

Free radical biology and medicine: it's a gas, man!

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Free radical biology and medicine: it's a gas, man! *Am J Physiol Regul Integr Comp Physiol* 291: R491–R511, 2006. First published April 20, 2006; doi:10.1152/ajpregu.00614.2005.—We review gases that can affect oxidative stress and that themselves may be radicals. We discuss O₂ toxicity, invoking superoxide, hydrogen peroxide, and the hydroxyl radical. We also discuss superoxide dismutase (SOD) and both ground-state, triplet oxygen (³O₂), and the more energetic, reactive singlet oxygen (¹O₂). Nitric oxide ([•]NO) is a free radical with cell signaling functions. Besides its role as a vasorelaxant, [•]NO and related species have other functions. Other endogenously produced gases include carbon monoxide (CO), carbon dioxide (CO₂), and hydrogen sulfide (H₂S). Like [•]NO, these species impact free radical biochemistry. The coordinated regulation of these species suggests that they all are used in cell signaling. Nitric oxide, nitrogen dioxide, and the carbonate radical (CO₃^{•-}) react selectively at moderate rates with nonradicals, but react fast with a second radical. These reactions establish “cross talk” between reactive oxygen (ROS) and reactive nitrogen species (RNS). Some of these species can react to produce nitrated proteins and nitrolipids. It has been suggested that ozone is formed in vivo. However, the biomarkers that were used to probe for ozone reactions may be formed by non-ozone-dependent reactions. We discuss this fascinating problem in the section on ozone. Very low levels of ROS or RNS may be mitogenic, but very high levels cause an oxidative stress that can result in growth arrest (transient or permanent), apoptosis, or necrosis. Between these extremes, many of the gasses discussed in this review will induce transient adaptive responses in gene expression that enable cells and tissues to survive. Such adaptive mechanisms are thought to be of evolutionary importance.

antioxidants; antioxidant defense and repair; nitric oxide; carbon dioxide; hydrogen sulfide; carbon monoxide; ozone; hydrogen peroxide; dissolved gases; oxidants

WE HERE DISCUSS GASES that have a profound influence on the chemistry and pathophysiology of free radicals and on oxidative stress. The first of these gases is oxygen (correctly, but less commonly, named dioxygen). Oxygen is essential for respiration and energy production; but oxygen has a Janus-like nature and also can be toxic.

The mechanism by which oxygen expresses its toxicity is a long-studied problem. It is now known that the superoxide radical ion (O₂^{•-}) is the key to understanding this classic problem. Therefore, we also discuss this radical ion, which is present in all aerobic cells. Superoxide can damage certain types of species directly. For example, certain easily oxidized molecules such as ascorbate and some phenols and thiols can

be oxidized by superoxide, and this can lead to inactivation of enzymes. Often, superoxide is converted to hydrogen peroxide by reduction or by dismutation, and then hydrogen peroxide is converted to the hydroxyl radical (HO[•]). This conversion most often involves metal ions, generally iron. The hydroxyl radical is one of the most reactive species known in chemistry, able to abstract an unactivated hydrogen atom at room temperature, and HO[•] reacts with most of the species it collides with at a rate constant very near the diffusion limit.

Superoxide can lead to the production of hydrogen peroxide, which is found in human fluids and exhaled breath (128, 133, 222, 232) but can be toxic. Hydrogen peroxide and superoxide may be separately regulated and, although each can cause pathological damage, both also function in normal cell regulation and signaling (48, 109, 196, 217, 218, 225). The interaction of superoxide-producing systems with iron is important in understanding superoxide chemistry, and we spend some time on this system.

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We also discuss $\cdot\text{NO}$, peroxyntrous acid, and its conjugate base, peroxyntrite. Nitric oxide, a stable free radical, is a hormone-like species that contains no carbon atoms. (This remarkable discovery was recognized in 1998 with the award of a Nobel Prize to Robert Furchgott, Louis Ignarro, and Ferid Murad.) Peroxyntrite ($\text{O-O-N}=\text{O}$) is formed by the combination of the two radicals, superoxide ($\text{O}_2^{\cdot-}$) and nitric oxide ($\cdot\text{NO}$). Peroxyntrite mediates some of the physiology and toxicity of nitric oxide and by oxidation and nitration can lead to altered protein function. In addition, carbon dioxide is a modulator of the biochemistry of peroxyntrite. This reaction begins as an addition of the peroxyntrite anion, $\text{O-O-N}=\text{O}$, to the CO_2 molecule, forming an adduct, $\text{O}=\text{N-O-O-C}(\text{O})\text{O}^-$ (135, 230). This species is unstable and rapidly decomposes to give nitrogen dioxide ($\cdot\text{NO}_2$) and the carbonate radical ($\text{CO}_3^{\cdot-}$).

We also discuss nitrogen dioxide, long studied in smog, but also formed *in vivo* when nitric oxide is present. It has recently been emphasized (210) that the combination of nitrogen dioxide ($\cdot\text{NO}_2$) and superoxide forms peroxyntrite ($\text{O}_2\text{N-O-O}^-$), the conjugate base of peroxyntritic acid ($\text{O}_2\text{N-OO-H}$).

There are two other forms of reactive oxygen: singlet oxygen, an excited form of oxygen in which all the electrons are paired, and ozone, O_3 , a nonradical that is a potent oxidant in photochemical smog. Ozone is known to greatly accelerate the air oxidation of biomolecules (50, 170, 178, 179, 182).

We also discuss carbon monoxide and hydrogen sulfide, which have fundamental cell signaling properties and effect oxidative stress. There are other gases that either respond to, or directly affect, the level of radicals, oxidants, and oxidative stress. Included in the list are ethylene (13, 25, 183) and a number of others. Perhaps this account will encourage others to take up the biochemistry of these other gases.

Our purpose here is to group together a discussion of these gases and to focus attention on the surprising fact that nature chose gases to perform vital biological functions involving oxidative stress.

Free radical biology has often been controversial. The word "radical" itself may be part of the problem, evoking visions of unkempt men stirring up revolution in coffeehouses. Vitamin E, nature's favorite antioxidant (19, 101), also has had a controversial history, since the original biodetection method, rat fetus resorption, led to the early association of vitamin E with human sexual function, despite the fact that vitamin E deficiency does not cause fetus resorption in mice, humans, or most other species.

1) Oxygen: Triplet And Singlet

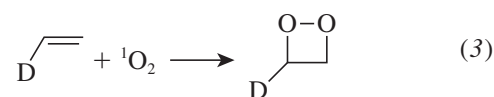
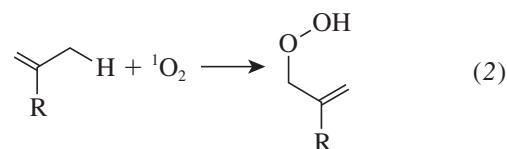
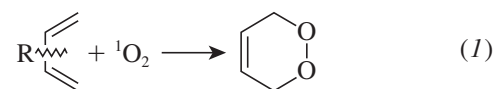
The electronic ground state of dioxygen is a triplet ($^3\text{O}_2$). Since it has two unpaired electrons, both with the same spin, it also is a diradical. Pairing these electrons to give one with "up" spin and one "down" gives a singlet state ($^1\text{O}_2$). This takes energy, and the singlet is 1 eV (23 kcal/mol or 94 kJ/mol) higher in energy than the ground state triplet.

The triplet state of oxygen (its ground state), $^3\text{O}_2$, acts as a diradical, a one-electron oxidant (albeit a fairly poor one), whereas singlet $^1\text{O}_2$ acts as a more potent, often two-electron, oxidant that adds to π bonds, undergoes ene reactions, and can insert into CH bonds. The nearly unique triplet ground state of oxygen occurs because the two highest energy electrons reside

in identical π^* molecular orbitals. In such a case, the triplet state is lower in energy because electrons with the same spin cannot occupy the same region of space (the Pauli exclusion principle). Electrons with opposite spin, as in the singlet state, can occupy the same region of space, but because the electrons are closer together on average, repulsion between these electrons is greater, and so the singlet state is higher in energy than the triplet.

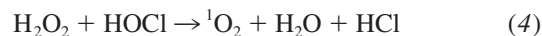
Oxygen atoms are present in a variety of minerals, and oxygen is the most abundant element in the Earth's crust (93). Triplet oxygen supports metabolism through respiration. In mammalian systems, oxygen binds to hemoglobin, a marvelously tuned carrier of oxygen; hemoglobin moves oxygen through the organism and uses cooperative binding to maximize uptake and release to tissues that require O_2 . When released, O_2 is involved in a series of complex redox cycles, ultimately being reduced to water and giving ATP.

Singlet oxygen is not produced by simple thermal processes from $^3\text{O}_2$, but requires intervention of high energy molecules. Singlet oxygen can be produced by energy transfer from the excited triplet states of aromatic dyes, like Rose Bengal, and also by fullerenes. These reactions occur with little or no activation enthalpy; Diels-Alder reactions, ene reactions, and 2+2 cyclo-additions with electron-rich alkenes (D = electron donor) are common singlet oxygen reactions, as shown in the three reactions below (reactions 1–3) (62).



Note these products are peroxides, and like typical peroxides these can generate radicals that can undergo subsequent reactions.

Singlet oxygen is produced in several physiological processes, but whether it has any natural function or physiological relevance has been a matter of controversy. Neutrophils produce hypochlorous acid, HOCl, and hydrogen peroxide, H_2O_2 , and these can react to generate singlet oxygen (reaction 4).



Singlet oxygen has a rather long lifetime in the gas phase but decomposes more rapidly in solution by a variety of nonradiative processes. Nevertheless, at one time, it was suggested that superoxide dismutase (SOD) was actually singlet oxygen decontaminase (161), a claim that has since faded away (204). SOD has nothing to do with singlet O_2 !

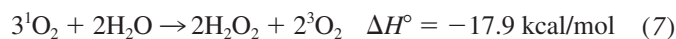
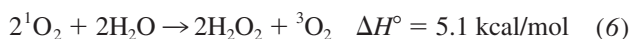
Singlet oxygen does have a role in medical interventions in biology, as the active species formed in photodynamic therapy. Sensitizers such as porphyrins in tumor cells are irradiated and

7sensitize the formation of singlet oxygen that ultimately kills tumor cells. A disease, porphyria, is related to a deposition of porphyrins that can cause deleterious formation of singlet oxygen upon exposure to sunlight on skin. The legend of vampires may have developed from porphyria-stricken people, who had to avoid sunlight to avoid photosensitized singlet oxygen generation.

Recently it was proposed by Wentworth et al. (242, 243) that singlet oxygen from neutrophils can oxidize water through antibody catalysis. These workers proposed that antibodies might not only have a long-reorganized pathogen recognition function but antibodies might also have once had a killing function through the generation of hydrogen peroxide (*reaction 5*) (242, 243).



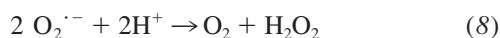
This reaction, as shown above, is endergonic by 28.1 kcal/mol, according to thermodynamic values in the 2005 NIST Chemistry WebBook database (2) or high-level quantum mechanical calculations (K. N. Houk and P. R. McCarren, unpublished observations). Because of unfavorable energetics, a research team at Scripps (242) has proposed that two or three singlet oxygens are required for each two hydrogen peroxide molecules produced (*reactions 6* and *7*). The energetics of these processes are:



Wentworth et al. (242) have performed calculations that show how water molecules oriented in an antibody could catalyze H_2O_2 formation. Interestingly, these reactions involve both hydroperoxyl and hydrotrioxyl radicals as intermediates. However, no mechanism has been proposed to explain how the conversion of two singlet oxygens to two triplet oxygens can be coupled to the reaction of singlet oxygen with water to form hydrogen peroxide. More recent publications (244, 245) have suggested that trioxygen species, including ozone, are produced by antibody catalysis. These reports are described below in the fourth section, titled *Ozone*.

2) Oxygen Toxicity and Superoxide

It has been known for many years that oxygen can be toxic, but the detailed mechanisms of oxygen toxicity are still under investigation. An enormously influential, watershed advance was made in 1969, when Joe McCord and Irwin Fridovich (139) reported the isolation of an enzyme from bovine red blood cells. They named the enzyme superoxide dismutase, SOD, and showed that it catalyzed the dismutation of superoxide (*reaction 8*) to form oxygen and hydrogen peroxide (139).



