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44. This value is a fixed parameter in the Florida State University ocean model but one that can be estimated from oceanographic data.
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46. The relation for off-equatorial ULT represents an implicit integration of Ekman pumping through time for one time step only. A more reasonable approach, where the effects of current and past forcing are both included to simulate the fact that off-equatorial Rossby wave activity represents an accumulation of forcing, does not change the results substantially.
47. Both T_{av} and FSU model ULT data show that the largest ULT anomalies associated with ENSO in the North Pacific are located between 12° and 16° latitude and require 1 to 2 years to propagate from the central Pacific to the maritime coast of Asia.
48. For example, "40 to 60" day oscillations [R. A. Madden and P. R. Julian, *J. Atmos. Sci.* **28**, 702 (1971)] and "west wind bursts" [D. S. Luther, D. E. Harrison, R. A. Knox, *Science* **222**, 327 (1983); K.-M. Lau and M.-T. Li, *Bull. Am. Meteorol. Soc.* **65**, 114 (1985)].
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DNA Damage and Oxygen Radical Toxicity

JAMES A. IMLAY* AND STUART LINN

A major portion of the toxicity of hydrogen peroxide in *Escherichia coli* is attributed to DNA damage mediated by a Fenton reaction that generates active forms of hydroxyl radicals from hydrogen peroxide, DNA-bound iron, and a constant source of reducing equivalents. Kinetic peculiarities of DNA damage production by hydrogen peroxide in vivo can be reproduced by including DNA in an in vitro Fenton reaction system in which iron catalyzes the univalent reduction of hydrogen peroxide by the reduced form of nicotinamide adenine dinucleotide (NADH). To minimize the toxicity of oxygen radicals, the cell utilizes scavengers of these radicals and DNA repair enzymes. On the basis of observations with the model system, it is proposed that the cell may also decrease such toxicity by diminishing available NAD(P)H and by utilizing oxygen itself to scavenge active free radicals into superoxide, which is then destroyed by superoxide dismutase.

THE APPEARANCE OF ATMOSPHERIC OXYGEN OFFERED TO the evolving biota the opportunity to utilize molecular oxygen as the terminal oxidant in respiration in order to gain energetic advantages over fermentation and respiratory pathways that rely on other oxidants. However, the presence of intracellular oxygen also allowed inadvertent redox reactions by oxygen radicals to damage critical biomolecules, and a variety of human disease states, including atherosclerosis, cancer, and aging have been attributed to such damage (*1*). This article describes the chemistry by which one oxidant, hydrogen peroxide (H_2O_2), generates toxic lesions in DNA and how the cell protects itself against such lesions.

Sources of Oxidative Stress

A variety of external oxidative stresses have toxic consequences in bacterial and eukaryotic cells. Hyperbaric oxygen, gamma radiation, near-ultraviolet (near-UV) radiation, ozone, peroxides, and redox-cycling drugs all exert deleterious effects through the intermediacy of oxygen species. We selected H_2O_2 for study of such phenomena because of the likelihood that it is central to the cytotoxic action of many of these agents and because the routine generation of H_2O_2 as a by-product of oxidative metabolism might cause it also to be an important endogenous oxidant in aerobic organisms. *Escherichia coli* was selected as an experimental organism because of its simple growth requirements, its ability to grow anaerobically or aerobically, and the extensive knowledge available concerning its genetics and enzymology.

Although molecular oxygen is strongly oxidative with respect to its fully reduced form, water, its oxidative potential is normally held in check by kinetic restrictions that are imposed by its two unpaired, spin-parallel electrons. However, consecutive univalent reductions of oxygen produce superoxide ($O_2\cdot^-$), H_2O_2, and hydroxyl radical (HO·), with the reaction potentials shown:

$$O_2 \xrightarrow{-0.33\ V} O_2\cdot^- \xrightarrow{+0.94\ V} H_2O_2 \xrightarrow{+0.38\ V} HO\cdot + H_2O \xrightarrow{+2.33\ V} H_2O \quad (1)$$

Each of these is exempt from the spin restriction and is kinetically and thermodynamically proficient at monovalent electron exchanges. The $O_2\cdot^-$ is notable in that it can act either as an oxidant or a reductant. Hydrogen peroxide is a relatively stable oxidant, but the HO· is an extremely powerful oxidant that reacts at nearly diffusion-limited rates with most organic substrates. The relatively mild oxidative capabilities of $O_2\cdot^-$ and H_2O_2 belie the severity of their direct oxidative effects on biomolecules; therefore, it has been assumed that their cytotoxic nature might be due to their ability to generate intracellular HO·.

