0023-6837/82/4705-0412$02.00/0
LABORATORY INVESTIGATION

Vol. 47, No. 5, p. 412, 1982
Printed in U.S.A.

Biology of Disease

Free Radicals and Tissue Injury

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A free radical is any molecule that has an odd number of electrons. Free radicals, which can occur in both organic (*i.e.*, quinones) and inorganic molecules (*i.e.*, $O_2^{\cdot-}$), are highly reactive and, therefore, transient. Free radicals are generated *in vivo* as byproducts of normal metabolism. They are also produced when an organism is exposed to ionizing radiation, to drugs capable of redox cycling, or to xenobiotics that can form free radical metabolites *in situ*. Cellular targets at risk from free radical damage depend on the nature of the radical and its site of generation. In this review we survey cellular sources of free radicals and the reactions they can undergo and discuss cellular defenses and adaptive mechanisms.

1. BIOLOGIC SOURCES OF FREE RADICALS

Free radical reactions are critical for the normal operation of a wide spectrum of biologic processes. The catalytic action of many cellular enzymes and electron transport processes involves one-electron transfers which yield free radical intermediates. Because of the ubiquity of molecular oxygen in aerobic organisms and its ability to readily accept electrons, oxygen-centered free radicals are often mediators of cellular free radical reactions.

There are diverse ways an individual can be exposed to free radicals other than through the processes of normal metabolism (Table 1). Oxygen radicals produced by activated phagocytes, which have been recently discussed in this review series (167), are microbicidal and can inadvertantly cause tissue damage. Many anthracyclic antineoplastic agents such as adriamycin, daunorubicin, doxorubicin, and other antibiotics that depend on quinoid groups or bound metals for activity are able to generate oxygen radicals. Many of the chemotherapeutic effects and cytotoxic side effects of these drugs have been ascribed to their ability to reduce oxygen to $O_2^{\cdot-}$, H_2O_2, and hydroxyl radical ($OH\cdot$). Irradiation of organisms with electromagnetic radiation (x-rays and γ rays) and particulate radiation (electrons, protons, neutrons, deuterons, and α and β particles) generate primary radicals by transferring their energy to cellular components such as water. These primary radicals, including e^-_{aq}, $OH\cdot$, and $H\cdot$, can then undergo secondary reactions with dissolved O_2 or with cellular solutes. In addition, a wide variety of environmental agents including photochemical air pollutants, hyperoxia, pesticides, tobacco smoke, solvents, anesthetics, and the general class of aromatic hydrocarbons also cause free radical damage to cells. These xenobiotics either already exist as free radicals or are converted to radical species by intracellular metabolic and detoxication processes.

Excellent articles have recently been published regarding free radical generation and cellular damage by antibiotics (38, 80), phagocytic cells (8, 167), radiation (13), and xenobiotics (29, 92–94). These free radical sources or initiators share many common mechanisms of inducing cell damage.

2. INTRACELLULAR SOURCES OF FREE RADICALS

A. Autoxidation of Small Molecules

A wide variety of soluble cell components, capable of undergoing oxidation-reduction reactions in a neutral aqueous milieu, are quantitatively important contributors to intracellular free radical production (Fig. 1). These include thiols (7), hydroquinones (104), catecholamines (113), flavins (9), and tetrahydropterins (42). In all cases, $O_2^{\cdot-}$ is the primary radical formed by the reduction of dioxygen by these molecules. Also, chelated Fe(III) can be reduced Fe(II) by thiols, ascorbate, and a host of other reductants. Fe(II) can then autoxidize, producing $O_2^{\cdot-}$ (102, 170). Hydrogen peroxide is a secondary product of one-electron autoxidations, via spontaneous or enzymatically catalyzed dismutation of $O_2^{\cdot-}$:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

The spontaneous dismutation of $O_2^{\cdot-}$ has a rate constant at pH 7.4 of approximately 2×10^5 M^{-1} sec^{-1}, whereas the reaction catalyzed by superoxide dismutase is about 10^4 times faster, having a rate constant of 2×10^9 M^{-1} sec^{-1} (52). Thus, cellular processes that yield $O_2^{\cdot-}$ will also produce H_2O_2 as an $O_2^{\cdot-}$ dismutation byproduct. Further reactions of $O_2^{\cdot-}$ and H_2O_2 will be discussed in subsequent sections.

B. Soluble Enzymes and Proteins

Numerous enzymes generate free radicals during their catalytic cycling. Xanthine oxidase, probably the most

studied free radical-producing enzyme, generates $O_2^{\cdot-}$ during the reduction of oxygen to H_2O_2. The relative proportion of $O_2^{\cdot-}$ and H_2O_2 released from the active site of the enzyme depends on pH, oxygen concentration, and substrate concentration (51). Interestingly, human xanthine oxidase serves *in vivo* as an NAD^+-dependent dehydrogenase and produces no free radical intermediates. Proteolytic modification of xanthine oxidase during purification or during *in vivo* ischemia converts the enzyme from the dehydrogenase form to the $O_2^{\cdot-}$ producing oxidase form (132, 134). Aldehyde oxidase, which is unique but structurally similar to xanthine oxidase, shares many of the same substrates and also generates $O_2^{\cdot-}$ (132). Dihydroorotate dehydrogenase (1), flavoprotein dehydrogenase (96), and tryptophan dioxygenase (69) also utilize $O_2^{\cdot-}$ during their catalytic cycle, deduced either from the observation that superoxide dismutase will inhibit enzyme activity or from electron spin resonance measurement of free radical intermediates during enzyme catalysis.

Studies of these enzyme sources of cellular free radical production have shown that modulation of enzyme activities, cofactor availability, substrate concentration, and oxygen tension can combine to affect rates of intracellular radical production. Thus, certain cellular metabolic states such as hyperoxia, ischemia, or antibiotic therapy can favor free radical production in excess of basal rates.

TABLE 1. COMMON FREE RADICAL SPECIES OR PRECURSORS ENCOUNTERED BY CELLS[a]

Species	Sources
$O_2^{\cdot-}$, H_2O_2, $OH\cdot$, $^1\Delta gO_2$	Oxygen metabolism—potentiated by hyperoxia, inflammation, radiation
NO_2, O_3, peroxyacylnitrates	Photochemical air pollution
Lipid peroxides	By-products of free radical propagation or prostanoid metabolism
Hypochlorite radicals	Inflammation
Semiquinones	Mitochondrial electron transport
Aromatic hydrocarbons	Environmental
Divalent metals	Heme and other metal-containing proteins, free and complexed metals

[a] A wide variety of substrates, metabolic intermediates, and environmental agents either exist as free radicals or can be converted to free radical species by cellular metabolic processes.

C. Mitochondrial Electron Transport

The mitochondrial production of H_2O_2 was first reported by Jensen (75) in 1966. Further studies have shown that most, if not all, mitochondrial H_2O_2 is derived from dismutation of $O_2^{\cdot-}$ (16, 36). Reduction of O_2 to H_2O by mitochondrial cytochrome *c* oxidase involves a four-electron transfer with no free radical intermediates, so this cytochrome is not a source of mitochondrial $O_2^{\cdot-}$ production (27). Superoxide radical generation by mitochondria is greatest when respiratory chain carriers located on the inner mitochondrial membrane are highly reduced (158). Thus, endogenous factors that influence mitochondrial radical production are those that regulate respiration. This includes the availability of NAD-linked substrates, succinate, ADP (which serves as a phosphate acceptor and relieves an ion gradient-established increase in respiratory chain reduction, (28)) and oxygen. If oxygen is present in concentrations that limit its reduction to H_2O by cytochrome oxidase (1 to 3 mm. Hg, (76)), increased respiratory chain reduction and an accumulation of reduced cofactors in cells may enhance $O_2^{\cdot-}$ production by electron transport components in ischemic cells.