A search for “superoxide” in the biological literature for 1968 and prior years gives 520 “hits,” with 56 publications in the year 1968 alone, the year of the publication of McCord and Fridovich. (The name perhydroxide, $\text{HOO}\cdot$, used in the early years, does gather some hits for 1968 and prior years; SciFinder Scholar gives 9 hits for this term for during this period.) At the beginning of 2006, there were 113,692 hits on

the word “superoxide”! The study of this species is now important in the fields of chemistry, biochemistry, biology, and medicine.

The very existence of SOD implied a revolutionary concept. The notion that superoxide plays a role in biology and medicine presupposes that oxygen, the prototypical oxidant in nature, can form superoxide, a species that usually reacts as a reductant. In the mid-20th century when SOD was discovered, radicals were regarded as too reactive and unselective to play any role in biology. (Radicals were thought to have a role that was limited to the chemistry that occurs in the stratosphere and in smog in the biosphere.) Radicals were believed to be too uncontrollable to occur in any reactions involving an enzyme; radicals in cells were viewed as a “bull in a china shop.”

Even the notion of organic “free” radicals had been controversial. The 19th century literature on the “impossibility” of radicals existing in nature is amazing to read in the light of current knowledge (167, 171). The existence of radicals was finally made real for organic chemists by the work of Gomberg on triphenylmethyl radicals in the years 1900–1910 and for physical chemists by the work of Paneth on small aliphatic radicals in 1929 (166). In the 20th century, early proponents of free radicals in biology argued that all redox reactions must occur one electron at a time (because coincidence of electron movement would be unexpected), and therefore all redox reactions must produce free radicals. For example see Michaelis (141, 168) and Szent-Györgyi and colleagues (67, 219–221). In the 1950s, Westheimer et al. (246) published a series of papers that implied that the oxidation of ethanol to acetaldehyde as catalyzed by the enzyme alcohol dehydrogenase occurred by a direct, stereospecific transfer of hydrogen (55) and therefore involved the hydride ion moving with two electrons at one time without free radical formation. This discovery, as well as the mistaken belief that all radicals are extremely reactive and very short lived, led to the acceptance of the incorrect idea that radicals¹ could not exist in vivo (169, 171).

This analysis overlooked the difference between the fleeting transfer of electrons one-at-a-time and actually observing and/or isolating free radicals, which would require a lifetime of more than just a few vibrations. Now that fast flash and pulse methods are available to identify very short-lived intermediates, the difference between the theoretical occurrence of one-electron intermediates and the actual observation and/or isolation of radical intermediates is understood.

Another problem with the early analysis is the assumption that all radicals are “a bull in a china shop.” In fact, radicals vary in reactivity (and therefore lifetimes) by some 10 orders of magnitude! Some radicals, such as the hydroxyl radical, react near the diffusion limit with the first molecule they bump into, whereas others, such as semiquinones, are stable for days, weeks, or months (172). So the “bull” may be reactive but also has his contemplative moments.

Hydrogen peroxide, which is an oxidant but not a radical, is produced in the SOD-catalyzed dismutation of superoxide (*reaction 8*). For this reason, catalase, which catalyzes the

¹ Frank Westheimer has said, “There was a time when I didn’t believe that any free radicals would ever show up in biology; I was obviously quite wrong”. Frank Westheimer, in a personal letter to W. A. Pryor dated November 26, 1996.

dismutation of hydrogen peroxide to oxygen and water (*reaction 9*), often is codistributed in tissue along with SOD.



The discovery of SOD did two things that had enormous impact. First, the ready availability of SOD allowed simple tests to be devised to prove the presence of the superoxide radical in biological systems and thus provided a powerful tool to resolve the long-standing debate on whether radicals could exist in cells. And second, as described in a review by Imlay (100), the role for superoxide in biology allowed the demonstration of the molecular mechanisms for oxygen toxicity and the involvement of superoxide.

The discovery of SOD was unquestionably an event of such enormous consequence and influence that it should have resulted in a Nobel Prize. Why has this Prize not been awarded? Initially the role of SOD was greeted with skepticism by some. Various arguments were advanced to support the idea that SOD must have another function, including the fact that superoxide dismutates spontaneously and does not "require" a catalyst (53). This argument is spurious; hydrogen peroxide is relatively unreactive as an oxidant, but there has never been any doubt that catalase exists to catalyze the dismutation of hydrogen peroxide to water and oxygen. The arguments by McCord and Fridovich (139), that SOD does not have the function assigned to it, have been demolished with a flood of publications on SOD, covering every aspect of its chemistry and biology. The tone of the debate on the subject of the role of SOD can be judged from a 1981 discussion of a paper presented by Fee (53).

In these early years when the role of SOD in biology was being debated, the role of nitric oxide and peroxynitrite in biology was not yet known. Now that the oxidizing power of peroxynitrite is known, it can be appreciated that SOD removes superoxide from systems containing nitric oxide and thus prevents the rapid reaction of nitric oxide and superoxide to form the potent oxidant peroxynitrite (discussed in more detail below) (180).

2.1) Oxidative stress. Sies stated, "A disturbance in the pro-oxidant/antioxidant systems in favor of the former may be denoted as an oxidative stress" (202). Many of the gases (and derived species) we review are biological oxidants that can initiate reactions that ultimately cause oxidative stress. Indeed, oxidative stress is a mechanism of toxicity (in many cases the major mechanism) for most of the gases we discuss. Oxidative stress can result from increased exposure to oxidizing gases or from a lessened protection against oxidants; both problems may occur simultaneously. Oxidative stress can cause damage to proteins, lipids, carbohydrates, and nucleic acids (21, 22, 43, 61, 83, 202). Thus cellular enzymes and structural proteins, membranes, simple and complex sugars, and DNA and RNA are all susceptible to oxidative damage (21, 22, 43, 61, 83, 202). Surviving an oxidizing environment is actually one of the greatest challenges faced by living organisms. The concept that we gradually surrender to a form of organic "rust" led to the free radical theory of aging (86, 159).

2.2) Antioxidant defense and repair systems. The first-level cellular response to oxidative stress is to use antioxidant defense and repair systems to minimize the damage that occurs and to remove or repair whatever cellular components were damaged (21, 22, 43, 61, 80, 83, 159, 202, 231, 237). Although

living organisms may eventually succumb to oxidant-induced aging, oxidatively damaged cellular constituents are maintained at very low levels for most of an organism's life span. Damage accumulation is kept low by multiple interacting systems of antioxidant compounds, antioxidant enzymes, damage-removal enzymes, and repair enzymes.

Antioxidant compounds include the vitamins C and E, ubiquinone, uric acid, glutathione, and others. Antioxidant compounds are sacrificially oxidized to protect more important cellular components (21, 22, 43, 83, 202). Antioxidant enzymes, such as superoxide dismutases, glutathione peroxidases, and quinone reductases, act catalytically to convert oxidants to less reactive species (43, 61). The essential cytoplasmic and nuclear proteolytic enzyme, proteasome, recognizes and selectively degrades oxidized proteins (43, 80, 159). Oxidized membrane lipids are recognized and selectively removed by lipases, particularly phospholipase A₂ (43, 159, 231). Oxidized DNA is subject to removal or excision repair by a wide series of DNA repair enzymes including endonucleases, glycosylases, polymerases, and ligases (43, 159, 237).

Oxygen concentration is tightly controlled in multicellular organisms, directly limiting oxidation of biopolymers by oxygen-centered oxidants. In humans, oxygen concentration falls from atmospheric levels (20%) in the lungs to 2–5% in the tissues. The very low Michaelis constant, *K_m*, of mitochondrial cytochrome oxidase for oxygen (23) assures that most oxygen in cells is bound and consumed by the cytochrome oxidase complex. Thus one component of oxidative stress defense is to keep cellular oxygen tension to a minimum. This fact is often overlooked by scientists performing cell culture experiments at atmospheric oxygen levels.

As described above, aerobic organisms have evolved a multitude of ways to protect themselves from the deleterious aspects of an aerobic existence. It has also become increasingly clear that cells have the capacity to adapt to the presence of oxidative stress via the activation of signaling pathways regulated by oxygen and oxygen-derived species. Moreover, it appears that oxygen-derived species are part of normal cell signaling processes. In the following discussion, examples of these types of effects are presented.

2.3) Mitogenic effects of low oxidant concentrations. Exposure of dividing mammalian cells in culture to very low concentrations of many oxidants actually stimulates cell growth and division. This fascinating mitogenic effect is seen, for example, with exposure of fibroblasts to hydrogen peroxide at 3–15 μM, i.e., 0.1–0.5 μmol/10⁷ cells (17, 18, 23, 247). Presumably such low oxidant concentrations do not cause a true oxidative stress. In all probability, low oxidant levels are signals for mitosis, although the mechanism is not fully understood. Interestingly, this growth-stimulatory effect of very low oxidant concentrations is also seen with bacteria (26, 46) and yeast cells (42).

2.4) Temporary growth arrest as a defense against mild-to-moderate oxidative stress. Although very low oxidant exposure is mitogenic, mild-to-moderate oxidative stress (e.g., 120–150 μM hydrogen peroxide, or 2–5 μmol/10⁷ cells) typically causes a temporary growth arrest in mammalian fibroblasts (247). This temporary growth arrest lasts for some 2–4 h and appears to be caused by expression of the *gadd45*, *gadd153* (12, 57, 58), and *adapt15* genes (37, 38). Many investigators have treated all growth-arrest responses as toxic outcomes of

oxidant exposure, but it is now clear that temporary growth arrest is actually a defense mechanism. During oxidant-induced temporary growth arrest, the expression of many housekeeping genes is halted, while expression of a select group of shock or stress genes is induced. Similarly, proliferating cells exposed to mild or moderate oxidative stress shut off expression of all but the most essential shock/stress genes, supercoil their DNA to protect it against oxidation, and conserve resources for future use, during temporary growth arrest.

In this review the term "mild-to-moderate oxidative stress" is defined by cellular responses, not by hydrogen peroxide concentrations. Mild stress involves little or no cell death (either by apoptosis or necrosis), and no long-term inhibition of cell division, DNA synthesis, or protein synthesis. In fact, cells exposed to mild-to-moderate oxidative stress exhibit adaptive responses, with increased expression of protective and repair genes.

2.5) Transient adaptation with mild-to-moderate oxidative stress. Very important early studies on transient adaptation to oxidative stress were performed by Spitz et al. (206) and by Laval (129). After 4–6 h of temporary growth arrest, many cells exposed to moderate oxidative stress (e.g., 120–150 μM hydrogen peroxide, or 2–5 $\mu\text{mol}/10^7$ cells) undergo further changes that can be characterized as transient adaptation to oxidative stress. In mammalian fibroblasts (32, 33, 35–38, 129, 130, 206, 240, 247) maximal adaptation is seen ~18 h after initial exposure to hydrogen peroxide, i.e., some 12–14 h after they exit from temporary growth arrest. In bacteria such as *Escherichia coli* and *Salmonella*, maximal adaptation is seen 20–30 min after oxidant exposure (26, 33, 46), whereas yeast cells require some 45 min (33, 42).