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DNA Damage

The characterization of cell damage caused by oxygen species has been complicated both by the variety of direct and indirect effects observed and by the diversity of oxidative stresses that have been used. Because the cell is particularly sensitive to DNA damage, genetic and physical effects of oxidative stresses upon cellular DNA are well documented. Hyperbaric oxygen, gamma radiation, H_2O_2, and $O_2\cdot^-$-generating drugs are mutagenic and cause chromosome deletions, dicentrics, and sister-chromatid exchanges (*2, 3*). Oxygen radicals may attack DNA at either the sugar or the base, giving rise to a large number of products (*4*) (Fig. 1). Attack at a sugar ultimately leads to sugar fragmentation, base loss, and a strand break with a terminal fragmented sugar residue. Such single-stranded DNA breaks accumulate during exposure of bacteria and mammalian cells to H_2O_2, $O_2\cdot^-$, gamma radiation, or ozone (*5–8*), and mutants of *E. coli* that are defective in recombinational DNA repair or the ability to excise such termini (strains lacking exonuclease III or endonuclease IV) are very sensitive to H_2O_2 (*9–11*). Near-UV radiation and a variety of antitumor drugs that are believed to exert their physiological effects partly by the production of partially reduced oxygen species also form single-stranded DNA breaks (*12, 13*). Those breaks formed in vitro by gamma rays or bleomycin contain 3′-phosphate or 3′-phosphoglycolate termini (*14, 15*) (see Fig. 1).

Hydrogen peroxide, osmium tetroxide, potassium permanganate, and gamma radiation also produce ring-saturated thymines, hydroxymethyluracil, thymine fragments, and adenine ring-opened products (*16*), presumably through oxygen radical attack at bases (Fig. 1). Enzymes exist for removing these damaged bases from DNA, so these lesions must be quantitatively important; however, to date there is no report of unusual sensitivities to oxidants of mutants lacking these enzymes, so that the relative contribution of base lesions as compared to strand breaks toward toxicity is unclear.

Oxidative damage to other biomolecules has also been observed, although the consequences for cell viability are more difficult to establish. Membrane damage from oxygen species is observable as an accumulation of lipid peroxides, the loss of a diffusion barrier to membrane-impermeable markers, and cell lysis (*17*). These phenomena have been reported to occur as a consequence of cell exposure to H_2O_2, $O_2\cdot^-$, and near-UV radiation (*18*). A variety of enzymes can be inactivated by exposure to H_2O_2 (*19*), and the sensitivity to $O_2\cdot^-$ of an enzyme necessary for branched chain amino acid biosynthesis may result in the growth impairment of *E. coli* in the presence of redox-cycling quinones or during hyperoxia (*20*).

H_2O_2 Toxicity in *E. coli* Is Mediated in Part by a Fenton Reaction

Exposure of logarithmically growing *E. coli* to H_2O_2 leads to two kinetically distinguishable modes of cell killing (*9*) (Fig. 2A). Mode-one killing is pronounced at low (1 to 3 m*M*) concentrations of H_2O_2 and is caused by DNA damage. Mutant strains that are defective in recombinational or excision DNA repair are especially sensitive to mode-one killing, and both mutagenesis and the induction of the SOS DNA repair regulon (indicators of DNA damage) accompany challenges at these doses (*21*). Mode-two killing requires a relatively high (>20 m*M* in Fig. 2) dosage, and the site (or sites) of the toxic injury has not been established.

Differences in the kinetics of production of these two classes of toxic damage indicate that they are not produced by a common oxidant. The damage that results in mode-two killing is generated at a rate that is linearly proportional to the concentration of H_2O_2, while the dose response for the generation of DNA lesions that cause mode-one killing is more complex (*9*) (Fig. 2). The rate of mode-one killing increases with H_2O_2 concentration to a maximum at about 1 m*M*, then drops off to a value at 10 m*M* that is approximately half-maximal and constant to at least 60 m*M*. The unusual dose response for mode-one killing is also observed when monitoring the efficacy of induction of the SOS response, mutagenesis, or filamentation (*9*). Various experiments have ruled out the induction of protective effects or transport phenomena to explain this response (*9*); instead, these kinetics reflect some aspect of the

