}_2 \xrightarrow{M^{n+}} 2\text{RSSR} + 2\text{H}_2\text{O} \tag{5.27}$$

In addition, in the reducing environment of the cell, redox-active metal ions catalyze a very efficient one-electron reduction of hydrogen peroxide to produce hydroxyl radical, one of the most potent and reactive oxidants known (Reactions 5.28 to 5.30).\(^\text{31}\)
$M^{n+} + Red^{-} \rightarrow M^{(n-1)+} + Red \tag{5.28}$

$M^{(n-1)+} + H_{2}O_{2} \rightarrow M^{n+} + OH^{-} + HO \cdot \tag{5.29}$

$Red^{-} + H_{2}O_{2} \rightarrow Red + OH^{-} + HO \cdot \tag{5.30}$

$\text{(Red}^{-} = \text{reducing agent)}$

Binding those metal ions in a metalloprotein usually prevents them from entering into these types of reactions. For example, transferrin, the iron-transport enzyme in serum, is normally only 30 percent saturated with iron. Under conditions of increasing iron overload, the empty iron-binding sites on transferrin are observed to fill, and symptoms of iron poisoning are not observed \textit{in vivo} until after transferrin has been totally saturated with iron. Ceruloplasmin and metallothionein may play a similar role in preventing copper toxicity.

It is very likely that both iron and copper toxicity are largely due to catalysis of oxidation reactions by those metal ions.

4. Systems for the Repair or Replacement of Damaged Materials

Repair of oxidative damage must go on constantly, even under normal conditions of aerobic metabolism. For lipids, repair of peroxided fatty-acid chains is catalyzed by phospholipase A$_2$, which recognizes the structural changes at the lipid-water interface caused by the fatty-acid hydroperoxide, and catalyzes removal of the fatty acid at that site. The repair is then completed by enzymatic reacylation.

Although some oxidatively damaged proteins are repaired, more commonly such proteins are recognized, degraded at accelerated rates, and then replaced. For DNA, several multi-enzyme systems exist whose function is to repair oxidatively damaged DNA.

For example, one such system catalyzes recognition and removal of damaged bases, removal of the damaged part of the strand, synthesis of new DNA to fill in the gaps, and religation to restore the DNA to its original, undamaged state. Mutant organisms that lack these repair enzymes are found to be hypersensitive to O$_2$, H$_2$O$_2$, or other oxidants.

One particularly interesting aspect of oxidant stress is that most aerobic organisms can survive in the presence of normally lethal levels of oxidants if they have first been exposed to lower, nontoxic levels of oxidants. This phenomenon has been observed in animals, plants, yeast, and bacteria, and suggests that low levels of oxidants cause antioxidant systems to be induced \textit{in vivo}. In certain bacteria, the mechanism of this induction is at least partially understood. A DNA-binding regulatory protein named OxyR that exists in two redox states has been identified in these systems.

Increased oxidant stress presumably increases concentration of the oxidized form, which then acts to turn on the transcription of the genes for some of the antioxidant enzymes. A related phenomenon may occur when bacteria and yeast switch from anaerobic to aerobic metabolism. When dioxygen is absent, these microorganisms live by fermentation, and do not waste energy by synthesizing the enzymes and other proteins needed for aerobic metabolism. However, when they are exposed to dioxygen, the synthesis of the respiratory apparatus is turned on. The details of this induction are not known completely, but some steps at least depend on the presence of heme, the prosthetic group of hemoglobin and other heme proteins, whose synthesis requires the presence of dioxygen.
Molecular Mechanisms of Dioxygen Toxicity

What has been left out of the preceding discussion is the identification of the species responsible for oxidative damage, i.e., the agents that directly attack the various vulnerable targets in the cell. They were left out because the details of the chemistry responsible for dioxygen toxicity are largely unknown. In 1954, Rebeca Gerschman formulated the "free-radical theory of oxygen toxicity" after noting that tissues subjected to ionizing radiation resemble those exposed to elevated levels of dioxygen.\textsuperscript{35} Fourteen years later, Irwin Fridovich proposed that the free radical responsible for dioxygen toxicity was superoxide, $\text{O}_2^-$, based on his identification of the first of the superoxide dismutase enzymes.\textsuperscript{36} Today it is still not known if superoxide is the principal agent of dioxygen toxicity, and, if so, what the chemistry responsible for that toxicity is.\textsuperscript{6}