Isolated mitochondria and submitochondrial particles prepared by sonication or alkaline treatment of isolated

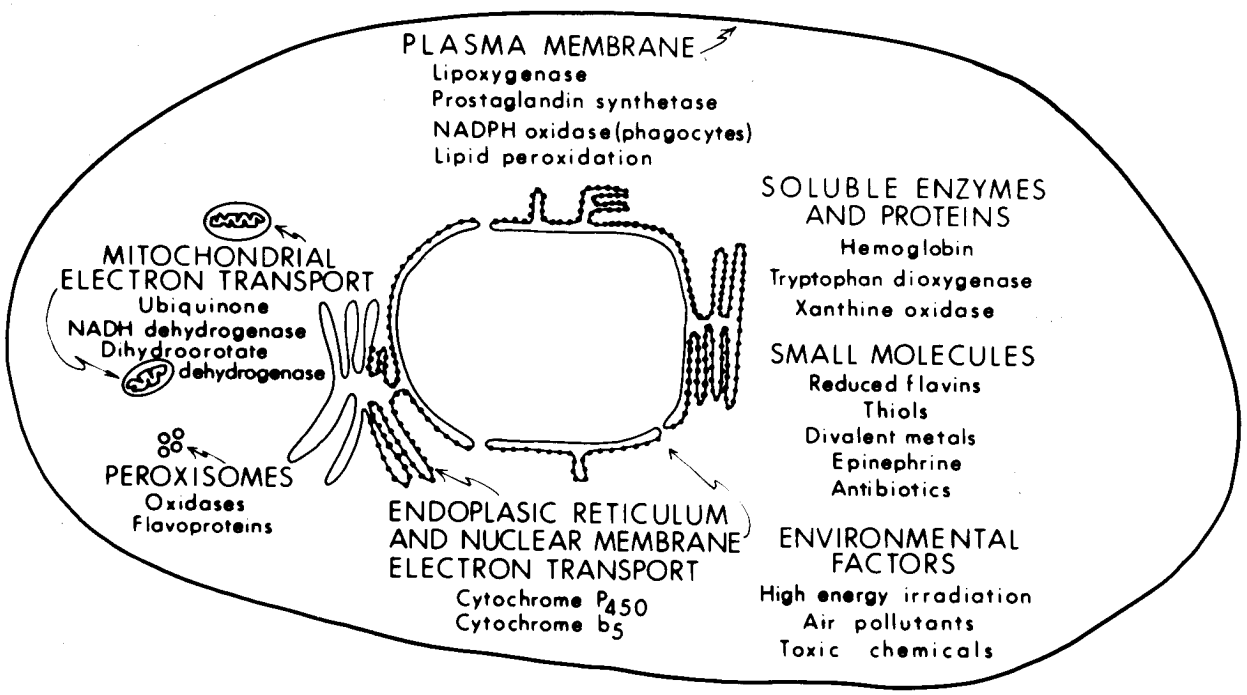

FIG. 1. Cellular sources of free radicals. Subcellular organelles, structural components, and cytoplasmic contents all contribute to generation of a wide variety of free radical species.

mitochondria have been used to measure sites and rates of respiratory chain $O_2^{\cdot-}$ and H_2O_2 production (Fig. 2). Intact mitochondria can be used to predict the rate of extramitochondrial H_2O_2 release into the cytosol (17, 157). Rates of intramitochondrial $O_2^{\cdot-}$ and H_2O_2 generation can only be estimated, using inner mitochondrial membrane fragments (submitochondrial particles) washed free of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. These enzymes, which metabolize $O_2^{\cdot-}$ and H_2O_2, have rate constants orders of magnitude greater than the probes used to quantify $O_2^{\cdot-}$ and H_2O_2. This necessitates removal or inhibition of these enzymes in biologic preparations in which $O_2^{\cdot-}$ and H_2O_2 are to be measured. Site-specific respiratory chain inhibitors, such as antimycin A, cyanide, and rotenone which were used in elucidating the steps of mitochondrial electron transport (25) also assisted in identification of $O_2^{\cdot-}$ producing respiratory chain components.

Studies of mitochondria isolated from bovine and rat heart (36, 156), isolated tumor cells (36), porcine lung, and rat lung (157, 158) all showed that the ubiquinone-cytochrome *b* region is the major site of $O_2^{\cdot-}$ production. The data suggested that $O_2^{\cdot-}$ generation in this region is due to autoxidation of ubisemiquinone. NADH dehydrogenase and dihydroorotate dehydrogenase are also autoxidizable electron carriers responsible for a portion of mitochondrial $O_2^{\cdot-}$ production (45, 156). There are species-to-species and organ-to-organ differences in mitochondrial respiratory chain component concentrations, which can contribute to differences in major sites and specific activities of $O_2^{\cdot-}$ and H_2O_2 production by mitochondria isolated from different sources (157). Recent reviews have extensively discussed mitochondrial $O_2^{\cdot-}$ and H_2O_2 generation (15, 27, 43).

Intact mitochondria can release H_2O_2 into the cytoplasm (17, 157), but it is controversial whether $O_2^{\cdot-}$ can escape intramitochondrial superoxide dismutase and wreak havoc in the cytosol (123, 157). Superoxide and H_2O_2 production normally accounts for 1 to 2 per cent of mitochondrial oxygen consumption under reduced conditions. The intramitochondrial concentration of $O_2^{\cdot-}$ has been estimated to be 8×10^{-12} M (159), and even though this calculation was based on a series of assumptions, it shows that mitochondrial superoxide dismutase maintains intramitochondrial $O_2^{\cdot-}$ at very low steady state concentrations. Thus, very little if any $O_2^{\cdot-}$ will enter the cytoplasm from mitochondria.

Hydroxyl radical production by mitochondria has also been reported (124). Formation of OH· and H_2O_2 accounts for many of the effects of $O_2^{\cdot-}$ generating systems already (and to be) discussed, since hydroxyl radical scavengers, in addition to superoxide dismutase and catalase, can protect free radical targets in many *in vitro* and *in vivo* test systems. The generation of the potent oxidant OH· is currently the object of intensive study (19, 102, 168) and seems to require not only $O_2^{\cdot-}$ or H_2O_2, but a transition metal such as iron. These substances can react by what has been termed an iron-catalyzed Haber-Weiss reaction:

$$Fe(III) + O_2^{\cdot-} \rightarrow Fe(II) + O_2 \quad (2)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH\cdot \quad (3)$$

Thus, $O_2^{\cdot-}$ reduces iron, which in turn reduces H_2O_2 to form OH·. Reductants such as ascorbate can also reduce Fe(III), implying that a source of peroxides in the presence of transition metals can generate OH· in the absence of $O_2^{\cdot-}$ (169). Measurement of hydroxyl radical generation by mitochondria shows that enough $O_2^{\cdot-}$ or H_2O_2 goes unscavenged to undergo further reactions yielding OH·.

D. Endoplasmic Reticulum and Nuclear Membrane Electron Transport Systems

Intramitochondrially derived free radicals must escape organelle antioxidant defenses to initiate cytosol damage. Free radicals produced by the endoplasmic reticulum and nuclear membrane can undergo both intraorganelle and cytosolic reactions. In the case of nuclear membrane-derived radicals, DNA would be particularly susceptible to free radical damage (See section 3.A.2).

According to experimeental observations summarized by Siekevitz *et al.* (147) in describing the concept of membrane spatial continuity, the endoplasmic reticulum and nuclear membrane share many of the same elements (Fig. 3). Both of these intracellular membranes contain the cytochromes P_{450} and b_5 that can oxidize unsaturated

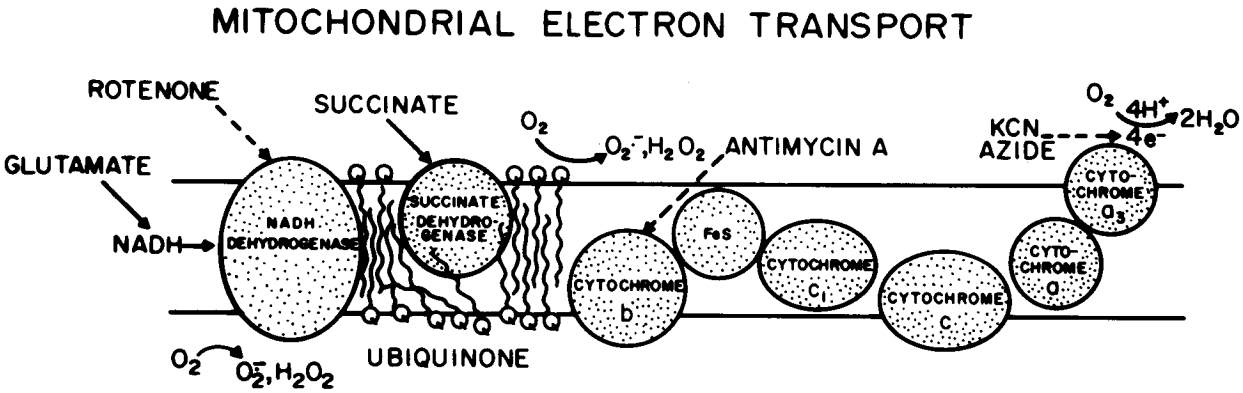

FIG. 2. Mitochondrial free radical generation. Major source of mitochondrial free radicals is electron transport chain located on inner mitochondrial membrane. Mitochondrial sources of $O_2^{\cdot-}$ have been studied using various electron transport inhibitors (rotenone, antimycin A, KCN, and azide, *dashed arrows*) and substrates (NADH-linked substrates and succinate, *solid arrows*). NADH dehydrogenase and ubiquinone-cytochrome b region have been shown to reduce oxygen to $O_2^{\cdot-}$, which in turn serves as precursor for H_2O_2 and OH·.