The adaptation referred to in the title of this section means increased resistance to mild-to-moderate oxidative stress, as measured by cell proliferation capacity. Furthermore, the adaptation is transient, lasting some 18 h in mammalian fibroblasts, 90 min in yeast, and only 60 min in *E. coli*. It is especially important to avoid selecting for preexisting resistant cells in the population, by repeatedly checking that transiently adapted cells can actually de-adapt. In both prokaryotes and eukaryotes, transient adaptation to oxidative stress depends upon transcription and translation. A large number of genes undergo altered expression during the adaptive response. Some genes are upregulated, some are downregulated, and some are modulated early in the adaptation, while the expression of others is affected at later times. Inhibiting either transcription or translation during the adaptive response greatly limits the development of increased H_2O_2 resistance. If both transcription and translation are inhibited, then little or no adaptation will occur. Therefore, the transient adaptive response to oxidative stress depends largely on altered gene expression but partly on increased translation of preexisting mRNAs. It further appears that message stabilization (for some mRNAs), increased message degradation (for other mRNAs), and altered precursor processing, are all involved in altered translational responses (32, 33, 35–38, 42, 43, 130, 240, 247). Studies in *E. coli* and *Salmonella* have shown that two particular regulons are responsible for many of the bacterial adaptive responses to oxidative stress: the oxyR regulon (214) and the soxRS regulon (79). In mammalian cells no "master regulation molecules" have been found, but at least 40 gene products are involved in the adaptive response. Several of the mammalian adaptive

genes are involved in antioxidant defenses, and others are damage removal or repair enzymes. Many classic shock or stress genes are involved very early in adaptive responses. As indicated in section 2.4 (*Temporary growth arrest as a defense against mild-to-moderate oxidative stress*) above, gadd153, gadd45, and adapt15 play important roles in inducing temporary growth arrest, which is a very important early portion of the adaptive response to oxidative stress (12, 37, 38, 57, 58, 247). The transcription factor, adapt66 (a mafG homologue) is probably responsible for inducing the expression of several other adaptive genes (36). A number of other "adapt" genes have recently been discovered, but their functions are not yet clear. One of these genes is the calcium-dependent adapt33 (240), and another is adapt73, which appears to also be homologous to a cardiogenic shock gene called PigHep3 (32). Adapt78 has also been called DSCR1 and RCAN1 (35, 130) and, in addition to its induction during oxidative stress adaptation, now appears to be involved in Down syndrome and Alzheimer disease (49, 87).

Numerous other genes have been shown to be inducible in mammalian cell lines following exposure to moderate oxidative stress that will cause transient adaptation. These include the protooncogenes c-fos and c-myc (30), c-jun, egr, and the platelet-derived growth factor-inducible "early" gene JE (147, 150, 199). Similar oncogene induction has also been reported following exposure to *tert*-butylhydroperoxide (147, 150). The induction of heme oxygenase by many oxidants may have a strong protective effect, as proposed by Keyse and Tyrrell (115). Other gene products that have been reported to be induced by relatively mild oxidative stress in dividing mammalian cell cultures include the following: the CL100 phosphatase (114); interleukin-8 (45); catalase, glutathione peroxidase, and mitochondrial manganese-superoxide dismutase (201); natural killer-enhancing factor-B (118); mitogen-activated protein kinase (81); and γ -glutamyltranspeptidase (252). Relatively low levels of nitric oxide have also been shown to induce expression of c-jun (102), c-fos (102, 144), and zif/268 (144). The list of oxidant stress-inducible genes is much longer than the space limitations of this review article will allow.

Investigators also have chronically exposed cell lines to various levels of oxidative stress over several generations and have selected for preexisting or mutant phenotypes that confer oxidative stress resistance, such as high catalase activity (207). Stable oxidative stress resistance may tell us a great deal about the importance of individual genes to overall cellular survival, and the value of such cell lines should not be underestimated. It should be clear, however, that transient adaptive responses in gene expression and stable stress resistance are quite different.

2.6) Permanent growth arrest at higher levels of oxidative stress. If dividing mammalian cells are exposed to higher oxidant levels than those that cause temporary growth arrest and transient adaptation, then they can be forced into a permanently growth-arrested state. Thus, cells exposed to H_2O_2 concentrations of 250–400 μM , or 9–14 $\mu\text{mol}/10^7$ cells, will never divide again (247).

Countless cytotoxicity studies have measured "cell death" by loss of proliferative capacity. These include studies with oxidizing agents, alkylating agents, heavy metals, and various forms of radiation. The common assumption of such investigations is that loss of divisional competence equals cell death. It is true that many cells exposed to sufficient stress will both

stop dividing and die (see next two sections, *sections 2.7 and 2.8*). It is clearly incorrect to conclude, however, that all permanently growth-arrested cells will die as a direct consequence of toxicant exposure; cells may survive an oxidative stress yet be permanently growth-arrested (24, 247).

Studies of both cell populations and individual cells have revealed that cultured mammalian cells (normal doubling time of 24–26 h) can survive for many weeks, or even months, following exposure to higher oxidative stress levels without dividing again (247). In the past, studies estimating percent cell viability by growth curves or colony formation measurements alone have completely missed this permanent growth-arrest response. Arrested cells still exclude trypan blue, maintain membrane ionic gradients, utilize oxygen, and make ATP; in other words, these cells are alive but do not divide. Interestingly, such permanently growth-arrested cells may make good cellular models for certain aging processes (24). Whether the cessation of proliferation induced by oxidative stress (or other stressful exposures) in some way mimics the loss of divisional competence typical of terminally differentiated cells remains to be seen.

2.7) Cell suicide or apoptosis at high levels of oxidative stress. A fraction of cells exposed to high levels of oxidative stress (e.g., H₂O₂ concentrations of 0.5–1.0 mM, or 15–30 μmol/10⁷ cells) will enter the apoptotic pathway. The mechanism of oxidative stress-induced apoptosis appears to involve loss of mitochondrial transmembrane potential (252), release of cytochrome *c* to the cytoplasm (187), loss of bcl-2 (106), downregulation and degradation of mitochondrial encoded mRNA, rRNA, and DNA (31, 34, 39), and diminished transcription of the mitochondrial genome (126). Current thinking about toxicant-induced apoptosis suggests that, in multicellular organisms, the repair of severely damaged cells represents a major drain on available resources for the “host” organism. To avoid this difficulty, individual cells within organisms (or organs or tissues) will “sacrifice” themselves for the common good of the many. Apoptotic cells are characterized by “blebbing”, nuclear condensation, and DNA laddering (6). Such cells are engulfed by phagocytes, which prevent an immune reaction and recycle usable nutrients (6, 31, 34, 39, 51, 106, 126, 164, 187, 252). Certain toxicants, such as staurosporine, can induce widespread apoptosis in fibroblast cell cultures, with greater than 80% cell suicide (34, 126). Even higher levels of apoptosis (98% or more) are routinely observed upon withdrawal of IL-2 from in vitro cultures of T lymphocytes (34, 126). In contrast, the highest levels of apoptosis induced in fibroblasts by H₂O₂ exposure never exceed 30–40% (34). The cause of such disparity is not at all clear, but it may suggest either slightly divergent pathways to apoptosis or different efficiencies of repair processes for various toxicants. The apoptotic pathway may be very important in several age-related diseases such as Parkinson disease, Alzheimer disease, and sarcopenia. Importantly, many mitochondrial changes, including loss of membrane potential (252) and downregulation and degradation of mitochondrial polynucleotides (31, 34, 39), are common to apoptosis directly induced by oxidants and to apoptosis induced by staurosporine or withdrawal of IL-2. Furthermore, overexpression of the p53 gene has been seen to result in induction of multiple “redox-related” gene products and initiation of apoptosis (164). These observations support a

strong involvement of oxidative stress mechanisms in general apoptotic pathways.

2.8) Cell disintegration or necrosis at very high levels of oxidative stress. At concentrations of hydrogen peroxide of 5.0–10.0 mM or 150–300 μmol/10⁷ cells, most cells simply disintegrate or become necrotic. Membrane integrity breaks down at such high oxidant stress levels, and all is then lost (100). Studies that purport to examine cellular responses to extremely severe oxidative stress are really not looking at the responses of cells but rather at the release of components those cells originally contained. At a sufficiently high level of oxidative stress, all mammalian cell cultures will turn into a necrotic “mess” (247). Oxidation-induced necrosis may play a significant role in ischemia-reperfusion injuries such as heart attacks, strokes, ischemic bowel disease, and macular degeneration. Unfortunately, necrotic cells cause inflammatory responses in surrounding tissues. Such secondary inflammation (also an oxidant stress) may be particularly important in many autoimmune diseases such as rheumatoid arthritis and lupus.

In most cases so far, we have discussed the hallmarks and biology of oxidative stress as it relates to the presence of oxygen and the generation of oxygen-derived species. In the following sections, we discuss in more detail the chemical nature of these oxidants and the possible mechanisms of their generation.

3) The Role of Iron Ions in Producing the Hydroxyl Radical Study of the reduction of hydrogen peroxide by ferrous ions dates to the work of Fenton, Haber, Weiss, and Willstatter at the end of the 19th century (124, 125, 236). Early concerns were the nature of the iron-hydrogen peroxide interaction and the intermediates produced. It was thought that an outer-sphere electron transfer was involved (236), as shown in *reaction 10*.



After a century of study, the mechanism of the reaction is still debated. Current thinking is that the net Fenton reaction is a simplification of a more complex process. The simplest way to envision the reaction is a single electron donation from Fe²⁺ to hydrogen peroxide (an outer sphere mechanism), as shown in *reaction 10*. However, this reaction is thermodynamically unfavorable, and it is thought an inner sphere mechanism is more likely (76).

The reaction in vivo is a chain reaction in which the ferric ion produced in *reaction 10* is reduced back to ferrous, as in *reaction 11*.



The nature of the electron donor in *reaction 11* has been the subject of a considerable amount of study. At first it was thought that the electron donor was superoxide, providing another rationalization for the existence of SOD (60, 139). However, the reduction of ferric ions by superoxide was shown to be far too slow (236). The reducing agent in *reaction 11* now is agreed to be the reducing agents that are ubiquitous in biosystems, including thiols and other more complex sulfur compounds and ascorbate and similar one-electron transfer agents.

Now that the chemistry of nitric oxide has caught up with the advances in superoxide chemistry, we recognize the vital role of SOD in preventing the combination of nitric oxide and

superoxide to form peroxyxynitrite, as in *reaction 12*. Thus, the need to protect biological systems against superoxide buildup is the result of both superoxide toxicity itself and the conversion of superoxide to peroxyxynitrite, a potent oxidant toward certain biological species.



4) *Ozone* Ozone, O_3 , is a gas in the stratosphere that protects organisms on Earth from the most harmful wavelengths of solar radiation ("good" ozone). Ozone also is a significant pollutant near the Earth's surface ("bad" ozone), where it is produced in the series of smog reactions and can oxidize bioorganic compounds and initiate the autoxidation of unsaturated fatty acids, leading to membrane destruction (228). Wentworth et al. (242–245) recently reported the startling claim that antibodies produce ozone in vivo. This group first reported this for the catalytic conversion of singlet oxygen to ozone and then in atherosclerotic plaques. Wentworth et al. (242–245) reported what they considered to be definitive evidence for ozone production through the identification of oxidation products that they propose are biomarkers for the presence of ozone.

Significant reservations about these conclusions have been expressed (203, 204). The suggestion that O_3 was involved in atherosclerosis arose from the discovery that 3β -hydroxy-5-oxo-5,6-secocholestanol-6-al and $3\beta,5$ -dihydroxy-5 β -B-norcholestane-6 β -carboxaldehyde are present in atherosclerotic plaques. The first compound, cholesterol secoaldehyde, exists in equilibrium with its intramolecular aldol condensation product (see Fig. 1). Wentworth et al. (244) identified these species as their 2,4-dinitrophenyl hydrazones and considered them proof for the participation of ozone in the systems they studied.

These biomarkers for the reaction of ozone with cholesterol probably can be, and we believe in this case likely are, formed by a pathway other than the reaction of ozone itself with cholesterol. Smith (204), a recognized expert on ozone, has suggested that singlet oxygen might be the oxidant, and Sies

(203), an expert on biological oxidations, also has commented on the problem.

In aqueous systems, such as occur in liposomes and biomembranes, the ozonation of double bonds gives poor yields of Criegee ozonides and instead favors formation of carbonyl products, produced by the hydrolysis of the ozone-derived products by water (211). This suggests that if ozone were to react with cholesterol in vivo, then cholesterol secoaldehyde (C-seco) and its aldol condensation product would be major products, and the Criegee ozonide (C-oz) would be only a minor product, since most of the cholesterol resides in the lipid bilayer but water traps most of the intermediate carbonyl-oxide before the fragments can recombine to form the Criegee ozonide (211). (See Fig. 2.) Thus, although the direct hydrolysis of C-oz, as well its reduction, does yield C-seco and its aldol condensation product, it is also possible that the reaction of the cholesterol ozonide with 2,4-dinitrophenylhydrazine gives the same hydrazone products as does the hydrazone's reaction with C-seco and its aldol condensation product.