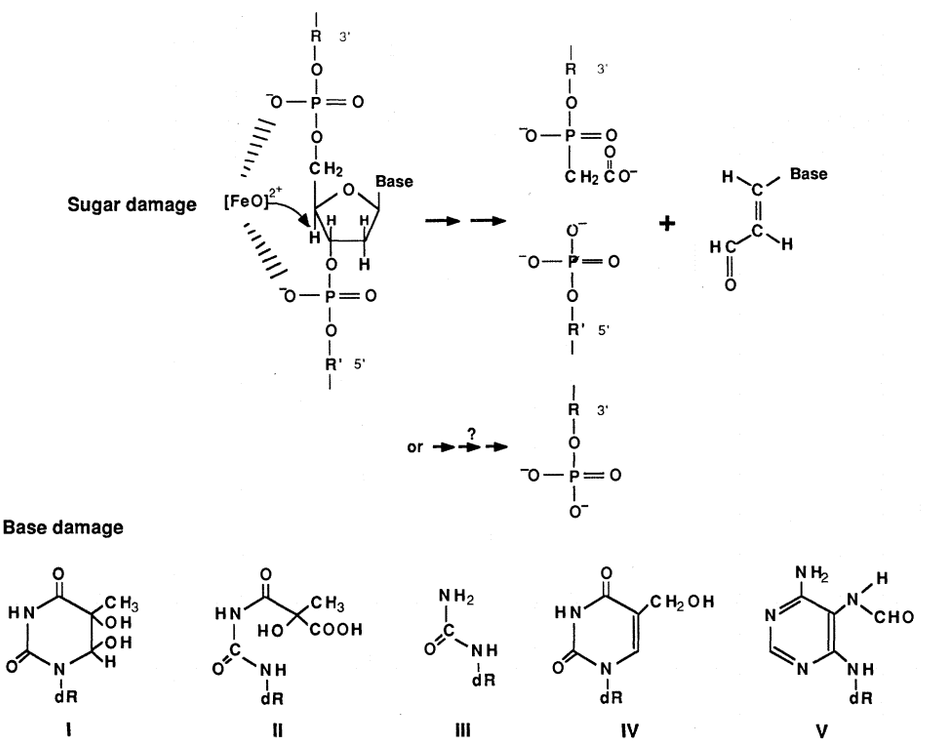

Fig. 1. DNA damage products formed by oxygen radicals. Only damages for which specific excision enzymes have been identified are shown. Sugar damage: phosphoglycolate and 3′-phosphates are removed by *E. coli* exonuclease III or endonuclease IV, and mutants lacking these enzymes are sensitive to oxygen radicals (*11*). In the former case, abstraction of a hydrogen from the C-4′ atom by a DNA-bound ferryl radical is hypothesized. Base damage: I, thymine glycol (cis or trans); II, methyltartronylurea; III, urea; IV, 5-hydroxymethyluracil; V, 4,6-diamino-5-formamidopyrimidine. I to IV are formed from thymine. I to III are removed by endonuclease III (bacteria) or γ endonuclease (mammalian cells), but bacterial mutants lacking the enzyme are not especially sensitive to oxygen radicals. IV is formed from thymine, and is apparently enzymatically excised by mammalian (*65*) but not bacterial cells. V is formed from adenine and is excised in both cell types. Enzymes excising *N*-oxypurines and cytosine derivatives are likely to exist but have not been reported.