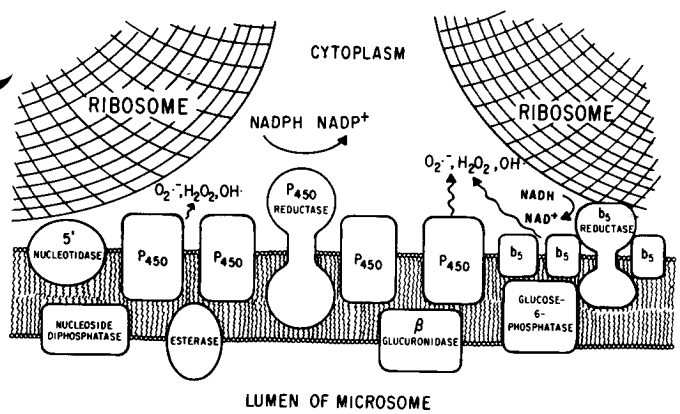

FIG. 3. Free radical generation in endoplasmic reticulum. Membrane-bound cytochromes (P_{450} and b_5) located in endoplasmic reticulum generate oxygen free radicals by autoxidation. Dissociation of cytochrome P_{450} complexes can form H_2O_2 and other peroxy intermediates. Wide spectrum of xenobiotics can be activated by P_{450} to produce free radical species (not illustrated).

fatty acids (23) and xenobiotics (29) and reduce dioxygen (6), among other substrates (Fig. 3). These mixed function oxidases convert nonpolar compounds to hydroxylated derivatives by electron transfer reactions. The more polar products are the better excreted by the kidney as glucuronides or other water soluble conjugated products. NADH or NADPH are required cofactors for these reactions. Flavoprotein-containing cytochrome reductases which provide the electrons for the cytochrome P_{450}- and b_5-mediated reactions are also capable of autoxidizing to produce $O_2^{\cdot-}$ and H_2O_2 (3). Microsomal and nuclear membrance cytochromes can directly form $O_2^{\cdot-}$ by one-electron transfer or will form H_2O_2 by dissociation of peroxy-cytochrome complexes (41).

Cytochromes P_{450} and b_5, which are critical for cellular demethylation, hydroxylation, and desaturation reactions, show the greatest activity in the presence of NADPH (cytochrome P_{450}) and NADH (cytochrome b_5), respectively, but are not absolutely specific for these cofactors and their associated reductases (125). Substrates for microsomal cytochrome P_{450} oxidative reactions have been shown to either stimulate (160) or inhibit (68) H_2O_2 formation. It is not well understood why some substrates "uncouple" cytochrome P_{450} and divert electron flow to dioxygen, thereby generating free radical byproducts, whereas other substrates are tightly coupled to cytochrome P_{450} electron transfer and do not increase free radical production.

Inhibition of microsomal cytochrome P_{450} by reaction of heme with metyrapone decreases H_2O_2 production only 25 to 40 per cent in the presence of NADPH (137), suggesting that microsomal components other than those related to cytochrome P_{450} are significant sources of H_2O_2. Thus, cytochrome b_5, which is present in rat liver microsomes in about 50 per cent of the concentration of cytochrome P_{450}, is probably another significant source of microsomal $O_2^{\cdot-}$ and H_2O_2 (11). Other sources of microsomal oxygen radical production include flavin-containing oxidases (97, 139).

Hydroxyl radicals are generated by rat liver microsomes, both in the absence (30) and presence (66) of cytochrome P_{450} substrates, and require the presence of NAD(P)H. Addition of azide increased OH· production by these systems. Azide inhibits catalase, thereby preventing H_2O_2 metabolism. This suggests that the microsomal OH· was derived from an iron-catalyzed Haber-Weiss reaction where H_2O_2 could have served as OH· precursor (Equations 1 to 3).

It is difficult to evaluate the relative contribution of endoplasmic reticulum and nuclear membrane-derived $O_2^{\cdot-}$ and H_2O_2 to the whole cell production of these species for several reasons. There are different forms of cytochrome P_{450}, each of which have distinct immunochemical and biochemical characteristics (144). These different forms of cytochrome P_{450} are present in microsomes prepared from a single organ, such as the lung or liver, and can also exhibit organ differences in substrate specificity and physical characteristics (88, 144). In complex organs, some cell types have a greater content of cytochromes P_{450} and b_5 than others, for example, we cite the very high cytochrome P_{450} content of Clara cells which are found in small airways of the lung (33). Thus, specific cell types may be either major or minor contributors to whole organ free radical production. There are also sex differences and circadian variations in microsomal drug-metabolizing activities in a variety of organs examined from rats and rabbits (154). Most *in vivo* measurements of mixed function oxidase activity do not employ cofactor concentrations approximating those found *in vivo*. This can lead to inaccurate estimation of *in vivo* rates of free radical production. Isolation of microsomes from cellular endoplasmic reticulum, which requires tissue homogenization, can also cause rearrangement of autoxidizable components. Microsomal electron flow diverted to O_2 to form $O_2^{\cdot-}$ and H_2O_2 *in vitro* may be utilized for tightly coupled oxidations *in situ*, thereby presenting less of a free radical threat to the cell than what would have been predicted from *in vitro* observations. Thus, a wide variability exists in the cellular content and *in vivo* free radical production potential of these autoxidizable hemoproteins. Similar arguments can also hold true for autoxidizable respiratory components of mitochondria, especially when inhibitors of the respiratory chain are used to study sites of $O_2^{\cdot-}$ production. Respiratory chain inhibition causes further reduction of respiratory chain components and increases the likelihood of respiratory chain autoxidation. This can lead to artifactually high estimates of *in vivo* rates of mitochondrial oxygen radical production.

E. Peroxisomes

Peroxisomes are potent sources of cellular H_2O_2 because of high concentrations of oxidases, none of which have been shown to generate $O_2^{\cdot-}$ as an immediate precursor of H_2O_2 (98). Some peroxisomal H_2O_2-generating enzymes include D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, and fatty acyl-CoA oxidase. Peroxisomal catalase is the enzyme that normally metabolizes most of the H_2O_2 generated by peroxisomal oxidases. The proportion of peroxisomal H_2O_2 that can diffuse out of peroxisomes into the cytoplasm ranges from a calculated 2 per cent (129) to a measured 11 to 42 per cent,

depending on the substrates used for determinations (18). Chance, Sies, and Boveris (27) proposed that the discrepancy between these calculated and observed rates of extraperoxisomal H_2O_2 diffusion may be due to increased peroxisomal membrane permeability after isolation for *in vitro* measurements. Peroxisomal metabolism of H_2O_2 infused into intact hepatocyte suspensions readily occurs (78), showing the ability of H_2O_2 to diffuse across at least two barrier membranes and through cytoplasm.

F. Plasma Membranes

The plasma membrane is a critical site of free radical reactions for several reasons. Extracellularly generated free radicals must cross the plasma membrane before reacting with other cell components and may initiate toxic reactions at the membrane. The unsaturated fatty acids present in membranes (phospholipids, glycolipids, glycerides, and sterols) and the transmembrane proteins containing oxidizable amino acids are susceptible to free radical damage. Also, increased membrane permeability caused by lipid peroxidation or oxidation of structurally important proteins can cause a breakdown of transmembrane ion gradients, loss of secretory functions, and inhibition of integrated cellular metabolic processes (Fig. 4).