The ozonation chemistry of cholesterol in the stratified aqueous and lipid milieu of a cell is complex and undoubtedly can give products detected as the hydrazones of C-seco and its aldol condensation product. But, we suggest that ozone may not be the only oxidant that can oxidize cholesterol to these products. Wentworth et al. (244) did not publish controls, which would have eliminated some alternative possibilities. In other publications, Wentworth et al. (243, 244) also used the reaction that converts the indigo carmine dye into isatin sulfonate as a signature reaction for ozone. Again, the appropriate controls to prove that this reaction is a unique signature for ozone were not published. For indigo carmine this is significant, since the superoxide radical, in aerobic organisms, can bleach indigo in a fast reaction. [The second-order rate constant for the reaction of the superoxide radical with indigo carmine is $9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ near physiological pH (Ref. 2).] Recently, Kettle et al. (113) confirmed that stimulated neutrophils, cells that produce superoxide, can convert indigo car-

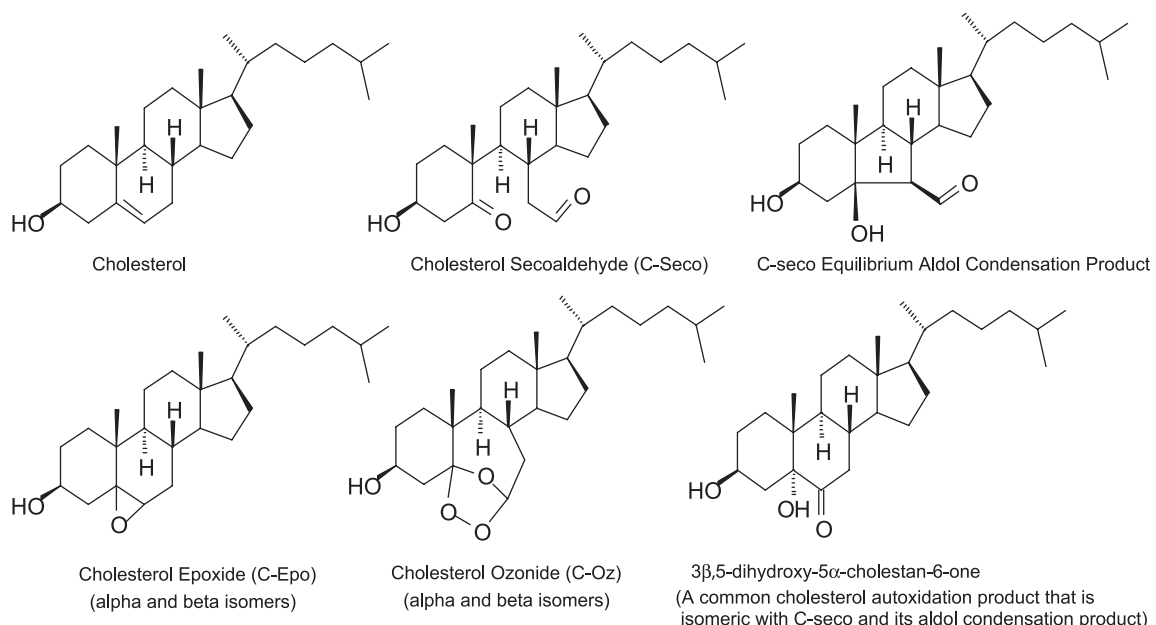


Fig. 1. Cholesterol and its oxidation products.

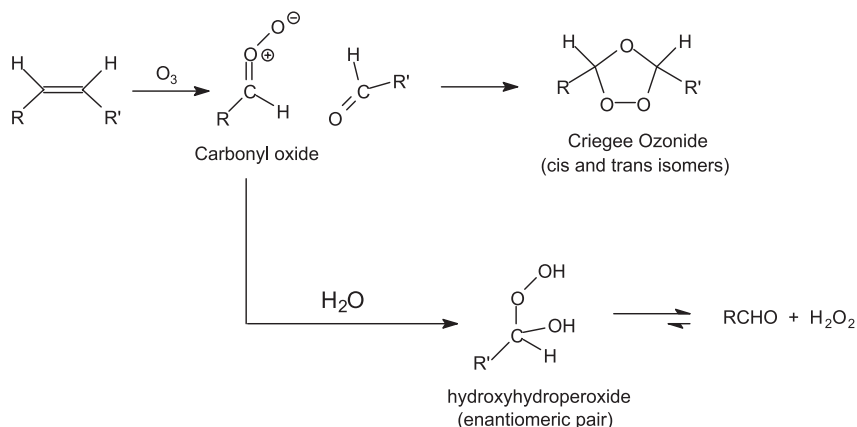


Fig. 2. Simplified mechanism for the ozonation of an alkene in an aqueous medium illustrating the trapping of the intermediate carbonyl oxide by water (211).

mine into isatin sulfonate even in the presence of singlet oxygen and ozone scavengers. The claim that the hydrazones of C-seco and its aldol condensation product are ozone reaction biomarkers is as yet unproven, as is the roll of ozone in the transformation of indigo carmine dye into isatin sulfonate.

Another concern in the report of Wentworth et al. (244) is that the hydrazone derivatives were identified by mass spectrometry by means of their $[M-H]^-$ negative ion at m/z 597. However, as remarked by Smith (204), the hydrazone derivative of 3 β ,5-dihydroxy-5 α -cholestan-6-one, an oxidation product of cholesterol that is formed even in the absence of ozone, will yield the same $[M-H]^-$ negative ion.

Yet another concern with the claim that cells produce ozone is that the formation of ozone requires substantial energy. The heat of formation of ozone in the gas phase is 34.1 kcal/mol; the gas phase reaction of three singlet oxygen molecules to form two ozone molecules (by an unknown mechanism) is exothermic by 1 kcal.

A new study reports that H₂O₃ is produced from "oxone," which is a mixture of ozone plus hydrogen peroxide used in water purification (151). These authors report that the reaction of ozone with hydrogen peroxide is of biological interest and that this reaction, wherein H₂O₃ is a reaction intermediate, is a source of singlet oxygen (151). Nieva and Wentworth (149) have proposed that H₂O₂, H₂O₃, ¹O₂, and O₃, all of which are highly reactive oxidants, can be endogenously produced by antibody catalysis of water oxidation. In advancing this hypothesis, these authors fail to compare, for example, the rate constants for the reactions of O₃ with H₂O₂ ($k = 0.065 \text{ M}^{-1}\cdot\text{s}^{-1}$) (1) with the rate constants for the reactions of O₃ with small molecular weight antioxidants, such as glutathione ($k = 7 \times 10^8 \text{ M}^{-1}\cdot\text{s}^{-1}$) and ascorbate ($6 \times 10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$) (68, 175). Taking into consideration that all these reaction rate laws are first order in O₃ and first order in these substrates and that these small molecular weight antioxidants are considerably more abundant than H₂O₂ in biological systems, one can conclude the reaction of O₃ with H₂O₂ is at least 10 billion times slower than the combined rate of O₃ with these antioxidants and that it will hardly occur in vivo. Thus, these claims are thought-provoking, but they remain highly controversial.

Lipid ozonation products (LOP) should be considered in the light of the observation of cholesterol oxidation (41, 59, 103, 104, 173, 181). LOP include ozone-derived lipid peroxides that can undergo homolysis and generate oxy-radicals that can oxidize unsaturated compounds. They oxidize polyunsaturated

lipids (PUFA) faster than lipids such as cholesterol, and unsaturated lipids probably are more plentiful than is cholesterol. Some LOP can initiate PUFA oxidation. It is possible that any oxidative stress (including ozone exposure) will result in cholesterol oxidation. So the oxidation/ozonation of cholesterol may well be the result of a complex, multistep process.

5) Nitric Oxide

The discussions above have, for the most part, focused on oxygen and oxygen-derived species. However, there is another relevant radical, nitric oxide, [•]NO. Nitric oxide is the first gaseous species unequivocally identified as an endogenously generated cell signaling/effector agent (65, 99, 160). This was a significant finding since it established a new paradigm in cell signaling whereby an endogenously generated, small, ordinarily gaseous molecule, which is toxic at moderate concentrations, is used as a cell-signal species. This discovery encouraged the suggestion that other small molecules (many of which were also known primarily for their toxicity) could also be biosynthesized and possess important physiological functions (see below). The first established physiological role for [•]NO was as a vasorelaxant. The ability of [•]NO to elicit vasorelaxation is due to its ability to increase intracellular levels of cGMP in smooth muscle tissue. Another important aspect of [•]NO, also due to increased intracellular cGMP levels, is its ability to inhibit cell (platelet) adhesion and aggregation. However, it appears that the effects and function of [•]NO go beyond its ability to regulate vascular tone and/or cell adhesion, as other aspects of [•]NO physiology and pathophysiology have been postulated (for a review, see Ref. 98). For example, [•]NO is made in the brain, as a part of immune response, and in mitochondria, and the role of [•]NO in these systems probably is not related to its specific effects on the vascular system or cell adhesion. Unlike the established role of [•]NO in vascular physiology, the function of [•]NO in these other systems is not well-understood. Below is a brief review of [•]NO biology and chemistry with focus on the role of [•]NO in biological free radical chemistry.

5.1) Nitric oxide biosynthesis and physiology. Nitric oxide ([•]NO) is biosynthesized by a family of enzymes referred to as the nitric oxide synthases (NOS) (for a review, see Ref. 216). There are three primary isoforms of NOS that originate from separate genes and differ in their subcellular localization and mode of regulation. They are typically designated as endothe-

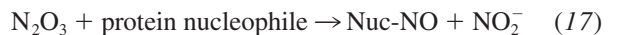
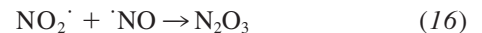
lial (eNOS), neuronal (nNOS), and immunological/inducible NOS (iNOS), although these designations do not strictly reflect their tissue expression or utility. eNOS and nNOS are constitutively expressed; nevertheless, their levels can change in response to a variety of physiological events (e.g., hormonal influences). As the name indicates, iNOS is highly induced in a variety of cells, often as a result of immune stimulation (e.g., lipopolysaccharide, cytokines). Both eNOS and nNOS are regulated primarily by Ca^{2+} via the actions of calmodulin. In contrast, iNOS is not regulated by Ca^{2+} .

Regarding the vascular effects of $\cdot\text{NO}$, it has been known for almost 140 years that some nitrogen oxide-containing species relieve the symptoms associated with coronary artery disease. For example, in 1867 it was reported that amyl nitrite could be used to alleviate the chest pain associated with angina (16). Although not known in these early studies, it is now established that the ability of "nitrovasodilators" such as amyl nitrite and nitroglycerin to relax coronary arteries is due to their biotransformation to $\cdot\text{NO}$ (for example, see Ref. 54). The mechanism by which $\cdot\text{NO}$ elicits vasorelaxation is known to occur via activation of the enzyme soluble guanylate cyclase (sGC) (for a review, see Ref. 89). sGC is a heterodimeric heme-containing soluble protein, which exists in the cytosolic fraction of virtually all mammalian cells and acts as a principal intracellular target for $\cdot\text{NO}$. (Homologous enzymes exist in lower species such as *Drosophila* and *Caenorhabditis elegans*.) Activation of sGC by $\cdot\text{NO}$ results in an increased conversion of GTP to the second messenger, cGMP, which governs many aspects of cellular function via interaction with cGMP-dependent protein kinases, cyclic nucleotide gated ion channels, or cyclic nucleotide phosphodiesterases (89, 195). As a consequence, sGC has become accepted as the primary $\cdot\text{NO}$ receptor, and the $\cdot\text{NO}$ -sGC-cGMP signal transduction pathway has been established as an important signal transduction system. Because of its ubiquitous nature, aberrations in sGC-mediated signaling may be fundamental to the etiology of a wide variety of pathological disorders; this is exemplified in the cardiovascular system by disease states such as sepsis and atherosclerosis (90, 91). Nitric oxide-mediated activation of sGC occurs by $\cdot\text{NO}$ ligation to the regulatory heme site on the protein. The heme of sGC does not directly participate in the catalytic chemistry of the enzyme and appears to serve a purely regulatory function. Nitric oxide is a unique ligand for heme proteins in that the ferrousnitrosyl adduct prefers to exist as a 5-coordinate, square pyramidal complex resulting in the release of an axial histidine ligand in sGC, which, presumably, is critical for enzyme activation (226, 227).