There is no question that superoxide is formed during the normal course of aerobic metabolism,\textsuperscript{121} although it is difficult to obtain estimates of the amount under varying conditions, because, even in the absence of a catalyst, superoxide disproportionates quite rapidly to dioxygen and hydrogen peroxide (Reaction 5.4) and therefore never accumulates to any great extent in the cell under normal conditions of pH.\textsuperscript{37}

One major problem in this area is that a satisfactory chemical explanation for the purported toxicity of superoxide has never been found, despite much indirect evidence from \textit{in vitro} experiments that the presence of superoxide can lead to undesirable oxidation of various cell components and that such oxidation can be inhibited by superoxide dismutase.\textsuperscript{38} The mechanism most commonly proposed is production of hydroxyl radicals via Reactions (5.28) to (5.30) with $\text{Red}^+ = \text{O}_2^-$, which is referred to as the "Metal-Catalyzed Haber-Weiss Reaction". The role of superoxide in this mechanism is to reduce oxidized metal ions, such as $\text{Cu}^{2+}$ or $\text{Fe}^{3+}$, present in the cell in trace amounts, to a lower oxidation state.\textsuperscript{37} Hydroxyl radical is an extremely powerful and indiscriminate oxidant. It can abstract hydrogen atoms from organic substrates, and oxidize most reducing agents very rapidly. It is also a very effective initiator of free-radical autoxidation reactions (see Section II.C above). Therefore, reactions that produce hydroxyl radical in a living cell will probably be very deleterious.\textsuperscript{6}

The problem with this explanation for superoxide toxicity is that the only role played by superoxide here is that of a reducing agent of trace metal ions. The interior of a cell is a highly reducing environment, however, and other reducing agents naturally present in the cell such as, for example, ascorbate anion can also act as $\text{Red}^+$ in Reaction (5.28), and the resulting oxidation reactions due to hydroxyl radical are therefore no longer inhibitable by SOD.\textsuperscript{39}

Other possible explanations for superoxide toxicity exist, of course, but none has ever been demonstrated experimentally. Superoxide might bind to a specific enzyme and inhibit it, much as cytochrome oxidase is inhibited by cyanide or hemoglobin by carbon monoxide. Certain enzymes may be extraordinarily sensitive to direct oxidation by superoxide, as has been suggested for the enzyme aconitase, an iron-sulfur enzyme that contains an exposed iron atom.\textsuperscript{122} Another possibility is that the protonated and therefore neutral form of superoxide, $\text{HO}_2^-$, dissolves in membranes and acts as an initiator of lipid peroxidation. It has also been suggested that superoxide may react with nitric oxide, NO, in the cell producing peroxynitrite, a very potent oxidant.\textsuperscript{123} One particularly appealing mechanism for superoxide toxicity that has gained favor in recent years is the "Site-Specific Haber-Weiss Mechanism."\textsuperscript{40,41} The idea here is that traces of redox-active metal ions such as copper and iron are bound to macromolecules under normal conditions in the cell. Most reducing
agents in the cell are too bulky to come into close proximity to these sequestered metal ions. Superoxide, however, in addition to being an excellent reducing agent, is very small, and could penetrate to these metal ions and reduce them. The reduced metal ions could then react with hydrogen peroxide, generating hydroxyl radical, which would immediately attack at a site near the location of the bound metal ion. This mechanism is very similar to that of the metal complexes that cause DNA cleavage; by reacting with hydrogen peroxide while bound to DNA, they generate powerful oxidants that react with DNA with high efficiency because of their proximity to it (see Chapter 8).

Although we are unsure what specific chemical reactions superoxide might undergo inside of the cell, there nevertheless does exist strong evidence that the superoxide dismutases play an important role in protection against dioxygen-induced damage. Mutant strains of bacteria and yeast that lack superoxide dismutases are killed by elevated concentrations of dioxygen that have no effect on the wild-type cells. This extreme sensitivity to dioxygen is alleviated when the gene coding for a superoxide dismutase is reinserted into the cell, even if the new SOD is of another type and from a different organism.42,43

Summary of Dioxygen Toxicity

In summary, we know a great deal about the sites that are vulnerable to oxidative damage in biological systems, about the agents that protect against such damage, and about the mechanisms that repair such damage. Metal ions are involved in all this chemistry, both as catalysts of deleterious oxidative reactions and as cofactors in the enzymes that protect against and repair such damage. What we still do not know at this time, however, is how dioxygen initiates the sequence of chemical reactions that produce the agents that attack the vulnerable biological targets in vivo.