Hydrogen peroxide can cross membranes almost as readily as can water. The charged $O_2^{\cdot-}$ molecule can cross membranes and enter cells via transmembrane anion channels (82). Also, the polyanionic cell surface attracts a high counterion concentration, much of which is solvent H^+, giving a microenvironment that has been estimated to be two to three pH units lower than surrounding tissue fluids. This protic environment will favor the formation of the protonated form of $O_2^{\cdot-}$, the perhydroxyl radical:

$$H^+ + O_2^{\cdot-} \rightarrow HO_2^{\cdot} \quad (4)$$

$HO_2^{\cdot}$ is a stronger oxidant than $O_2^{\cdot-}$ and would be expected to better partition into lipid and the hydrophobic core of proteins and exert toxic effects. Thus, cell surfaces are capable of serving as both targets of reactive free radicals and as a gating mechanism that provides a barrier to charged species and can modify other radical species to a more permeable and reactive form.

The phagocytic cell plasma membrane NADPH oxidase-mediated production of free radicals is an important biologic source of free radicals recently reviewed in this series (167). Phagocyte-derived free radicals can damage both the source cell and cells in close apposition to stimulated phagocytes.

Free radical production by microsomal and plasma membrane-associated enzymes such as lipoxygenase and cyclooxygenase is of current interest because of many recent discoveries regarding arachidonic acid metabolism, the predominant substrate for these enzymes which is converted into biologically potent products. These products include prostaglandins, thromboxanes, leukotrienes, and slow-reacting substances of anaphylaxis (Fig. 5). How these enzymatic processes that are involved in arachidonate metabolism can lead to autocatalytic lipid peroxidation is currently an area of active investigation.

The enzymatic oxidation of arachidonic acid by membrane-bound cyclooxygenase involves free radical intermediates, one of which has been shown by electron spin resonance to be a carbon-centered free radical. This radical was produced by cyclooxygenase-mediated abstraction of one of the methylene hydrogens of arachidonic acid, producing a fatty acid free radical species

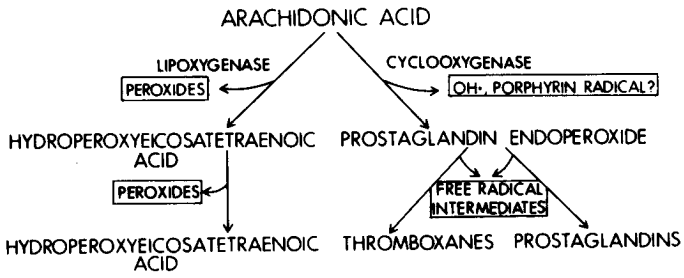

FIG. 5. Free radical by-products of arachidonic acid metabolism. During conversion of arachidonic acid to bioactive products, wide spectrum of oxygen-, carbon-, and hemoprotein-free radical intermediates are produced that can lead to tissue injury.

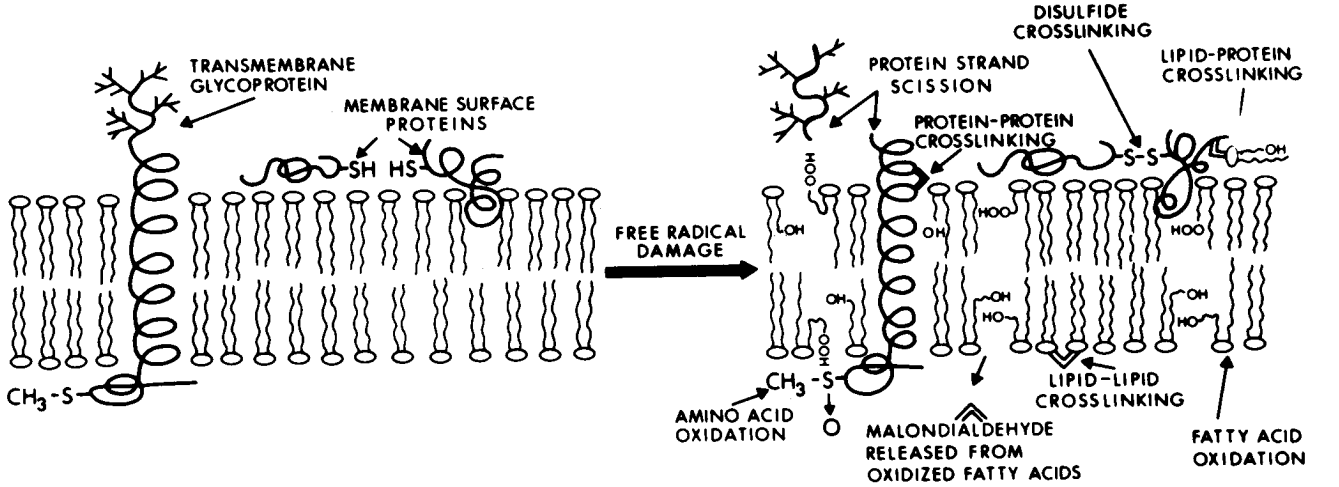

FIG. 4. Free radical damage to membranes. Free radicals can affect lipids by initiating peroxidation, which leads to short chain fatty acyl derivatives and the by-product malondialdehyde. Variety of cross-linking reactions can be mediated by malondialdehyde reactions. Free radicals can also catalyze amino acid oxidation, protein-protein cross-linking, and protein strand scission.

(95). Also, during cyclooxygenase-catalyzed arachidonic acid metabolism, an oxygen-centered radical which can be scavenged by methional or phenol is produced during the breakdown of the hydroperoxide on PGG_2 (40). This radical has been proposed to be OH· (40), possibly hemoprotein derived (121). The nature of this free radical is controversial and has recently been proposed to be a cyclooxygenase hemoprotein radical, distinct from oxygen-centered free radicals such as OH· (79). In any event, the formation of a free radical intermediate could account for the irreversible oxidative self-deactivation of cyclooxygenase during catalysis which can be prevented by free radical scavengers (121). The production of hydroxyl radical or another radical species during prostaglandin synthesis could lead to feedback regulation of cyclooxygenase, modulate both the rate and extent of prostaglandin biosynthesis, and participate in secondary messenger and cytotoxic effects after prostaglandin synthesis. Cyclooxygenase is also capable of metabolizing xenobiotics to more toxic species. Cyclooxygenase can convert sulfite and bisulfite to the sulfur trioxide radical anion, which in turn can react with oxygen to yield the sulfur pentoxide radical anion (115). Sulfite and bisulfite are hydrated and ionized species of sulfur dioxide (SO_2), which is found in photochemical air pollution and used as a food preservative and bleaching agent.

Thromboxane synthesis in platelets is inhibited by the radical scavengers imidazole and nordihydroguiaretic acid, suggesting that a free radical reaction is involved in the conversion of prostaglandin endoperoxide (PGH_2) to thromboxanes (141). Peroxides generated by lipoxygenase are also capable of modulating oxidant-sensitive cyclooxygenase activity (67). Thus, it appears that the biosynthesis of prostaglandins and thromboxanes results in hemoprotein-, oxygen-, and carbon-centered free radicals capable of reacting with the biosynthetic enzymes themselves and other cell components. All of these effects are in addition to the demonstrated chemotactic, vasoactive, and platelet-aggregating effects of prostaglandins.

It is important to note that, because of the proximity of arachidonic acid-metabolizing enzymes and other plasma membrane free radical sources to cell and organelle surfaces, metabolic byproducts can affect cytosolic, membrane, and extracellular components, depending on product solubility and by-product diffusion distances. The reactivity of by-products has a major influence on diffusion distances. For example, OH· has a high and indiscriminate reactivity, so this free radical is not likely to diffuse away from cellular sites of production—it will react nearby. Less reactive free radicals may be capable of reacting distally from sites of generation. Superoxide, much less reactive with cell components than OH·, could potentially diffuse further away from sites of generation, were it not for the high concentration of superoxide dismutase in cells (estimated to be 3×10^{-5} M in liver cells (52)), which maintains $O_2^{\cdot-}$ at 10^{-11} to 10^{-12} M (159). Unless $O_2^{\cdot-}$ becomes protonated, this charged molecule will not diffuse through nonpolar microenvironments in a cell. Hydrogen peroxide, much less reactive than OH· and $O_2^{\cdot-}$, is maintained under normal conditions at concentrations of 10^{-7} to 10^{-9} M by intracellular catalase and peroxidases (126). Hydrogen peroxide has been shown to diffuse across mitochondrial membranes (157), peroxisomal membranes (27, 78), and across the plasma membrane (143, 163), thus potentially exerting toxic effects at a distance from its site of generation.