5.2) Physiological and pathophysiological chemistry of $\cdot\text{NO}$. As mentioned above, $\cdot\text{NO}$ is a unique ligand for heme proteins, and, indeed, the distinctive coordination chemistry of $\cdot\text{NO}$ allows it to be an especially potent activator of sGC. Nitric oxide possesses other unique and important chemical properties that are also critical to aspects of its biology. Nitric oxide has one unpaired electron located in an anti-bonding orbital and therefore is a radical. Nitric oxide is almost exclusively a monomeric radical species at room temperature and pressure, so its reactions with other radicals (such as $\text{O}_2^{\cdot-}$ or alkyl radicals) are extremely facile. The inherent radical nature of $\cdot\text{NO}$ and its reactions with other free radicals present in biological systems are important to some of its possible biological actions. For example, it has been proposed that $\cdot\text{NO}$ can

be toxic through reaction with superoxide ($\text{O}_2^{\cdot-}$) to generate peroxynitrite ($\text{O}=\text{NOO}^-$), an oxidizing agent capable of modifying a variety of biological molecules (see above and Ref. 180). An interesting concept is that $\cdot\text{NO}$ protects against the toxicity of $\text{O}_2^{\cdot-}$ by scavenging it to form $\text{O}=\text{NOO}^-$, which in part spontaneously decomposes to NO_3^- . Further comments on the toxicity of $\text{O}=\text{NOO}^-$ can be found in Ref. 64.

The reaction of $\cdot\text{NO}$ with O_2 generates species such as nitrogen dioxide (NO_2^{\cdot}) and dinitrogen trioxide (N_2O_3) that may have biological significance. Dinitrogen trioxide is a potent nitrosating agent that can alter protein function via nitrosation of critical nucleophilic residues (*reactions 13–17*).



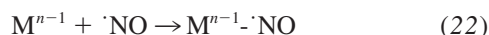
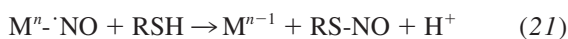
Note that *reaction 17* can lead to protein nitrosation and altered protein function.

The NO_2^{\cdot} formed in *reaction 15* is also a free radical species that, unlike $\cdot\text{NO}$, is a fairly potent oxidant [E° for the $\text{NO}_2^{\cdot}/\text{NO}_2^-$ couple = 1.04 V, vs. normal hydrogen electrode (NHE)]. There are a variety of potential reaction pathways by which NO_2^{\cdot} can cause oxidation to biological molecules: hydrogen atom abstraction, addition to unsaturated bonds and electron transfer reactions (for review of NO_2^{\cdot} oxidation chemistry, see Ref. 96). The kinetics for the $\cdot\text{NO} + \text{O}_2$ reactions (first order in O_2 and second order in $\cdot\text{NO}$ to form NO_2^{\cdot}) requires fairly high concentrations of $\cdot\text{NO}$ for this reaction to be physiologically relevant. However, it has been postulated that the lipophilicity of $\cdot\text{NO}$ and O_2 allows their concentration to be high enough for this reaction to occur in cell membranes (132).

The chemistry of $\cdot\text{NO}$ and derived species with thiols appears to be an important aspect of $\cdot\text{NO}$ biology (212). Modification of biological molecules by $\cdot\text{NO}$ may occur via reaction with a thiol function; for example, nitrosothiols (RSNO) can be formed by reaction with $\cdot\text{NO}$ or, more likely, $\cdot\text{NO}$ -derived species. RSNO formation can be accomplished by *reaction 17* (with a nucleophilic thiol). Also, RSNO formation has been postulated to occur via direct reaction of $\cdot\text{NO}$ with a thiol, a reaction first proposed by Pryor and coworkers (174) followed by reaction of the thiol- $\cdot\text{NO}$ intermediate with O_2 (*reactions 18 and 19*) (77).



This route to RSNO formation has not, however, been established to be physiologically relevant. Finally, RSNO formation can occur via metal-mediated processes whereby the metal binds $\cdot\text{NO}$ and acts as an electron acceptor when reacted with a thiol (see, for example, Refs. 56, 82, 235, 241) (*reactions 20 and 21*). This chemistry can be accomplished by, for example, ferric heme proteins, ultimately resulting in the generation of a ferrous nitrosyl adduct (*reaction 22*, $\text{M}^n = \text{Fe}^{3+}$ -heme, $\text{M}^{n-1} = \text{Fe}^{2+}$ -heme) in a process referred to as "reductive nitrosylation."



Thus, there are a variety of potential physiologically relevant mechanisms that lead to the generation of *S*-nitrosothiols, and there is little doubt that they are formed *in vivo*. Once formed, RSNO compounds can react further with a variety of biological species. For example, RSNO can react with another thiol, R'SH, to transfer the equivalent of a nitrosonium ion, NO⁺ (10, 11) (*reaction 23*).



RSNO can also react with thiols to give, instead, the corresponding disulfide and HNO (*reaction 24*) (249).



The factors governing the site of attack on the RSNO functional group have not been established.

Modification of protein or peptide thiols by $\cdot\text{NO}$ and $\cdot\text{NO}$ -derived species is of possible biological significance to metal metabolism/metal homeostasis, since the binding of metals to proteins often involves thiol ligation. Thus, nitrogen oxide-mediated thiol modification can potentially disrupt metal-protein interactions, leading to possible metal release and subsequent metal-derived free radical chemistry. Thiols ligated to metals may be specific targets for modification by $\cdot\text{NO}$ and would indicate an ability of $\cdot\text{NO}$ to disrupt/regulate metal metabolism even in the presence of high levels of non-metal-bound thiols such as glutathione (see, for example, Ref. 200).

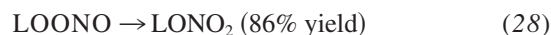
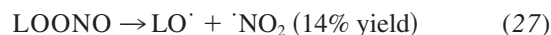
5.3) Nitric oxide as an oxidant and antioxidant. As indicated above, $\cdot\text{NO}$ can react with O₂ and O₂^{•-} to generate potentially deleterious oxidants such as O=NOO⁻ and $\cdot\text{NO}_2$. Indeed, it has been hypothesized that much of the toxicity associated with high levels of $\cdot\text{NO}$ is a result of formation of these oxidants. However, the ability of $\cdot\text{NO}$ to react with radicals also predicts that it can have antioxidant properties. That is, $\cdot\text{NO}$ can combine with another radical leading to termination of radical chain reactions. Probably the best example of the antioxidant properties of $\cdot\text{NO}$ is the effect it can have on lipid peroxidation (see, for example, Refs. 94, 192, 193, 215, 248).

Free radical chain processes occur in membranes because the membrane PUFA are susceptible to radical initiation processes and undergo the well-known PUFA radical chain autoxidation (166, 170). Lipid alkoxy (LO \cdot) and peroxy (LOO \cdot) radicals are important intermediates in these lipid autoxidation processes. Nitric oxide can behave as an antioxidant or as a prooxidant in lipid autoxidations, depending on the experimental conditions (88, 152, 153). The antioxidant action of $\cdot\text{NO}$ occurs by chain-breaking termination reactions of $\cdot\text{NO}$ with LO \cdot and LOO \cdot radicals, as in *reactions 25* and *26*.



Thus, depending on conditions, $\cdot\text{NO}$ can act as an oxidant or antioxidant. The reaction of nitric oxide with LOO \cdot results in the formation of an alkyl peroxyxynitrite, LOONO, which can homolyze to generate a geminate radical pair, $\cdot\text{NO}_2$ and an alkoxy radical, LO \cdot . Both of these radicals can initiate further

radical reactions (for example, see Refs. 75 and 255). About 86% of these radical pairs from LOONO rapidly recombine to give unreactive alkyl nitrates, LONO₂ (75), indicating that $\cdot\text{NO}$ can be an effective antioxidant. However, the remaining 14% of the radical pairs formed in the homolysis of LOONO become free $\cdot\text{NO}_2$ and LO \cdot radicals (see *reactions 27* and *28*).



Thus, 14% of the $\cdot\text{NO}$ and LOO \cdot radicals that react to form LOONO get effectively converted into $\cdot\text{NO}_2$ and LO \cdot , a much more reactive pair. For instance, in abstracting a hydrogen atom from a doubly allylic position, the rate constants for LOO \cdot and LO \cdot are 31 M⁻¹·s⁻¹ and 1 × 10⁷ M⁻¹·s⁻¹ (4), respectively, and although $\cdot\text{NO}$ cannot abstract a hydrogen atom from a doubly allylic position in a PUFA, the rate constant for the reaction of $\cdot\text{NO}_2$ with linoleic acid is 2 × 10⁵ M⁻¹·s⁻¹ (165). In summary, through the sum of *reactions 26–28*, 86% of the LOO \cdot and $\cdot\text{NO}$ radicals go on to form the stable product LONO₂, and 14% go on to form the more reactive radicals LO \cdot and $\cdot\text{NO}_2$, revealing the Janus-like behavior of $\cdot\text{NO}$.

The rate constant for the homolysis of LOONO with L = CH₃ can be estimated using group increment rules, using a procedure similar to the procedure applied by Richeson et al. (189) to estimate the corresponding rate constant for HOONO. Thus, using a basis set containing Δ_fH° and S° for HOONO (-1 ± 1 kcal/mol and 68 ± 1 cal/K·mol, respectively, from Ref. 189), and H₂O₂, CH₃OH, CH₃OOH, CH₃OOCH₃, and CH₃ONO (from Ref. 2), Δ_fH° (CH₃OONO) can be computed by replacing H by CH₃, H by CH₃O, or H by NO in various ways, affording a mean value of Δ_fH° (CH₃OONO) = 1 ± 1 kcal/mol. Similarly, S° (CH₃OONO) can be computed from the entropy gain for H → CH₃, H → CH₃O, or H → NO as a mean value of S° (CH₃OONO) = 78 ± 1 cal/K·mol. Combining these values with CH₃O \cdot and $\cdot\text{NO}_2$, one can compute thermodynamic values for the equilibrium shown in *equation 29* as ΔH₂₉° = 11 ± 1 kcal/mol, ΔS₂₉° = 36 ± 1 cal/K·mol, and ΔG₂₉° = 0 ± 1 kcal/mol.



The gas phase high-pressure limit, independent of temperature, for *k*₋₂₉ (with L = CH₃) has been determined to be 1.2 × 10¹⁰ M⁻¹·s⁻¹. Accordingly, the preexponential A₂₉ at 298 K can be computed from A₂₉/s⁻¹ = A₋₂₉(0.224T)⁻¹exp(ΔS₂₉°/R) = (1.2 × 10¹⁰)(1.5 × 10⁻²)(7.37 × 10⁷) = 1.3 × 10¹⁶. Since ΔH₂₉°‡ = ΔH₂₉° = 11 ± 1 kcal/mol, the rate constant for homolysis of CH₃OONO in the gas phase is computed as *k*₂₉ = (1.3 × 10¹⁶)exp(-11/RT) = 1.5 × 10⁸ s⁻¹. If one assumes a retarding solvent cage effect (142) of a factor of about 10² and small solvent effects for the homolysis process, then one would expect an overall solution rate constant of decomposition for CH₃OONO on the order of 10⁶ s⁻¹, contrasting sharply with the rate of decomposition for HOONO of about 1 s⁻¹. These calculations are in good agreement with a recent report by Goldstein et al. (75), who estimated this rate constant at about 5 × 10⁵ s⁻¹. Recent calculations by Zhao et al. (255) predict a ΔH‡ for homolysis of HOONO of 18 kcal/mol beginning from the *cis* conformer. The corresponding activation energy for homolysis of CH₃OONO is 12 kcal/mol.