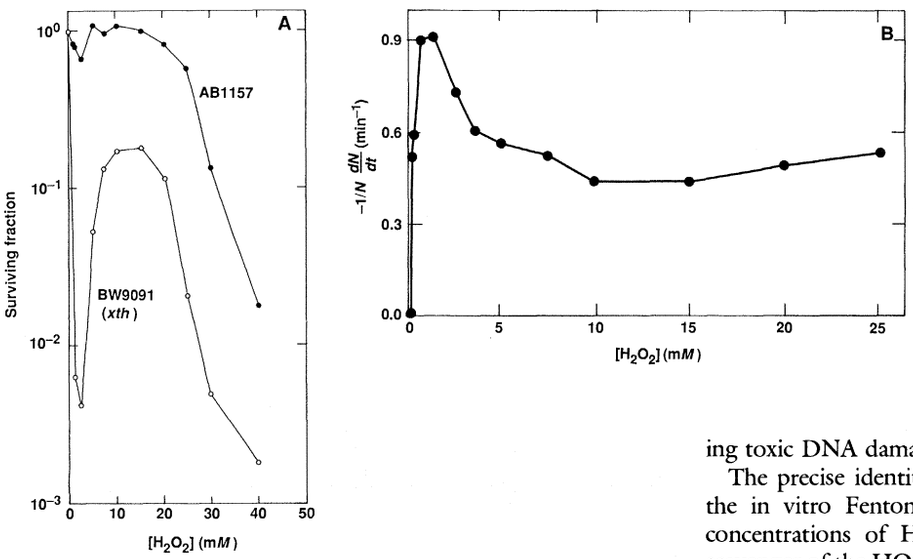

Fig. 2. Killing of *E. coli* by exposure to H_2O_2. (**A**) Logarithmically growing cells were exposed to the indicated concentrations of H_2O_2 for 15 min and then diluted and plated; survival was determined by colony formation after 24 hours. Solid circles: DNA repair–proficient strain AB1157; open circles: exonuclease III–deficient strain BW9091 (DNA excision repair deficient). (**B**) Initial linear killing rates of strain BW9091 (exonuclease III–deficient) were determined in time-course experiments (*9*). Note the factor of 2 difference in killing rate between 1.25 and 10 mM which gives rise to the large "dip" seen in (A). With these initial rate observations (6 min or less) mode-two killing occurs only above 50 mM H_2O_2.

chemistry by which H_2O_2 is converted into an oxidant that is capable of damaging the DNA.

Single-stranded DNA breaks that contribute to mode-one killing in DNA repair–deficient strains result from the collapse of the deoxyribose ring after abstraction of a hydrogen atom (Fig. 1) (*4, 14, 15*). Hydrogen abstraction requires a powerful oxidant; since the HO· can be generated by monovalent reduction of H_2O_2, this species could be a reasonable source of DNA damage. The chemistry of the reduction of H_2O_2 was explored first by Fenton (*22*) and subsequently by Haber and Weiss (*23*) in their studies of the reductive decomposition of H_2O_2 by reduced metals:

$$Me^{N+} + H_2O_2 \rightarrow Me^{(N+1)+} + HO\cdot + H_2O \quad (2)$$

Indeed, the participation of transition metals in H_2O_2-induced cell damage is indicated by the protective effects of cell-permeable metal chelators. Desferal and *o*-phenanthroline prevent the appearance of sister-chromatid exchanges, single-stranded DNA breaks, and cell killing in a variety of mammalian cells during exposure to H_2O_2 (*2, 6, 7, 24*). Similarly, mode-one killing does not occur in either wild-type or repair-deficient strains of *E. coli* if *o*-phenanthroline, dipyridyl, or desferal is present during H_2O_2 challenge (Table 1) (*25*).

Although copper and cobalt are capable of serving in Fenton reactions, the relative preponderance of cellular iron and its high affinity for the protective chelators suggest that it is likely to fulfill this role in vivo. The inhibitory effects of these chelators may be due either to their sequestering the metal away from the DNA or to their occupancy of metal coordination sites, thereby obstructing interaction with oxygen species (*26*). Mode-two killing is not blocked by these agents, confirming that this other type of oxidative damage is produced by a distinct mechanism.

The mixing of H_2O_2 and reduced transition metals results in rapid metal oxidation and the production of HO· (Eq. 2). This short-lived oxygen species can be detected by its immediate reaction with a dye such as *p*-nitrosodimethylaniline and by competition experiments with other substrates such as ethanol, which react at known rates with the HO· (*25*). H_2O_2 and ferrous iron [Fe(II)] also generate radicals that can produce inactivating DNA lesions in bacteriophage λ and single-stranded breaks in purified duplex DNA (*25*) (Fig. 3). Intriguingly, the maximum extent of DNA nicking occurs at low concentrations of H_2O_2, and higher concentrations suppress about half of the damage (Fig. 3). Thus the dose response for DNA nicking during the iron-based in vitro Fenton reaction (Fig. 3) reproduces a characteristic aspect of the dose response for generating toxic DNA damage in vivo (Fig. 2B).

The precise identities of the direct DNA oxidants generated by the in vitro Fenton reactions are unclear. DNA nicking at all concentrations of H_2O_2 is biphasically inhibited by ethanol, a scavenger of the HO·. Approximately half of the damage is prevented by the inclusion of micromolar concentrations of ethanol (*25*). Kinetic analysis of the ethanol-sensitive nicking indicates that ethanol and DNA compete for the oxidant and that their relative reaction rates are the same as their relative rates of reaction with HO·. By contrast, several hundred times as much ethanol is required to competitively reduce the remainder of the DNA nicking. Thus, about half of the DNA damage generated in vitro is apparently due to a diffusible HO·, while the remaining nicks (such as those generated in the experiment shown in Fig. 3) are due to a different oxidant. The latter might be an HO· formed directly on the DNA surface, or a distinct radical species.