3. Reactions of Free Radicals

It is difficult to generalize about the biologic reactions of free radicals because of the diversity of radical species an organism must face during life processes (Table 1). Free radicals are typically molecules that contain one or more unpaired electrons. The formation of a free radical is termed initiation, one of a series of reactions in which free radicals may participate. As discussed earlier, electron transfer from transition metals to oxygen species is an important example of cellular free radical initiation. Free radicals can then undergo atom transfers as a part of the propagation sequence of radical reactions. Most commonly, a radical will abstract univalent atoms, such as hydrogen atoms or halides:

$$R\cdot + XH \rightarrow RH + X\cdot \quad (5)$$

$$R\cdot + CCl_4 \rightarrow RCl + Cl_3C\cdot \quad (6)$$

Another important radical reaction in cells involves radical addition to unsaturated bonds, such as those present in fatty acids and aromatic rings (Table 2). Free radical reactions can proceed further via free radical intermediates, termed propagation reactions, wherein cellular damage can occur. These propagation reactions can continue indefinitely or can be terminated by a variety of free radical scavenging species, some of which are essential to cellular integrity and if depleted can lead to cytotoxicity. Other scavengers of free radicals fall under the general category of antioxidant defenses, which evolved to assist survival of organisms subjected to free radicals.

Table 2. Cellular Free Radical Targets[a]

Target	Consequence
"Small" molecules	
Unsaturated and thiol-containing amino acids	Protein denaturation and cross-linking, enzyme inhibition Organelle and cell permeability changes
Nucleic acid bases	Cell cycle changes, mutations
Carbohydrates	Cell surface receptor changes
Unsaturated lipids	Cholesterol and fatty acid oxidation Lipid cross-linking Organelle and cell permeability changes
Cofactors	Decreased nicotinamide and flavin-containing cofactor availability and activity, ascorbate oxidation porphyrin oxidation
Neurotransmitters	Decreased neurotransmitter availability and activity, including serotonin, epinephrine
Antioxidants	Decreased availability, includes α-tocopherol and β-carotene
Macromolecules	
Protein	Peptide chain scission, denaturation
DNA	Strand scission, base modification
Hyaluronic acid	Change in synovial fluid viscosity

[a] Virtually all cell components are capable of reacting with free radicals. Chemical modification of these molecules leads to metabolic and structural modifications of cells which can ultimately cause cell death.

A. Cellular Components at Risk from Free Radical Damage

1. Proteins. Because of the reactivity of unsaturated and sulfur-containing molecules with free radicals (130), proteins containing the amino acids tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine can undergo free radical-mediated amino acid modification. Enzymes such as papain (21) and glyceraldehyde-3-phosphate dehydrogenase (87), which depend on these amino acids for reactivity, will be inhibited by exposure to free radicals or radical-generating agents. Cytoplasmic and membrane proteins can also be cross-linked into dimers or larger aggregates after exposure to a number of oxidizing agents, including ozone (49) and protoporphyrin IX (56). These cross-links may be mediated either by interprotein disulfide formation or by more irreversible reactions between free radical-damaged amino acid residues.

Due to the extreme reactivity of some free radicals such as OH·, protein constituents normally modification-resistant, such as peptide bonds or amino acids such as proline and lysine, may be affected by reduced oxygen derivatives. Proline and lysine hydroxylation can also occur nonenzymatically when these amino acids are exposed to reaction conditions that generate $O_2^{\cdot-}$, H_2O_2, and OH· (155).

Reaction of free radicals with proteins may also generate by-products that would amplify the damage of the initial reaction. For example, oxidation of tryptophan will produce *N*-formyl kyneurenine and H_2O_2 as products (112). *N*-formyl kyneurenine can combine by a Schiff's base reaction with amino-containing compounds to form cross-linked species between lipids and/or proteins. Hydrogen peroxide can react directly with cell components or initiate further radical production according to Equation 3. Another way radical damage can be potentiated is by abstraction of an electron from molecules such as thiols, which can then form an intermediate (X·) capable of attacking other molecules, as shown in Equation 5.

The susceptibility of proteins to free radical damage depends on their amino acid composition, the importance and location of susceptible amino acids that mediate protein conformation and activity, and whether the damaged protein can be repaired (*i.e.*, reduction of radical-induced disulfide or methionine sulfoxide formation (20)). The cellular location of proteins and the nature of the threatening free radical also influence the extent of protein damage.

2. Nucleic acids and DNA. Deposition of energy in cells by radiation, which can include ultraviolet and visible light, heat, γ, and x-ray irradiation creates ions, free radicals, and excited molecules. Ionizing radiation produces radicals and electrons as primary species that decay to produce charged and neutral free radicals (138). Cell mutation and death from ionizing radiation is primarily due to free radical reactions with DNA (117, 164) (Fig. 6). Moreover, OH· has been implicated as the agent responsible for greater than 80 per cent of the radiation-induced cell killing in both prokaryotic and eukaryotic cells. Cytotoxicity in large part is a consequence of chromosomal aberrations arising from either nucleic acid base modifications or DNA strand scission (165). Cell death and mutations arising from free radicals generated during

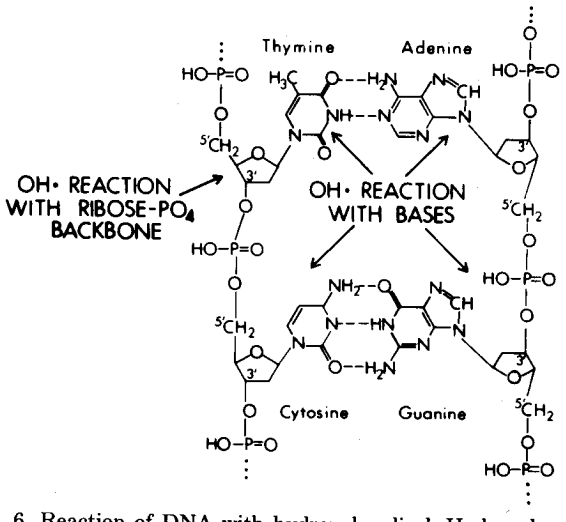

FIG. 6. Reaction of DNA with hydroxyl radical. Hydroxyl radical can react with and modify DNA bases. DNA strand scission also results from OH· reaction with ribose-phosphate backbone.

normal metabolism, hyperoxia, and environmental sources such as photochemical air pollutants have also been ascribed to reactions with DNA (114, 173).

Studies in dilute aqueous solutions have shown that OH· reacts readily with and modifies deoxyribose and base moieties (84). However, in double-stranded DNA *in situ*, bases sheltered in the double helix may be more sterically protected from reaction with OH·. Strand scission, which results from radical reaction with the sugar-phosphate backbone, occurs in DNA preparations exposed to γ radiation (136), potassium superoxide, H_2O_2 (85), phenanthroline-copper complex (131), and an enzymic source of oxygen radicals (xanthine + xanthine oxidase (19)). In all cases, OH· scavengers inhibited DNA strand scission. When sources of $O_2^{\cdot-}$ and H_2O_2 were examined in the previous models, superoxide dismutase, catalase, and metal-chelating agents prevented strand scission in addition to OH· scavengers. This reaffirms that $O_2^{\cdot-}$ and H_2O_2 can interact by a metal-catalyzed process to generate OH· (Equations 2 and 3) which induces strand scission. Enzymatic scavenging of $O_2^{\cdot-}$ and H_2O_2 protects DNA by decreasing the concentration of OH· precursors.

3. Membrane lipids. The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals and undergo peroxidation. This process can become autocatalytic after initiation and will yield lipid peroxide, lipid alcohol, and aldehydic by-products (Fig. 7). Lipid epoxides have also been measured in animals exposed to oxidants and are proposed to be of peroxidatic origin (111). Lipid peroxidation has recently been extensively reviewed (22, 37, 47, 162), so we will only summarize hallmarks of cell damage that can be induced by lipid peroxidation.