In contrast to LONO, LNO₂, and LONO₂, which are relatively stable, LOONO is not stable, and the rapid homolysis of LOONO into LO[•] and [•]NO₂ will be reflected in the oxidative effects of [•]NO. Another important aspect of lipid-nitrogen oxide interaction is the finding that lipid nitration, likely via [•]NO₂, generates a nitrolipid (LNO₂), which has been reported to have biological signaling properties that are cGMP-dependent and -independent (29). Recently, nitrolipids had been found in red blood cell membranes in surprisingly high levels and appear to represent a novel class of lipid-derived signaling mediators. The mechanism by which these nitrolipids are formed in vivo is not known with certainty. However, [•]NO₂ had been found to yield vinylnitro- as well as allylnitro-lipids under a variety of conditions (66) (Fig. 3).

Thus, [•]NO₂ can induce *cis-trans* isomerization of the *cis*-double bonds in unsaturated fatty acids by a reversible addition reaction, as shown in Fig. 3. The radical intermediate formed by addition of an [•]NO₂ to the alkene can rotate around the C-C bond and lead to the lower-energy *trans* isomer of the fatty acid. The radical intermediate can react with other radicals to yield an α -substituted nitro-fatty acid, where X could be, for example, -OH, -OOH, -ONO, -NO₂, -ONO₂, etc. This α -substituted nitro-fatty acid could then undergo elimination of HX and yield a vinylnitro-fatty acid. Nitrogen dioxide (or a more reactive radical) also can react with unsaturated fatty acids by abstracting an H atom from an allylic position yielding an allylic radical which can then react with [•]NO₂ to yield an allylnitro-fatty acid. Finally, vinyl- and allylnitro-fatty acids may isomerize and equilibrate, as has been reported for several homologous compounds (223). Of course, lipid radical intermediates can also lead to biologically active isoprostanes (191).

5.4) *Oxidation and nitration in nitric oxide-producing systems.* Nitric oxide can play a role in cell physiology, for example, by acting as a cell signaling agent. Signaling by [•]NO is usually accompanied by some degree of oxidative and

nitritative stress in which the superoxide radical and dissolved gases (e.g., oxygen and carbon dioxide) can play modulatory roles. Moreover, as we have shown, peroxyxynitrite, peroxyxynitrate, and their esters, which are known to play a role as reactive intermediates in smog, can also occur downstream from the formation of [•]NO in vivo. The delicate interplay of these reactions is briefly reviewed below.

5.5) *Peroxyxynitrite, CO₂, and selectivity among free radicals.* The discovery in 1995–1996 (47, 134, 230) that CO₂ reacts rapidly with peroxyxynitrite and alters its reactivity was vital in understanding its reactions. In the absence of CO₂, peroxyxynitrite slowly decomposes to form the free radicals HO[•] and [•]NO₂ and is a powerful but slow oxidant and a poor nitrating reagent (208). In humans, fluids and tissues contain 1–2 mM CO₂, and the reaction of peroxyxynitrite with CO₂ is 50 to 100 times faster than its rate of decomposition to form HO[•] and [•]NO₂. Furthermore, other biological molecules, such as heme proteins, also react with peroxyxynitrite at considerable rates. Thus, in vivo, peroxyxynitrite does not decompose to form HO[•] and [•]NO₂, because this reaction is too slow.

The modulation of peroxyxynitrite reactivity by CO₂ described above is shown in *reactions 30* and *31*. In the absence of CO₂, peroxyxynitrite slowly decomposes to give the HO[•] and [•]NO₂ radicals, but in its presence peroxyxynitrite generates the CO₃^{•-} and [•]NO₂ radicals in a fast reaction.



As discussed above, some but not all free radicals are short-lived and reactive. Certain free radicals, such as HO[•], react with virtually all biomolecules as fast as they collide and consequently live only microseconds. For this reason, biological damage by the HO[•] radical is random, inefficient, and widespread, and generally does not affect critical cellular targets. In contrast, other radicals, such as CO₃^{•-} and [•]NO₂, display various degrees of selectivity for biological molecules (7, 208, 210). Furthermore, when CO₃^{•-} and [•]NO₂ react in concert, as when these radicals are produced together from the reaction of peroxyxynitrite with CO₂, these two radicals constitute an efficient nitrating system that is pivotal to biological nitration of protein tyrosine residues. More than 50 human diseases show elevated 3-nitrotyrosine levels (78, 84).

The different reactivities of the free radicals HO[•], CO₃^{•-}, and [•]NO₂ are shown in Table 1, where second-order rate constants for the reactions of these free radicals with selected biological target molecules are given. These rate constants were taken either from published work and databases of rate constants (72) or, when not available, were estimated from the reactivities of related compounds (69, 117). Thus, the HO[•] radical reacts at or near the rate limit for diffusion with most biomolecules, whereas the CO₃^{•-} and [•]NO₂ radicals react more slowly and consequently have longer diffusion distances. Furthermore, unlike the HO[•] radical, the CO₃^{•-} and [•]NO₂ radicals have a wide range of reactivities.

The order of reactivity is HO[•] > CO₃^{•-} > [•]NO₂, and unlike the HO[•] radical, CO₃^{•-} and [•]NO₂ can be quite selective for certain biomolecules. For example, the CO₃^{•-} radical reacts at least 100,000 times faster with tyrosine, tryptophan, or cysteine than it does with alanine or glycine, effectively targeting only some of the amino acids in a protein. Nitrogen dioxide, [•]NO₂,

Cis-Trans Isomerization and Vinylnitro Products

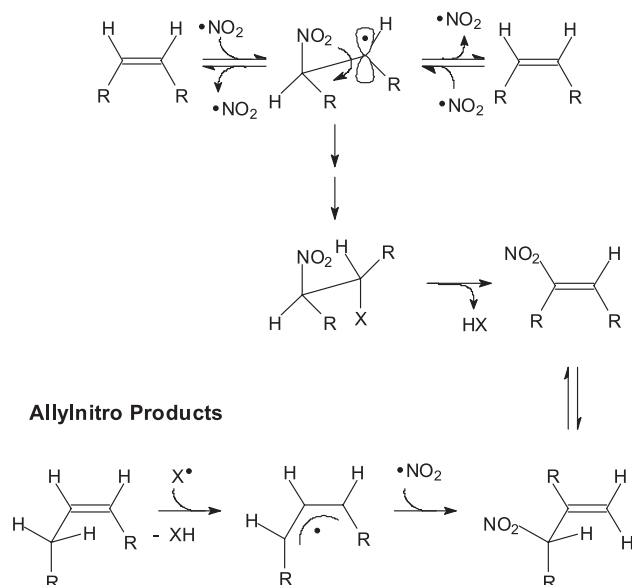


Fig. 3. [•]NO₂-induced formation of vinyl- and allylnitro-lipids.

Table 1. Comparison of second-order rate constants for the reactions of selected biological molecules with the free radicals HO[•], CO₃^{•-}, and [•]NO₂

Biomolecule	HO [•]	CO ₃ ^{•-}	[•] NO ₂
Tyrosine	1.3×10 ¹⁰	4.5×10 ⁷	3.2×10 ⁵
Tryptophan	1.3×10 ¹⁰	7×10 ⁸	<5×10 ⁵
Arginine	3.5×10 ⁹	9×10 ⁴	very slow reaction
Alanine	4.3×10 ⁸	<1×10 ³	very slow reaction
Glutathione	1.3×10 ¹⁰	5.3×10 ⁶	2×10 ⁷
Glycine	1.7×10 ⁷	<1×10 ³	very slow reaction
Cysteine	4.7×10 ¹⁰	1.6×10 ⁷	5×10 ⁷
Glutathione disulfide	0.9–2.3×10 ¹⁰	1.3×10 ⁶	very slow reaction
Oleic acid	~1×10 ¹⁰	<1×10 ³	<1
Linoleic acid	~1×10 ¹⁰	1×10 ¹⁰ >k>2×10 ⁵	~2×10 ⁵
Glucose	1.5×10 ⁹	7×10 ⁴	very slow reaction
NADH	2.0×10 ¹⁰	1.4×10 ⁹	4.0×10 ³
Ascorbate	~1×10 ¹⁰	1.1×10 ⁹	3.5×10 ⁷

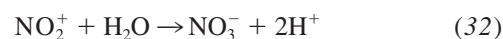
Rate constants are expressed as M⁻¹·s⁻¹.

also has been found to react selectively with the amino acid residues tyrosine, tryptophan, and cysteine in proteins (117).

The modulation of peroxynitrite reactivity caused by carbon dioxide is dramatic. It leads to the less reactive CO₃^{•-} and [•]NO₂ radicals, which have longer diffusion distances than the HO[•] radical. Beckman et al. (14) proposed in 1990 that peroxynitrite will be formed in biological systems in which both [•]NO and superoxide are produced. Yet, the mechanism for the reaction of peroxynitrite with CO₂ was not understood until 1995–1996, and the impact that this reaction has in the oxidative biochemistry of peroxynitrite was not immediately recognized. As a result, the role of CO₂ was ignored for several years, and the reactions of peroxynitrite in biological and model systems were not investigated in solutions that contained CO₂ (usually achieved by using bicarbonate buffers). Given that the chemistry of peroxynitrite is so different in the absence or the presence of CO₂, and that only the latter conditions are biologically relevant, literature from this time period should be interpreted with care. However, since CO₂ is ubiquitous, is present in indoor air, and spontaneously dissolves in solutions, even in those cases where bicarbonate buffers were not used, sufficient dissolved CO₂ probably was present so that the reaction with CO₂ still was the predominant reaction of peroxynitrite. For experiments where peroxynitrite is used in relatively low concentrations (probably up to 50–100 μM), it is likely there is sufficient adventitious CO₂ contamination to modulate the chemistry of peroxynitrite just as would have occurred with physiological levels of CO₂. This is true because CO₂ behaves as a catalyst and is partly recycled in the decomposition of peroxynitrite (177). As a result, much research that ignored a role for CO₂ may have afforded meaningful biological data because there was enough dissolved adventitious CO₂ to channel the decomposition of peroxynitrite through the peroxynitrite/CO₂ adduct.

5.6) Nitrogen dioxide and nitration of tyrosine residues. As we have seen, nitrogen dioxide is formed from the reaction of peroxynitrite with CO₂. In addition, peroxidases such as myeloperoxidase and eosinophil peroxidase can generate [•]NO₂ using nitrite as a substrate. Additionally, environmental [•]NO₂ can affect organs like the skin and the lung. Nitrogen dioxide induces both oxidation and nitration in cell membranes (233), and the presence of oxygen has been found to increase the yields of oxidation over nitration products (66).

Nitrated biological molecules, such as nitrotyrosine-containing proteins (184) and nitrolipids (9), are widely distributed in many organisms, and [•]NO₂ is the most likely nitrogen species that can play a role in their formation. Participation of the classic nitration intermediate, the nitronium ion (NO₂⁺) is unlikely because in biological systems it would react with water to form nitrate (*reaction 32*) much faster than it could nitrate tyrosine. Water can reach targets deep in the interior of biological membranes. For example, nearly 90% of the intermediate carbonyl-oxide is trapped by water during the ozonation of oleate-containing phospholipids in liposomes, where the double bond is originally positioned 9 carbons deep into the membrane (211). Other types of nitrating intermediates, such as transition metal ion complexes that may function as carriers of NO₂⁺ have been proposed, but their participation in biological systems has not yet been confirmed.



It is widely accepted that formation of nitrotyrosine residues in proteins takes place in a two-step reaction in which a free radical, such as CO₃^{•-}, abstracts a H atom from a tyrosine residue, generating a tyrosyl radical which then reacts with a [•]NO₂ in a radical-radical recombination reaction (7, 20, 74, 131, 136, 208) (Fig. 4).

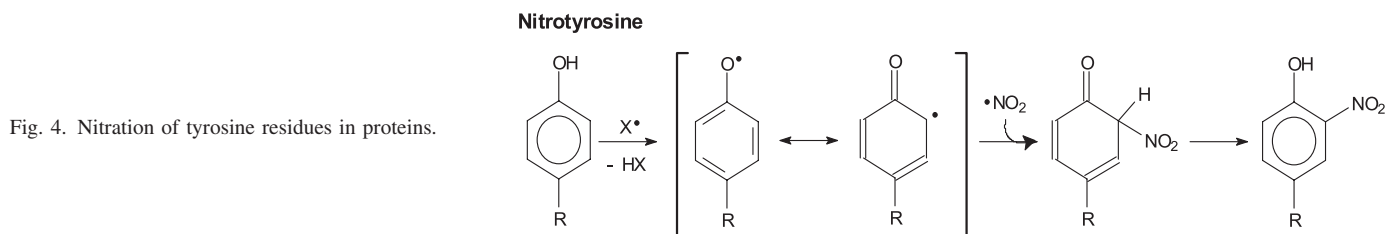


Fig. 4. Nitration of tyrosine residues in proteins.