Since high concentrations of H_2O_2 reduce even the ethanol-resistant DNA nicking by approximately 50%, the suppressions by high concentrations of H_2O_2 and by ethanol are not directly related. The peroxide-mediated suppression is not due to inhibition of the extent of the Fenton reaction, since the limiting amount of Fe(II) is totally oxidized and the amount of peroxide decomposition is independent of the peroxide concentration. Instead, the suppression appears to be due to a reduction of the efficiency with which an oxidant brings about toxic DNA damage. In sum, the identity of the ethanol-resistant, peroxide-sensitive DNA oxidant, which is observed in vitro and appears to cause DNA damage in vivo, is unknown.

Others have also observed oxidants formed by iron-, copper-, or cobalt-based Fenton reactions that differ from HO· generated by ionizing radiation. For example, HO· scavengers such as ethanol do not block hydrogen abstraction from methionine or hydroxylation of salicylate (*27–29*). Such oxidants have been proposed to be an oxygen radical complexed to the metal, the organic substrate, or both (*29–31*). The formation of such complexes could either alter the oxidative potential of the oxygen species, or impose steric restrictions upon the reaction with competing substrates. Koppenol and colleagues have concluded that the ferryl ion, an iron-oxygen chelate that is charge-equivalent to a Fe(III)-complexed HO·, is probably less oxidizing than is the unbound HO· (*32*). The suppression of DNA damage by high concentrations of H_2O_2 may then be due to reaction of H_2O_2 with the ferryl radical:

$$Fe^{3+}\text{–}[HO\cdot] + H_2O_2 \rightarrow Fe^{3+} + H_2O + O_2\cdot^- + H^+ \quad (3)$$

Conversely, a Fe(II)-EDTA–dependent Fenton system also produces lethal DNA lesions, but in this case high concentrations of H_2O_2 do not suppress nicking, possibly because steric factors might reduce the availability of the radical center for reaction with peroxide.

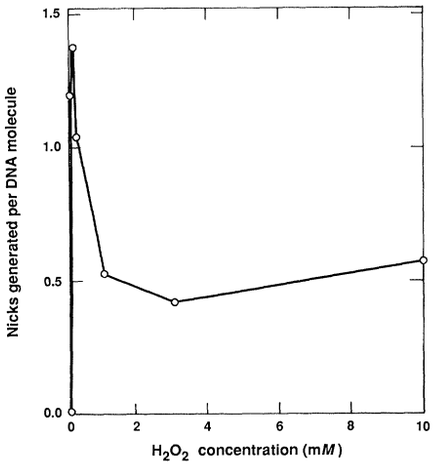

Fig. 3. DNA damage by Fenton reactions in vitro. Phage PM2 DNA (17 μM nucleotide) was mixed at room temperature with 80 nM $FeSO_4$, 10 mM ethanol, 0.8% NaCl, and the indicated concentrations of H_2O_2. After 7.5 min, reactions were terminated and DNA single-stranded breaks were quantitated by a nitrocellulose filter-binding assay (*9*). NaCl is included in this and subsequent experiments to provide physiological ionic strength; it is unnecessary for the progress of the Fenton reaction or the generation of DNA damage. Buffers were excluded because of their tendency to perturb the system by binding the metal or scavenging the oxyradicals. The measured *p*H in these reactions was between 6 and 7.

The Source of Reducing Equivalents for an Ongoing Fenton Reaction

To maintain an ongoing Fenton reaction, an electron source must be available to regenerate the reduced metal. Thiols, ascorbate, superoxide, NAD(P)H, or radicals generated by redox-cycling drugs have been used in vitro for this purpose (*28, 33, 34*). As mode-one toxicity in *E. coli* is apparently mediated by Fe(II) and continues for as long as 30 min a cellular electron donor must exist.

Treatments that deplete or enlarge the pool of reducing equivalents in *E. coli* alter the level of Fenton reactions in vivo (*25*). Starved cells are not subject to mode-one toxicity by H_2O_2 (Table 1), but re-addition of glucose to these cells immediately restores their sensitivity. The blocking of respiration chemically by cyanide or genetically by inactivating the NADH dehydrogenase gene (*ndh*) dramatically sensitizes *E. coli* to killing by H_2O_2 (Table 1). Sensitization is immediate upon the addition of cyanide, and desensitization follows rapidly upon its removal. Thus the redox status of the iron involved in mode-one DNA damage or of its immediate electron donor appears to be in equilibrium with the pool of reducing equivalents.