Lipid peroxidation can be indicated by a variety of means. Increased absorbance of lipid extracts at 233 nm. indicates conjugated diene formation, a consequence of hydrogen abstraction and bond migration in unsaturated fatty acids (Fig. 7). Loss of cell membrane unsaturated fatty acids, formation of lipid peroxides, and oxygen uptake by lipid preparations all indicate peroxidation.

Peroxidation of fatty acids containing three or more double bonds will produce malondialdehyde. The presence of this oxidation by-product can be measured with thiobarbituric acid which, although not a specific or quantitative indicator of fatty acid oxidation, correlates with the extent of lipid peroxidation. The thiobarbituric acid reaction, critically discussed by Donato (37), needs to be cautiously interpreted because acidic reaction conditions cause thiobarbituric acid to react with sugars and lipid peroxides, in addition to malondialdehyde. To further complicate matters, malondialdehyde is a volatile product that is metabolized *in vivo* and will react with other cell lipids and proteins.

The reaction of malondialdehyde with primary amines also yields fluorescent conjugated Schiff's base products that can be detected at 470 nm. following 365 nm. excitation (35). Age pigments, termed lipofuscin, probably result from lysosomal accumulation of insoluble conjugated Schiff's bases formed from reaction of malondialdehyde with lipid and protein during lipid peroxidation. Another useful estimation of the extent of lipid peroxidation involves measurement of ethane and pentane (34). These volatile hydrocarbons are metabolic by-products of cellular hydroperoxide metabolism and can be detected by sensitive and noninvasive gas chromatography.

Plasma membrane and organelle lipid peroxidation can be stimulated by all of the previously mentioned sources of free radicals and is potentiated by the presence of metals. These metals can serve as redox catalysts and also catalyze the conversion of $O_2^{\cdot-}$ and H_2O_2 to more potent oxidants (70, 152). Lipid peroxides and lipid peroxy radicals can exert their toxicity by reacting with many of the same cellular components as O_2-derived free radicals. Because of the hydrophobic nature of the lipid radicals, most of the reactions will take place with membrane-associated molecules. After peroxidation of membrane fatty acids, the presence of shortened-chain fatty acids containing R-OOH, R-COOH, R-CHO, and R-OH groups may seriously affect membrane permeability and microviscosity (72). Oxidized phospholipid fatty acids are readily cleaved by phospholipase A_2 (145), presumably as a repair process or a step in prostaglandin synthesis.

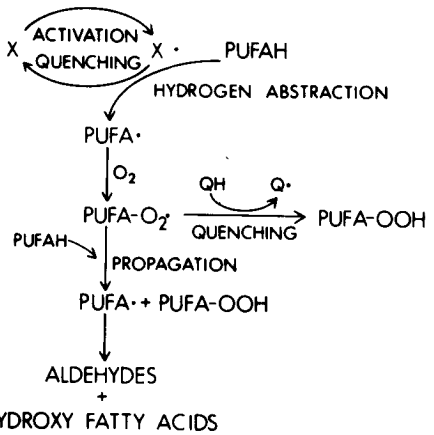

FIG. 7. Scheme of lipid peroxidation. Polyunsaturated fatty acids (PUFAH) undergo hydrogen abstraction (PUFA·) and reaction with molecular oxygen to form peroxy acyl intermediates (PUFA-O_2). Lipid peroxidation can be terminated by scavenging reactions or continue by autocatalytic propagation reactions. Final products of lipid peroxidation can include short chain aldehyde and hydroxy fatty acid derivatives.

Malondialdehyde produced by peroxidation can cause cross-linking and polymerization of membrane components (71, 118). This can alter intrinsic membrane properties such as deformability, ion transport, enzyme activity, and the aggregation state of cell surface determinants (Fig. 4). Because malondialdehyde is diffusible, it will also react with nitrogenous bases of DNA (37). All of these effects may explain why malondialdehyde is mutagenic (116), genotoxic to cultured cells (14), and carcinogenic (146).

4. Cytosolic Molecules. Many soluble cell components act as free radical scavengers and are considered to be expendible. These molecules will be discussed in section 4.A.1 and 2. Cytosolic proteins can undergo modification by cytoplasmic free radicals as discussed in section 3.A.1. In the case of hemoproteins, such as oxyhemoglobin, either $O_2^{\cdot-}$ or H_2O_2 can react with the iron substituent to form methemoglobin (166):

$$O_2^{\cdot-} + Hb - Fe^{2+} - O_2 \xrightarrow{2H^+} Hb - Fe^{3+} + H_2O_2 + O_2 \quad (7)$$

$$H_2O_2 + 2Hb - Fe^{2+} - O_2 \xrightarrow{2H^+} 2Hb - Fe^{3+} + 2H_2O + O_2 \quad (8)$$

This suggests that a wide spectrum of hemoproteins can be damaged by oxygen-derived free radicals. Another important cytoplasmic hemoprotein, catalase, is inhibited by $O_2^{\cdot-}$ (83). Superoxide served to convert catalase to the ferroxy (compound III) and ferryl states (compound II), which are inactive forms of the enzyme.

Hydrogen peroxide, the dismutation product of superoxide, can inhibit CuZn superoxide dismutase by reducing enzyme-bound Cu^{2+} to Cu^{+1} and then reacting with Cu^{+1} to give a potent oxidant, probably OH·. The OH· attacks an adjacent active site histidine residue necessary for catalytic activity (73). This particular example of free radical destruction of proteins is a two-step process, in which a protein-reactive species was generated by metalloprotein "activation" of H_2O_2. The aforementioned examples of hemoglobin and catalase damage involve a direct reaction of oxygen species with the metal ligand.

5. Extracellular effects. The anti-inflammatory nature of superoxide dismutase (74) was recognized before the enzymatic activity of this protein was described as a superoxide dismutase (103). Free radicals play an important role in modulating the extent of an inflammatory response and consequent tissue damage. Extracellular tissue components which are especially at risk from inflammatory cell-mediated free radical damage include collagen and hyaluronic acid, which have been shown to be affected in inflammatory osteoarthritis (59). Collagen, a major constituent of cartilage, can be damaged by $O_2^{\cdot-}$ thereby preventing gelation (61). Collagen gelation involves the interaction of single collagen peptide chains by hydrogen bonding to form triple peptide chain helices. Superoxide dismutase will protect soluble collagen from $O_2^{\cdot-}$ mediated inhibition of gelation. Hyaluronic acid, necessary for maintaining joint synovial fluid viscosity, can be depolymerized by $O_2^{\cdot-}$ (60). Scavengers of $O_2^{\cdot-}$, H_2O_2, and OH· will prevent hyaluronic acid depolymerization by a $O_2^{\cdot-}$ generating system (100). Since extracellular fluids have very low superoxide dismutase and

catalase activities, small amounts of reduced oxygen species can cause extensive damage in this compartment.

Superoxide and other reduced oxygen species produced by activated inflammatory cells can react with a plasma component to generate chemotactic factor(s) that cause further inflammatory cell infiltration. Generation of this plasma factor by leukocytes, which cochromatographs with serum albumin on gel filtration chromatography, can be inhibited by superoxide dismutase (107). The factor may be an oxidized fatty acid bound to albumin, since defatted serum albumin did not become chemotactic when exposed to $O_2^{\cdot-}$. This free radical-generated plasma chemotactic factor may also explain why superoxide dismutase can blunt the extent of an inflammatory reaction. Examples of the anti-inflammatory effects of superoxide dismutase include inhibition of the reverse passive Arthus reaction, inhibition of carrageenan-induced foot edema (106), and decreased activated leukocyte-dependent lung capillary endothelial cell damage and pulmonary edema (108, 140).

4. Cellular Defenses against Free Radical Damage

The large increase in atmospheric oxygen concentration that paralleled the evolution of photosynthetic organisms 2 to 3 billion years ago (12) permitted the concomitant evolution of organisms dependent on aerobic energy metabolism. Since this time, the survival of both prokaryotic and eukaryotic life forms in an oxygen-containing atmosphere has depended upon the elaboration of a system of biochemical defenses that protect organisms from the free radical damage which is possible in an oxidizing environment. These biochemical defenses include both low molecular weight free radical scavengers and complex enzyme systems (Fig. 8). These defenses serve to lower the steady state concentrations of free radical species, which might otherwise cause excessive damage to cell components. Proofs that these defense mechanisms are critical for cell survival in aerobic environments have recently been presented (27, 44, 53, 62). Free radical scavengers have also been used to characterize the production, nature, and toxicity of free radical species in *in vitro* and *in vivo* systems. The utility and inherent problems of this approach for studying free radical metabolism will be discussed in section 4.D.