Some enzymes contain tyrosyl radicals in their active sites, and these radical centers are targets for reaction with $\cdot\text{NO}_2$ because the reaction of these two radicals occurs near the diffusion limit. Thus, preferential nitration of active-site tyrosyl radicals in prostaglandin H₂ synthase-1 (PGSH-1) has been suggested, since the degree of nitration is found to correlate with the loss of enzyme activity, whereas substitution of residues not in the active site, for example, of tyrosine for phenylalanine, does not result in loss of activity (44). Oxidative stress will likely raise the level of protein tyrosyl radicals due to oxidation of tyrosine residues that are not redox active during the normal function of the protein and this will result in amplified tyrosine nitration. In this regard, the reduction of protein tyrosyl radicals by hydroxamic acids has been suggested as a mechanism to explain the hydroxamic acid-mediated inhibition of tyrosine nitration in a manner that is not related to the reactivity of the hydroxamic acid with $\cdot\text{NO}_2$ or to its metal ion chelating properties (127). Thus, the tyrosyl radical is a key intermediate in protein tyrosine nitration.

5.7) *Peroxynitrate: the consequences of a less reactive $\cdot\text{NO}_2$.* Although $\cdot\text{NO}_2$ is a more reactive and a more powerful oxidant than is $\cdot\text{NO}$, reactions of $\cdot\text{NO}_2$ with closed-shell molecules are relatively slow compared with those of $\text{HO}\cdot$ and $\text{CO}_3^{\cdot-}$ (see Table 1). However, $\cdot\text{NO}_2$ reacts rapidly with other radicals. This is one reason nitrotyrosine is formed from the reaction of $\cdot\text{NO}_2$ with protein tyrosyl radicals (210).



Just as the rate of reaction of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$ to form peroxynitrite is fast and close to the diffusion limit (70, 97, 123), so the reaction of $\cdot\text{NO}_2$ with $\text{O}_2^{\cdot-}$ to form peroxynitrate also is fast (137) (Table 2). This implies that when both $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}_2$ are present in the same environment, they will most likely react to form peroxynitrate.

Because of the higher reactivity of $\cdot\text{NO}_2$ relative to $\cdot\text{NO}$, the formation of peroxynitrate might be somewhat less likely than the formation of peroxynitrite. Nevertheless, peroxynitrate appears to be formed under a variety of experimental conditions. Others (5, 71, 92, 122) and Pryor and collaborators (229) have implicated peroxynitrate during the *in vitro* decomposition of peroxynitrite in the presence of certain substrates that lead to the formation of $\text{O}_2^{\cdot-}$ and $\cdot\text{NO}_2$. Since $\text{O}_2^{\cdot-}$ is ubiquitous in aerobic organisms and $\cdot\text{NO}_2$ can be formed endogenously by several pathways, the formation of peroxynitrate could be more widespread than presently recognized, and possible roles for peroxynitrate in oxidative biology should be studied further.

The biochemistry of peroxynitrate is very different from that of peroxynitrite. For example, in contrast to peroxynitrite, with peroxynitrate it is the conjugate base (O_2NOO^-) that is kinetically unstable. Again, in contrast to what is observed with peroxynitrite, CO_2 does not catalyze the decomposition of peroxynitrate. Biological oxidations by peroxynitrate would then result from reactive intermediates that are formed during

its decomposition and/or from direct oxidations by peroxynitrate. For example, theoretical and experimental data suggest that singlet oxygen (*reaction 34*) may be produced during the unassisted decomposition of peroxynitrate (73, 116, 138, 140, 154).



Peroxynitrate is a more powerful two-electron oxidant than peroxynitrite; their reduction potentials are $E^\circ(\text{pH } 7) = 1.59 \text{ V}$ vs. $E^\circ(\text{pH } 7) = 1.37 \text{ V}$ vs. NHE, respectively (73).

Knowledge of the reaction kinetics of peroxynitrate with biological molecules is very limited. The kinetics of the reaction of peroxynitrate with methionine were studied recently, affording $k = 34 \text{ M}^{-1}\cdot\text{s}^{-1}$, at pH 7.4 and 25°C (209), which compares with $181 \text{ M}^{-1}\cdot\text{s}^{-1}$ for the reaction of methionine with peroxynitrite under similar conditions (176).

We are only beginning to understand the delicate interplay of the radical reactions and the generation of secondary reactive species downstream from the formation of $\cdot\text{NO}$ and how these reactions can integrate with biochemical processes.

7) Carbon Monoxide

Thus far, most of our discussion has focused on biologically relevant gaseous species that are radicals ($\cdot\text{NO}$) or diradicals (O_2), along with derived molecules, some of which are also radicals and/or potent oxidants. As such, these species participate in biochemical processes that are radical in nature and/or involved in oxidative stress. However, there are several other endogenously generated gaseous species that are not themselves radicals or oxidants but have been proposed to be biologically important and may impact the biology and biochemistry of the other gaseous signal/effecter species. The first of these nonradical gases to be discussed is carbon monoxide (CO). This gas is probably best known as a toxic air pollutant. Exposure to as little as 0.4% CO in air (vol/vol) can cause death in less than 1 h (157).

Carbon monoxide is not a radical, but it is endogenously generated, and some of its proposed biological actions appear to involve modulation of other radical species or radical-mediated processes (see below). Like $\cdot\text{NO}$ and O_2 , CO is a simple diatomic, colorless gas at room temperature and pressure. The water solubility of CO is similar to $\cdot\text{NO}$ and O_2 with a maximum concentration of $\sim 2.5 \text{ mM}$ at standard temperature and pressure. However, unlike $\cdot\text{NO}$ and O_2 , the biological chemistry of CO is relatively simple (for a review, see Ref. 162). Carbon monoxide does not react with O_2 or $\cdot\text{NO}$ (under physiological conditions) and is generally stable in most mammalian cell environments. There have been reports that CO can be oxidized to CO_2 in mitochondria with subsequent generation of two electrons (251), presumably via a cytochrome *c* oxidase-CO interaction. However, the physiological relevance of this process remains to be established. Probably the most important reactions of CO in biology involve its ability to form coordination complexes with metalloproteins and compete with $\cdot\text{NO}$ and O_2 for these sites. For example, the binding of CO to iron heme proteins differs significantly from $\cdot\text{NO}$ in that CO will only bind reduced, ferrous heme, whereas $\cdot\text{NO}$ has the ability to bind both ferric and ferrous heme. Also, the binding geometry of CO is typically distinct from $\cdot\text{NO}$ and O_2 ; CO prefers to bind metals in a linear fashion, whereas $\cdot\text{NO}$ can

Table 2. Comparison of the reactivities of $\cdot\text{NO}$ and $\cdot\text{NO}_2$ with the superoxide radical

Reaction	Product	$k, \text{M}^{-1}\cdot\text{s}^{-1}$	Reference
$\cdot\text{NO} + \text{O}_2^{\cdot-}$	ONOO^-	$3.8\text{--}6.7 \times 10^9$	70,97,123
$\cdot\text{NO}_2 + \text{O}_2^{\cdot-}$	O_2NOO^-	4.5×10^9	137

bind in either a linear or bent mode (depending on the electronic requirements of the complex; Ref. 190), and O₂ typically adopts a bent geometry. To date, CO complexes with almost all heme proteins with open coordination sites have been observed in vitro. Carbon monoxide complexes with myoglobin, hemoglobin, cytochrome *c* oxidase, guanylate cyclase, NOS, cytochrome *P*-450, catalase, cystathionine β-synthase (CBS, discussed later), and others have been examined and characterized in vitro. In most cases, the binding of CO is thought to be inhibitory to the natural function of the heme protein. The exception to this is guanylate cyclase, where CO has been reported to elicit slight activation (although this is controversial; Ref. 90). The most well-studied reaction of CO with a heme protein is that with deoxyhemoglobin to form carboxyhemoglobin (COHb). The affinity of CO for deoxyhemoglobin is about 220 times greater than that for O₂. Thus, low levels of CO (for example, 50 ppm) will result in 5% COHb. The half-life of the COHb complex is 200–400 min in humans in air. The high affinity of CO for deoxyhemoglobin is largely responsible for the toxicity of CO. The COHb complex alters the kinetics of O₂ binding to other, vacant deoxyhemoglobin sites. The presence of CO confers higher cooperative binding for O₂ binding to the remaining sites. That is, O₂ “extraction” from hemoglobin by sites depleted in O₂ is less when CO is bound. Therefore, CO not only lessens the O₂ binding capacity of hemoglobin (by occupying an oxygen-binding site) but also inhibits O₂ release from the remaining sites.

As mentioned above, carbon monoxide binds only reduced (not oxidized) iron heme proteins and competes with O₂ and, presumably, NO. Thus, under hypoxia, where O₂ levels are low and the cell is highly reducing, it may be expected that CO complexes are prevalent. Moreover, under oxidative stress, where NO levels may be low due to reactions with O₂ and oxygen-derived species, CO may favorably compete for binding sites. Thus, the relative chemical stability of CO in mammalian cells allows opportunistic binding to sites that might otherwise bind O₂ or NO.

7.1) Carbon monoxide biosynthesis, regulation, and activity. The majority of CO made in mammalian cells originates from the activity of the heme oxygenase enzymes (HOE) (234). There are three known mammalian isoforms of HOE; HO-1, which is highly inducible and constitutively expressed in tissue where extensive heme catabolism occurs (spleen, liver, etc.); HO-2, which is not inducible, is present primarily in the testes, endothelium, the gastrointestinal tract, and brain and is thought to be involved in specific cell signaling processes; and HO-3, an isoform which has only recently been discovered in rat brain by cDNA cloning and possesses extremely low catalytic activity (for reviews, see Refs. 145, 163, 198). HO-1 is induced by a variety of signals and stresses such as hypoxia, hyperoxia, heat shock (HO-1 is also known as HSP32), endotoxin, hydrogen peroxide, heavy metals, arsenic, UV light, cytokines, heme, and NO (145).

A common thread linking most all of the inducers of HO-1 is that they can all either cause, contribute to, or exacerbate oxidative stress. Thus, it would appear that HO-1 is induced for the purpose of coping with oxidative stress. Indeed, many researchers view HO-1 expression as a major “antioxidant” response.

As the name implies, HOE oxidize “free” heme, resulting in the generation of CO, iron, and biliverdin. Interestingly, HOE

utilize iron heme as both a substrate and prosthetic group. HOE bind free iron heme and then use it to bind and activate O₂ (see, for example, see Refs. 155 and 250). Further discussion of the catalytic mechanism of HOE is beyond the scope of this review. It is clear, however, that the complexity and chemical difficulty in generating CO from heme oxidation strongly suggests that CO is not merely a toxic waste byproduct of heme catabolism, but rather an entity that has been synthesized for a purpose.

The induction of HO-1 expression is an important aspect of an anti-inflammatory, anti-apoptotic response to cellular stress. For example, HO-1 expression is protective against myocardial reperfusion toxicity (85), inhibits transplanted organ rejection (85), prevents hypoxia-induced pulmonary hypertension (27), and inhibits fas-mediated apoptosis (110) and TNF-α-mediated endothelial cell apoptosis (15). Significantly, many of the actions of HO-1 can be mimicked by administration of low levels of CO. For example, low-level CO administration can protect against hyperoxic lung injury (158), ischemic lung injury (63), inhibit proinflammatory cytokine expression (156), and inhibit apoptosis (15). Thus, it is apparent that much of the biology of HOE activity is a result of CO generation (although it is evident that biliverdin/bilirubin and released iron possess significant and important activity as well; see, for example, Ref. 28).