The observation that *ndh* mutants, which accumulate NADH as a consequence of the inability to donate electrons to respiratory pathways, are hypersensitive to mode-one killing (Table 1) indicates that the metal reductant lies outside of the respiratory chains. Glutathione synthesis and glutathione reductase mutants exhibit normal sensitivity to mode-one killing (*21, 35*), so glutathione is not the reductant. The presence of the flavin radical, FAD·, has been reported, so monovalent metal reduction could occur by means of a flavin-dependent enzyme. However, the rapidity of iron oxidation by H_2O_2 and the high reactivity of the DNA oxidant are such that it is unlikely that either the reduced metal or the oxidant would diffuse from an enzyme crevice to DNA before reacting with other biomolecules. It therefore appears that the Fenton-active metal is reduced on the surface of the DNA where it is probably chelated to the phosphodiester backbone, and that the metal reductant is a small diffusible molecule, such as NAD(P)H or $O_2\cdot^-$.

The ability of $O_2\cdot^-$ to drive an iron-dependent Fenton reaction has been established, and the toxicity of superoxide has been attributed to this reaction. The presence of superoxide dismutases (SODs) in aerobic organisms suggests that $O_2\cdot^-$ contributes to oxidative toxicity, and mutants of *E. coli* which totally lack this activity are sensitive to mode-one killing by H_2O_2 (Table 1). This sensitivity could indicate a role for $O_2\cdot^-$ in the production of the Fenton oxidant via metal reduction. However, although exposure of *E. coli* to H_2O_2 under anaerobic condition prevents $O_2\cdot^-$ formation because of the absence of its precursor, oxygen, mode-one killing occurs (Table 1). In fact, killing is markedly enhanced. This experiment was performed with a catalase-deficient strain to avoid oxygen generation from H_2O_2 by catalase.

We therefore have investigated whether NADH could drive the DNA-damaging Fenton reaction. Whereas NADH participates in enzymatic reactions only as a two-electron donor, it can participate in chemical reactions as a monovalent reductant. Carlson *et al.* have used ferrocenium compounds to determine that the half-reaction potential of monovalent NADH oxidation is −0.93 V; iron would therefore be a feasible oxidant (*36*). In fact, at acid *p*H, which maintains Fe(III) solubility in water, NADH and iron form a 2:1 complex that results in iron reduction (*37*). These observations suggest that NADH might drive the in vivo Fenton reaction by the monovalent reduction of DNA-bound iron.

To study such a reaction in vitro, iron-EDTA complexes were used to ensure the solubility of oxidized iron at neutral *p*H. NADH oxidation by Fe(III)-EDTA alone is extremely slow; however, the reaction is accelerated more than 100-fold by the inclusion of H_2O_2 (Fig. 4). The decomposition of H_2O_2 is likewise stimulated by the

Table 1. Mode-one killing rates (*63*). The time-averaged killing rate is calculated as $-(1/N)(dN/dT)$ after a 15-min challenge with 2.5 mM H_2O_2 as described in the legend to Fig. 2.

Strain*	Killing rate (min^{-1})
Wild-type	0.03
+ Chelator†	0.00
Starved	0.00
+ KCN‡	0.54
+ KCN‡ + chelator†	0.00
ndh	0.68§
Scavenger-defective	
sodA	0.08
sodB	0.08
sodA sodB	0.29
katE katG	0.03
Anaerobic	0.77
gshB	0.02
*oxyR*Δ	0.02
DNA repair–defective	
xth	0.49
+ Chelator†	0.00
Starved	0.00
recA	0.45
xth recA	2.6§
nfo	0.03
xth nfo	1.0§
polA	1.7§
lexA(Ind^-)	0.38
nth	0.03
uvrA	0.02

* The wild-type strain used for these studies was *E. coli* AB1157. The identities and genotypes of mutant strains have been described (*9, 21, 25*). The relevant genes listed carry mutations that totally eliminate protein activity except for *polA*. These corresponding activities are *ndh*, NADH dehydrogenase; *sodA*, Mn SOD; *sodB*, Fe SOD; *katE katG*, catalase; *gshB*, glutathione synthase; *oxyR*Δ, lacks the induction of increased levels of scavengers on exposure to peroxide; *xth*, exonuclease III; *recA*, recA protein; *nfo*, endonuclease IV; *polA*, DNA polymerase I (this mutant allele was *polA1*, and the deletion allele is more sensitive); *lexA*(Ind^-), does not induce the SOS response; *nth*, endonuclease III; *uvrA*, uvrA protein. †Chelators (0.3 mM *o*-phenanthroline, 1.0 mM dipyridyl, or 20 mM desferrioxamine mesylate) were added 5 min before the H_2O_2 challenge. ‡KCN (3 mM) was added to cells 5 min before the H_2O_2 challenge. §The extent of killing at 15 min was estimated by extrapolation from shorter exposures, since killing becomes nonlinear with time. The 2.5 mM H_2O_2 time course of reference (*9*) was used as the basis for extrapolation.