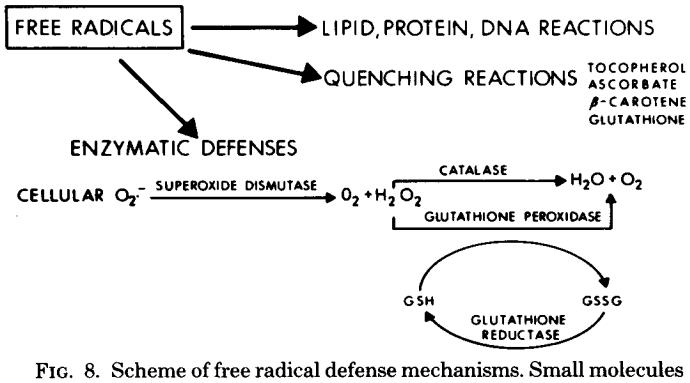

FIG. 8. Scheme of free radical defense mechanisms. Small molecules and enzyme systems have evolved to maintain low steady state concentrations of intracellular free radicals. Free radicals can undergo three major reactions in a cell. Reactions with lipid, protein, or DNA may lead to cytotoxicity. Free radicals may be quenched by reactions with small molecules located in cytoplasm or membranes. Finally, a series of enzymes have evolved that scavenge superoxide, hydrogen peroxide, and lipid peroxides.

A. Low Molecular Weight Free Radical Scavengers

1. Lipid soluble. A variety of molecules that preferentially partition into membranes function by reducing lipophilic free radical species to a less toxic form. Vitamin E (a series of isomers of tocopherol) will reduce $O_2^{\cdot-}$, OH·, singlet oxygen, lipid peroxy radicals, and other radical species (120, 127). Ascorbate is proposed to have similar properties and may serve to maintain tocopherols in the reduced active form (153). Ascorbate serves as a water-soluble reductant and radical scavenger. β-Carotene is an efficient singlet oxygen scavenger and inhibits lipid peroxidation (81).

Any molecule that reacts with a free radical can be termed "scavenger"; thus, cell components such as sugars, unsaturated amino acids, sulfur-containing amino acids, and unsaturated fatty acids can also scavenge free radicals. Products of these reactions may or may not be less toxic to cells than the original free radical.

2. Cytoplasmic. The tripeptide glutathione (GSH), in concert with its reductant NADPH and enzymatic catalysts, can reduce H_2O_2, lipid peroxides, disulfides, ascorbate, and free radicals (Fig. 8). A class of enzymes, termed GSH peroxidases, catalyze peroxide reduction. These enzymes are differentiated from heme-containing peroxidases by selenium content, physical characteristics, and substrate specificity (55, 99, 161). The product of the reaction of GSH with peroxides and disulfides is glutathione disulfide (GSSG) or a GSH adduct of lipid or protein. Glutathione reductase will reduce disulfides using NADPH as a cofactor. A secondary manifestation of cellular free radical stress is the depletion of NADPH needed for GSSG reduction (24). Cellular transhydrogenases serve to maintain NADH and NADPH in equilibrium; thus, a free radical stress can significantly lower the concentration of all reduced pyridine nucleotides in a cell and will affect countless integrated metabolic processes (26).

Another toxic manifestation of cellular oxidant stress is an increased concentration of intracellular GSSG, which can deleteriously affect the conformation or activity of thiol-containing proteins by disulfide interchange:

$$\text{G-SS-G} + \text{R-SH} \rightarrow \text{GSH} + \text{R-SS-G} \qquad (9)$$

The active transport of GSSG out of erythrocytes subjected to GSH oxidants (150) and the appearance of GSSG in the perfusate of isolated lungs or liver treated with hyperbaric oxygen (119, 148) shows that cells have evolved a GSSG clearance mechanism to avoid these potentially harmful disulfide interchange reactions.

Lactobacillus plantarum and related lactic acid bacteria accumulate high concentrations of nonprotein Mn(II). This metal appears to act as a superoxide dismutase (4) by catalytically scavenging $O_2^{\cdot-}$ and forming H_2O_2 as a product. Although a quantitatively similar role for Mn(II) in mammalian cells is unlikely because (Mn(II) is not present in as high a concentration, metal-catalyzed nonenzymatic disproportionation of radicals may serve a minor scavenging role.

Uric acid, present in the plasma at about 300 μM, protects hemoglobin from peroxide oxidation (Equation 8) and red cells from peroxidative damage to lipids. Because of its ability to react with singlet oxygen and OH·, urate can be an effective free radical scavenger (2).

B. Enzymatic Free Radical Scavengers

Catalase and peroxidases can be considered free radical scavengers even though H_2O_2 is not a radical species (Fig. 8). They help lower the steady state concentrations of H_2O_2, which is a precursor of more potent radical species (Equation 3). Thus, the cytotoxic potential of H_2O_2 is in large part a function of intracellular catalase and peroxidase activities that scavenge H_2O_2 and the concentration of transition metals that can reduce H_2O_2 to OH·. The subcellular distribution of catalase and GSH peroxidases is not completely defined (122), although it is known that peroxisomes have a very high catalase activity (89).

Superoxide dismutases are metalloproteins that function to dismute $O_2^{\cdot-}$ to H_2O_2 (Equation 1). Mammalian cells have a dimeric 32,000-dalton CuZn superoxide dismutase and a tetrameric Mn superoxide dismutase made of four identical 21,500-dalton subunits (52). Manganese superoxide dismutase has been reported to be located in mitochondria of rat liver (128). Human liver contains 20 times the concentration of Mn superoxide dismutase. Some of this activity may be cytoplasmically located since centrifuged human liver homogenates have 70 percent of the Mn superoxide dismutase activity remaining in the soluble fraction (101). Specific localization of free radical-protective enzymes by immunocytochemical techniques, at the electron microscope level, would provide valuable information about subcellular distribution of cellular antioxidant defenses from cell type-to-cell type, organ-to-organ, and among species.

Circulating $O_2^{\cdot-}$ metabolizing proteins have been reported, including ceruloplasmin and a recently described copper-containing tetrameric 134,000-dalton superoxide dismutase found in plasma and purified from human lung (91). Ceruloplasmin does not dismute $O_2^{\cdot-}$ by a disproportionation reaction and is 25 to 50 thousand times less active toward $O_2^{\cdot-}$ than superoxide dismutases (10). The role of ceruloplasmin as a plasma $O_2^{\cdot-}$ scavenger is unclear, since it is not active enough to protect erythrocytes from hemolysis by enzymatically generated $O_2^{\cdot-}$ (122). Nevertheless, there is a low superoxide dismutase activity present in human serum (90), some of which may be the recently described 134,000-dalton superoxide dismutase. Erythrocyte enzymes may also scavenge $O_2^{\cdot-}$ and H_2O_2 that diffuse into cells after generation by plasma sources.

C. Interactions of Radical Scavengers

From kinetic considerations, H_2O_2 would be expected to be preferentially metabolized by GSH peroxidase, because of its lower K_m for H_2O_2 than catalase (31). Catalase has a greater activity toward H_2O_2 at higher H_2O_2 concentrations (27). Thus, cells may avoid GSH depletion and GSSG accumulation by depending more on catalase during increased rates of cellular H_2O_2 production. More direct studies of this interenzyme cooperativity have been reported using isolated hepatocytes, in which either peroxisomal or endoplasmic reticular H_2O_2 production was stimulated by selective addition of H_2O_2-generating substrates or by addition of H_2O_2 (77, 78). The results supported the hypothesis that catalase function increases as the rate of H_2O_2 production is enhanced. It is also clear that the functions of catalase and GSH peroxidase in H_2O_2 metabolism also depend on intracellular enzyme localization and the source of H_2O_2.