As mentioned above, an inducer of HO-1 expression is hypoxia. Since HO-1 is an oxygen-requiring enzyme, it may seem odd that hypoxia would induce this enzyme. However, the kinetics of oxygen utilization by HO-1 makes its activity even under moderate hypoxia possible. The apparent *K_m* of oxygen for HO-1 in liver is reported to be only 12 μM (162). In comparison, NOS have about 2–4 times greater *K_m* for oxygen, indicating that HO-1 will favorably compete for oxygen (188). Thus, the kinetics of HO-1 catalysis under hypoxic conditions appears to favor generation of CO over, for example, NO.

The signaling pathways stimulated subsequent to HO-1 expression (or more specifically by CO exposure) have been examined extensively (15, 194, 205). It is clear that CO is capable of inhibiting the expression of proinflammatory genes (IL-1β, TNF-α, MIP-1α) while, at the same time, enhancing the expression of anti-inflammatory genes (e.g., IL-10). It appears that these effects are independent of cGMP and mediated through selective activation of the p38-MAP kinase pathway. Thus, some of the downstream effects associated with HO-1 generation of CO can be explained through activation of specific MAP kinase pathways. Recently, Morse and coworkers (146) found that the effect of CO was mediated via the JNK signaling pathway and the transcription factor AP-1, indicating multiple signaling pathways (e.g., p38-MAPK and/or JNK) depending on the various models. Regardless, the mechanistic details of how CO activates these pathways are wholly unknown.

8) Hydrogen Sulfide

Another endogenously generated gaseous species receiving recent attention is hydrogen sulfide, H₂S. This species, like CO and NO, can be toxic. It is, in fact, more toxic than hydrogen cyanide and carbon monoxide; fatality can occur to exposure to as little as 300 ppm in air for 30 min. Hydrogen sulfide is the

most recent small endogenously generated species touted as a biological signal species (for recent reviews, see Refs. 108, 143, 238, 239). Like CO, H₂S is not a radical but has the apparent ability to interact with and disrupt/modulate the actions of other radicals, making its appearance in this review appropriate. Hydrogen sulfide has a pK_a of 6.8, making the anionic species the predominant form under most physiological conditions.

8.1) H₂S biosynthesis, regulation, and biological activity. In mammalian systems, the generation of H₂S occurs via the actions of primarily two enzymes (105). In the brain, H₂S is synthesized from cysteine via the actions of cystathionine β-synthase (CBS) (3, 213). This enzyme normally catalyzes the reaction between homocysteine and serine to make cystathionine, an intermediate in the cysteine biosynthesis pathway from methionine/homocysteine. However, it is now apparent that CBS can also catalyze the reaction of cysteine with other thiols to generate H₂S and the corresponding thioether. CBS is a pyridoxyl phosphate-requiring enzyme that is at least partially regulated by Ca²⁺ (120) and S-adenosylmethionine (SAM) (3). Calcium elicits a three- to fourfold increase in activity via binding of the Ca²⁺-calmodulin complex to CBS. Significantly, constitutively expressed NOS isoforms, nNOS and eNOS, are also regulated by Ca²⁺-calmodulin, indicating that cells containing both CBS and these NOS isoforms could generate significant amounts of [•]NO and H₂S simultaneously when high cytosolic Ca²⁺ levels are present. SAM increases enzyme activity by as much as twofold via binding to an allosteric regulatory site on CBS. One of the most provocative and intriguing aspects of CBS is the fact that it also contains an iron heme (112). This is highly unusual, since the catalytic activity of CBS does not require any heme chemistry (i.e., no redox, O₂-binding, or O₂-activation processes). This is reminiscent of the biological target for [•]NO, sGC, and likewise the heme component of CBS appears to be primarily regulatory. Interestingly, it has been demonstrated that both CO and [•]NO are capable of inhibiting enzyme activity via heme binding (224). However, unlike most other heme proteins, CO binds with significantly higher affinity compared with [•]NO. This may indicate that CO can regulate CBS activity *in vivo* since [•]NO is unlikely to reach high enough concentrations *in vivo* to cause CBS inhibition. One important note: the regulation and/or inhibition of CBS activity by the above-mentioned agents is with respect to the formation of cystathionine from homocysteine and serine. It is not known whether these agents have similar or different effects on the H₂S-forming processes.

In the vascular system, H₂S biosynthesis is not carried out by CBS, but rather by a distinct enzyme, cystathionine γ-lyase (CSE, also referred to as cystathionase; for example, see Refs. 254). Like CBS, CSE also is a pyridoxyl phosphate-requiring enzyme. This enzyme converts cystine (oxidized cysteine) to pyruvate, ammonia, and thiocysteine. Thiocysteine then reacts with other thiols to generate H₂S and the disulfide. Interestingly, [•]NO donors have been shown to increase CSE expression in cultured smooth muscle cells (254) and increase CSE activity (239), implicating [•]NO-mediated regulation of this enzyme. CSE contains multiple cysteines that may be sites for [•]NO-mediated modification (i.e., thiol nitrosation).

Physiological levels of H₂S can be quite high (at least compared with the other endogenously generated gaseous sig-

naling/effector species) (239). In mammalian brain tissues, H₂S levels can be as high as 50–160 μM, and in the vascular system, blood levels of 10–100 μM have been reported. Considering that CO and [•]NO levels are not likely to exceed single-digit micromolar levels (even under pathophysiological conditions), physiological H₂S levels typically will far exceed even the highest levels of some of the other small signaling molecules.

One of the predominant roles for H₂S (or sulfide, S²⁻) in biology is as a component of iron-sulfur (FeS) clusters. The sulfide for the biosynthesis of FeS clusters is generated by pyridoxal phosphate-dependent cysteine desulfuration that occurs in mitochondria (148). However, the chemistry and biology of FeS clusters are not particularly relevant to this discussion, as we are focusing on the actions of free H₂S; thus the role of sulfide in FeS chemistry will not be reviewed. Recent discoveries of biological H₂S activity further underscore the importance and prevalence of endogenously generated, gaseous signaling molecules. Thus far, it is reported that H₂S has both vascular and CNS functions. In the vascular system, H₂S causes vasorelaxation (254). The mechanism of this activity is, however, distinct from that of [•]NO in that it is cGMP-independent. However, H₂S can enhance the vasorelaxant effects of [•]NO (95). The actions of H₂S as a vasorelaxant have been determined to be at least partially due to its ability to activate K_{ATP} channels on smooth muscle cells. However, there also appears to be an endothelium-dependent effect (239). In the brain, H₂S can have numerous effects (238). One effect demonstrated under physiological concentrations is the ability of H₂S to act as a neuromodulator enhancing *N*-methyl-D-aspartate (NMDA) receptor responses. Indeed, physiological concentrations of H₂S elicit long-term potentiation (3). The ability of H₂S to enhance NMDA receptor responses appears to be related to cAMP production (119). As there is an established connection between elevated cAMP and modulation of the NMDA receptor, this is not surprising. It has been reported that H₂S can have antioxidant properties, protecting neurons from oxidative stress (121). The antioxidant effect of H₂S is attributed to its ability to raise the intracellular glutathione (GSH) concentration by as much as twofold, without increasing oxidized GSH (GSSG) levels, as well as increasing the levels of the GSH biosynthetic enzyme γ-glutamylcysteine synthase. It has also been demonstrated that H₂S can increase the ability for the antioxidant enzyme SOD to scavenge superoxide (197).

As with many of the actions of [•]NO and CO, the chemical basis for the biological actions of H₂S in the vasculature and in the brain is not established. However, one of the common chemical themes relating many of the signaling molecules mentioned herein (e.g., [•]NO, O₂, CO, H₂S) is that they all have the ability to interact with similar metals and/or metalloproteins. That is, metals/metalloproteins that have the capacity to bind one of these species often have the capacity to bind at least some of the others (although an oxidation state change may be required). For example, cytochrome *c* oxidase can bind [•]NO, CO, O₂, and H₂S. Thus, it is intriguing to speculate that metallo-receptors may be an important aspect to the physiological signaling associated with H₂S and the other gaseous species and that common sites of activity exist.

9) Carbon Dioxide

In this review, we do not specifically address many aspects of a very important and endogenously generated gaseous molecule, carbon dioxide (CO₂) or its hydrated derivatives, carbonic acid (H₂CO₃), bicarbonate (HCO₃⁻), and carbonate (CO₃²⁻). The omission of many of these topics is not because they are not important and vital signaling species/biochemical agents but, rather, because of the fact that many of their roles and functions in biology are mechanistically distinct or unrelated to the majority of the species discussed herein (with a few exceptions, below). For example, in mammalian systems, the role of CO₂ as a pH modifier (the basis for much of its signaling properties), its interaction with hemoglobin (via carbamoyl acid formation), its biosynthesis (for example, by the citric acid cycle or the pentose phosphate pathway), and its role in fatty acid and purine biosynthesis is covered in most elementary biochemistry textbooks. Thus, we have omitted these topics from this review. However, the chemistry and interaction of CO₂ and derived species with free radicals (or precursor species) is potentially important. Indeed, we devoted a significant portion of this review to the interaction of CO₂ with peroxynitrite, a reaction that is predominant in the fate of this oxidant. As discussed above, the reaction of CO₂ with peroxynitrite leads to the generation of the carbonate radical (CO₃^{·-}), an important oxidant. It is also worthwhile noting that other pathways, independent of nitrogen oxide chemistry, have been reported for the generation of CO₃^{·-}. Of significant recent interest are the reports of CO₃^{·-} generation from the reaction of hydrogen peroxide and bicarbonate with Cu,Zn-SOD (for example, see Refs. 107, 186, and 253). This chemistry appears not to be limited to SOD, as it can be catalyzed by copper ions in solution (185). As a strong one-electron oxidant ($E^\circ = 1.59$ vs. NHE), CO₃^{·-} can react with a variety of biological species such as guanine (40) or tyrosine and tryptophan (7). One of the most intriguing and potentially important aspects of CO₃^{·-} is that it is a somewhat selective oxidant able to diffuse in biological systems. That is, unlike hydroxyl radical, which is so reactive that it will not diffuse any significant distance from its point of generation, CO₃^{·-} is able to diffuse longer distances than the hydroxyl radical and react with more specific species. This is one mechanism by which the reactivity of a potent oxidant, generated in a specific location and in the presence of HCO₃⁻, can be translated to more distant species (albeit the reactivity of CO₃^{·-} will be less than that of the initiating oxidant). Thus, like the other gaseous species discussed herein, CO₂ and its derivatives may also play an important role in free radical biology.

10) Summary

As the ultimate electron acceptor, O₂ is the basis for respiration and fundamentally important to bioenergetics. However, it is now clear that endogenously generated gases (e.g., [·]NO, CO, H₂S, CO₂), ubiquitously present gases (O₂, CO₂), and the species derived from these gases (e.g., [·]NO₂, ONOO⁻, H₂O₂) also are important as physiological signaling agents, in the etiology of numerous diseases, and as biological effectors. One of the most intriguing aspects of the biology and biochemistry of many of these species is their participation in, or initiation of, free radical processes and their effect on cellular redox biochemistry. Nature's selection of these small molecule spe-

cies as specific signaling agents and/or effector molecules is due to their unique chemistry. However, at the same time that life evolved around the desired biochemical properties of these species (e.g., O₂, [·]NO), mechanisms to cope with their potentially deleterious reactions had to evolve. (For an interesting discussion of our O₂, see Refs. 93 and 111).

Indeed, the Janus-faced biology of [·]NO, O₂, H₂O₂, and other species mentioned here is now a well-recognized and accepted aspect of their biochemistry. Moreover, misregulated or adventitious generation of many of these species leads to disease states often characterized by oxidative stress and free radical damage. Although we have come a long way in our understanding of small molecule biology/pathology and now recognize the importance of free radical biochemistry, many significant and fundamental discoveries remain. It is clear that this field will continue to "be a gas, man" for many years to come.

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The subtitle of our manuscript has its origin in a painting that has hung in W. A. Pryor's office for 40 years. The painting shows Dizzy Gillespie, in beret and horn-rim glasses, playing a glass reflux condenser as if it were a trumpet. The legend states: Thelonious Avogadro's Law: "These molecules are real gassers." This story of gases was inspired partly by those inventive, iconoclastic masters of modern music, Bird and Diz.

C. S. Foote died on June 13, 2005 after fighting a brain tumor for a year. His extraordinary contributions to our knowledge of singlet oxygen and his warm and wonderful friendship to many of us live on.

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