Table 2. Involvement of $O_2^{\cdot -}$ in the aerobic NADH-driven Fenton reaction. The 3.5-ml reactions contained 25 μ*M* $FeCl_3$, 38 μ*M* Na_2H_2 EDTA, 200 μ*M* NADH, 200 μ*M* H_2O_2, 0.8% NaCl, and 50 m*M* potassium phosphate (*p*H 7.8). Where indicated, 20 U/ml of the *E. coli* manganese-dependent SOD was included. Anaerobic reaction solutions were degassed by bubbling water-saturated argon through them for 2 hours prior to the addition of H_2O_2; degassing was subsequently continued during the reaction. After 110 min, NADH concentrations were determined by absorbance at 339 nm, and H_2O_2 concentrations were determined by a peroxidase assay (*64*). The omission of potassium phosphate in other experiments had no qualitative effect on the results.

Condition	Consumed	
	H_2O_2 (μ*M*)	NADH (μ*M*)
Aerobic	157	177
Aerobic + SOD	4	10
Anaerobic	124	127
Anaerobic + SOD	109	107

addition of NADH; NADH oxidation and H_2O_2 decomposition exhibit a 1:1 stoichiometry throughout the course of the reaction. The ultimate product of NADH oxidation by Fe(III)-EDTA and H_2O_2 is NAD^+, as determined by the coelution of 90 percent of the oxidized NADH with NAD^+ on reversed-phase high-performance liquid chromatography and by its enzymatic reduction back to NADH by alcohol dehydrogenase. Not surprisingly, NADPH or reduced nicotinamide mononucleotide can replace NADH in these reactions, whereas NAD^+ cannot.

In vivo, H_2O_2 toxicity via DNA damage is maximal with 1 m*M* H_2O_2 (Fig. 2). Remarkably, for the in vitro system containing Fe(III)-EDTA, the rate of NADH oxidation also is a maximal at 1 m*M* H_2O_2 (Fig. 4). The reaction rate is first-order in both NADH and Fe(III)-EDTA concentrations (insets in Fig. 4), but the profile of Fig. 4 is essentially unaffected by variation in NADH and metal concentrations.

When DNA is included in the NADH–Fe(III)-EDTA–H_2O_2 system, single-stranded breaks ensue. The appearance of this damage requires the presence of all reactants (Fig. 5), and NAD^+ is ineffective. The rate of DNA nicking, like the rate of NADH oxidation, increases to a limit with H_2O_2 concentration (Fig. 5) (*38*).

The single-stranded DNA breaks that accumulate in *E. coli* mutants lacking exonuclease III or endonuclease IV during H_2O_2 exposure contain 3′ sugar fragments (*39*). Because these fragments block the 3′-OH group necessary for DNA synthesis, such breaks do not support synthesis by *E. coli* DNA polymerase I unless the sugar fragments are first removed by *E. coli* exonuclease III or endonuclease IV. This requirement presumably explains the synergism between *xth* and *nfo* mutations in sensitizing cells to mode-one killing (*11, 25*). The DNA nicks generated in vitro during the Fe(III)-EDTA–NADH–H_2O_2 reaction similarly cannot support nucleotide incorporation unless the DNA is treated with exonuclease III (Fig. 6). Thus, while we do not know the state of iron chelation in the cell, Fe-EDTA seems to be an appropriate model.

These results support the hypothesis that NADH can drive the DNA-damaging, iron-mediated Fenton reaction by reducing the catalytic iron. In addition, studies with free-radical scavengers indicate that the DNA oxidant is likely to be the same as that generated with the Fe(II)-H_2O_2 system (*40*). However, the detailed chemistry is complex, because the presence of SOD in an aerobic NADH–Fe(III)-EDTA–H_2O_2 system prevents NADH oxidation, H_2O_2 decomposition (Table 2), and DNA damage. This inhibition is due to the enzyme activity of the SOD, since heat-inactivated enzyme has no effect.

Under anaerobic conditions, however, the reaction proceeds normally even in the presence of SOD (Table 2). Therefore, it appears that molecular oxygen can reversibly remove unpaired electrons from some member of the reaction pathway to form $O_2^{\cdot -}$, and, in the presence of SOD, this scavenging is irreversible and the Fenton reaction is effectively blocked. This behavior mirrors the ability of cellular SOD to prevent mode-one killing by H_2O_2 despite the fact that killing is enhanced under anaerobic conditions.