A recent innovation useful for studying the role of GSH in free radical metabolism is an inhibitor of γ-glutamylcysteine synthetase, buthionine sulfoximine (64). This compound, when used with 1-chloro-2,4-dinitrobenzene which covalently binds GSH (65), effectively depletes cells of GSH. This technique has been used to study rates of cellular GSH turnover (63) and altered sensitivities of cells to free radical species upon GSH depletion (5). GSH is the primary low molecular weight cellular thiol, a major reserve of cellular cysteine, and is released from cells during oxidant stress. The interorgan metabolism and resorption of GSH must also be important in order to avoid a futile cycle of GSH synthesis, disulfide formation, and subsequent release (109). Use of buthionine sulfoximine in kinetic measurements of GSH metabolism during free radical studies will prove useful in defining the metabolism and protective role of this tripeptide.

The inhibition of superoxide dismutase by H_2O_2 and catalase by $O_2^{\cdot-}$ described in section 3.A.4. implies that there may be regulatory interactions between cellular free radical metabolizing enzymes. Product inactivation of superoxide dismutase (by H_2O_2) could potentially be a problem, yet under normal metabolic conditions other peroxide-metabolizing systems may protect superoxide dismutase from H_2O_2. Indeed, red cell superoxide dismutase activity has been reported to remain constant throughout the life of the red cell (151). Other investigators, however, have reported from combined immunochemical and biochemical measurements that the specific activity of superoxide dismutase either remains unchanged, increases, or decreases as a function of increasing cell age. This suggests there may be cellular variations in cell age-related or radical-induced changes in superoxide dismutase activity (39, 135, 149, 171). Red cells exposed to 1,4-napthoquinone-2-sulfonic acid, which autoxidizes to yield $O_2^{\cdot-}$ (hence H_2O_2), suffered decreased superoxide dismutase and catalase activity when cells were incubated in glucose-free media (110). No inhibition of these enzymes was observed in the presence of glucose, suggesting the importance of the NADPH-GSH system in scavenging toxic partially reduced species of oxygen which could otherwise inhibit superoxide dismutase and catalase.

The relative importance of superoxide dismutase and catalase in protecting cells from free radical damage has been studied using enzyme inhibitors such as diethyldithiocarbamate for CuZn superoxide dismutase and 3-amino-1,2,4-triazole(aminotriazole) for catalase. Catalase-H_2O_2 complexes (compound I) are inactivated by aminotriazole; thus, the extent of catalase inactivation by aminotriazole can indicate rates of cellular H_2O_2 production. When guinea pigs were injected with diethyldithiocarbamate and aminotriazole, liver catalase inhibition by aminotriazole was 8 times less than when guinea

pigs were treated with aminotriazole alone (54). This suggests that in liver CuZn superoxide dismutase may be a significant source of H_2O_2 production. Oxygen toxicity, a consequence of increased cellular free radical production (48, 50), was increased in both rats (46) and cultured cells (86) pretreated with diethyldithiocarbamate.

Pharmacologic manipulation of free radical damage using diethyldithiocarbamate and aminotriazole can be difficult to interpret if rigorous controls are not used. For example, low doses of diethyldithiocarbamate (approximately 100 to 200 mg per kg) protect rats from 95 percent oxygen toxicity, whereas higher doses (>250 mg. per kg.) potentiated the toxicity of 95 percent oxygen (32). This phenomenon was attributed to an increase in lung glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase activities, all important in NADPH-GSH-mediated H_2O_2 metabolism, in the low-dose diethyldithiocarbamate-pretreated animals. To further complicate matters, greater concentrations of diethyldithiocarbamate (1.2 gm. per kg.) will inhibit lung glutathione peroxidase (58). Ambiguous results may also be obtained using aminotriazole as a catalase inhibitor because aminotriazole can react with and inhibit other hemoproteins as well.

The results outlined in this section suggest that free radical scavenging processes in a cell can be both cooperative and antagonistic. The reactions can be cooperative in the sense that catalase and glutathione peroxidase combine to metabolize H_2O_2 produced by different subcellular sites, with catalase contributing more to H_2O_2 metabolism at higher H_2O_2 concentrations. An example of a potentially antagonistic interaction could be the generation of H_2O_2 during superoxide dismutase metabolism of $O_2^{\cdot-}$. Hydrogen peroxide can deplete cellular GSH and reduced nicotinamides during glutathione peroxidase-catalyzed H_2O_2 reduction, serve as a precursor for OH·, and may also inhibit cytoplasmic CuZn superoxide dismutase. Bacterial studies have shown, however, that superoxide dismutase generally serves a protective rather than an antagonistic role in defending against overall free radical damage to cells, as indicated by decreased cytotoxicity in stressed cells that have augmented superoxide dismutase activity (62, 172).

D. Use of Radical Scavengers to Delineate Mechanisms of Free Radical Toxicity

When studying complex biologic systems, care must be taken extrapolating knowledge derived from the reaction of a radical scavenger or inhibitors in a well-defined chemical system to the workings of a cell or a multicellular system. As mentioned in the previous section diethyldithiocarbamate can inhibit not only CuZn superoxide dismutase (58) but will also affect a number of enzymes involved in H_2O_2 reduction. The free radicals being scavenged, or their by-products, may inhibit activities of enzymes added to scavenge H_2O_2 or $O_2^{\cdot-}$; thus, it is advisable to redetermine scavenger concentrations or activities at the end of an experiment.

When studying the effect of high concentrations of low molecular weight scavengers on the protection of target molecules from free radical damage, it may be difficult to ascribe an observed effect on only OH·, singlet oxygen, $O_2^{\cdot-}$, H_2O_2, or other organic radical species. The problem lies in the fact that most scavengers react nonspecifically. Mannitol, formate, dimethylsulfoxide, α-tocopherol, β-carotene, ascorbate, uric acid, and thiols can react with more than one radical species. Thus, inhibition of a free radical-induced effect does not implicate only one species as the culprit. Conversely, the lack of a protective effect by a scavenger does not conclusively show that the primary radical(s) of interest were not involved. The free radical and the scavenger may have been sterically prevented from interacting. Also, a secondary radical may have been formed after reaction with the added scavenger, which may in itself react as a toxic specie. An example of this is the generation of methyl radical and methylperoxy radical after reaction of OH· with dimethylsulfoxide (133).

Another complication exists when studying free radical reactions with intact cells. To inhibit the radical-mediated damage, the added scavenger must have access to the cellular sites of free radical reactions. Extracellular addition of enzymatic $O_2^{\cdot-}$ or H_2O_2 scavengers will have little effect on intracellular reactions of H_2O_2 and $O_2^{\cdot-}$ because of the membrane impermeability of these macromolecules. Even if the enzymes are added to an extracellular source of $O_2^{\cdot-}$ or H_2O_2 (*i.e.*, an activated leukocyte), the scavengers may not intercept H_2O_2 or $O_2^{\cdot-}$ passing from one membrane through another if the cells are in close apposition. Thus, it is not valid to conclude that a certain free radical species does or does not play a role in radical-induced cytotoxicity unless rigorous tests are performed. A good example of this dilemma was illustrated by studies of McCord and Salin (105) and Salin and McCord (142). Self-directed cytotoxicity of activated leukocytes could only be partially inhibited by 300 units per ml. bovine CuZn superoxide dismutase. When activated leukocytes were incubated with 2 units per ml. of human Mn superoxide dismutase, more than 100 times less enzyme, the cytotoxic effect of self-generated $O_2^{\cdot-}$ was completely eliminated (105). Net charge of the enzymes at pH 7.4 was the main difference between the two superoxide dismutases, which both had similar specific activities in pure preparations (approximately 3000 units per mg.). Native or derivatized enzymes that had positive charges at pH 7.4 were more effective in protecting cells from $O_2^{\cdot-}$ mediated lysis, suggesting that electrostatic interactions between superoxide dismutase and negatively charged cell surfaces are important in superoxide dismutase protection of cells from proximally generated $O_2^{\cdot-}$.

SUMMARY

Free radicals affect virtually all aspects of biologic existence by reaction with and modification of structural, metabolic, and genetic material. Protective mechanisms have evolved to defend cell components from free radical damage, but disease states, xenobiotics, and other environmental stresses can overwhelm defense mechanisms and cause cytotoxicity.

Acknowledgments: We thank Dr. Irwin Fridovich for his kind assistance and penetrating comments.

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