The most surprising aspect of these results is that SOD blocks NADH oxidation. If NADH were to act solely as a metal reductant, subsequent scavenging of the transferred electron should not prevent NADH oxidation (see Fig. 4). Therefore, NADH appears to be involved in propagating a free-radical chain which it has initiated; blockage of that chain would then prevent significant NADH oxidation. The following reactions describe such a sequence:

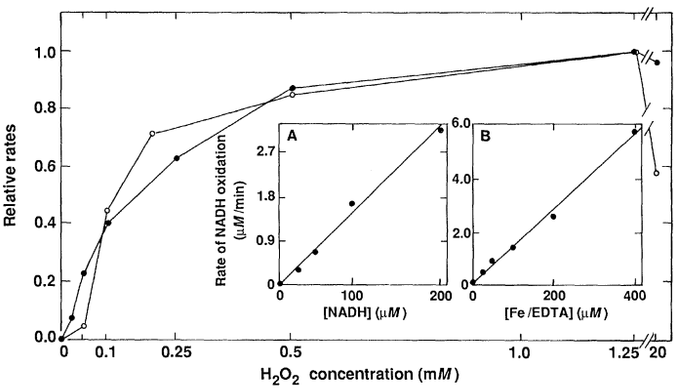

Fig. 4. NADH oxidation by a Fe(III)-EDTA–dependent Fenton system exhibits the same dependence upon sub-millimolar H_2O_2 concentration as does cell killing. NADH oxidation (●) was monitored at 339 nm in reactions containing 100 μ*M* NADH, 100 μ*M* $FeCl_3$, 150 μ*M* EDTA, 0.8% NaCl, and the indicated concentrations of H_2O_2. The initial rates are normalized to the rate at 1.25 m*M* H_2O_2 (1.9 μ*M* NADH oxidized per minute). The initial rates of cell killing (○) were determined previously in an exonuclease III–deficient strain (*21*). At H_2O_2 concentrations over 5 m*M*, the rate of cell killing decreased by about a factor of 2, while the NADH oxidation rate remained maximal (note 20 m*M* H_2O_2 points). (**Inset A**) The NADH oxidation rate is linearly proportional to NADH concentration. Reactions contained 10 m*M* H_2O_2, 100 μ*M* $FeCl_3$, 150 μ*M* EDTA, and 0.8% NaCl. (**Inset B**) The NADH oxidation rate is linearly proportional to Fe(III)-EDTA (1:1.5) concentration. Reactions contained 10 m*M* H_2O_2, 100 μ*M* NADH, and 0.8% NaCl.

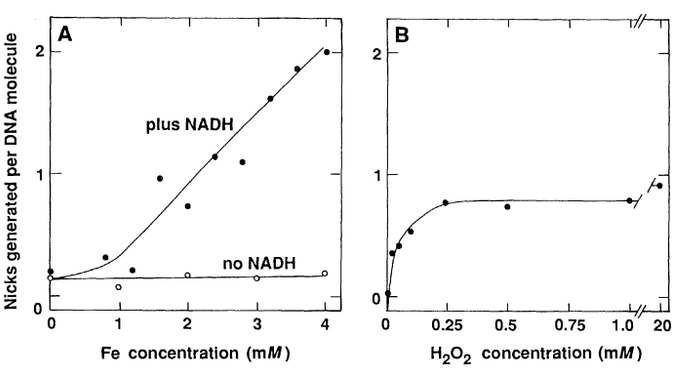

Fig. 5. DNA damage generated by an NADH-driven Fenton system. (**A**) Reactions contained 20 μ*M* PM2 DNA (nucleotide concentration), 1 m*M* H_2O_2, 0.8% NaCl, and various concentrations of $FeCl_3$-Na_2H_2 EDTA (1:1.5). Where indicated, 200 μ*M* NADH was included. (**B**) Reactions contained 20 μ*M* PM2 DNA, 2.4 μ*M* $FeCl_3$, 3.6 μ*M* Na_2H_2 EDTA, 200 μ*M* NADH, and various concentrations of H_2O_2. Incubations were for 9 min at room temperature.

Initiation

$$Fe^{3+}(EDTA) + NADH \rightleftharpoons Fe^{2+}(EDTA) + NAD\cdot + H^+ \quad (4)$$

Propagation

$$H^+ + Fe^{2+}(EDTA) + H_2O_2 \rightarrow Fe^{3+}(EDTA)\text{–}[HO\cdot] + H_2O \quad (5)$$

$$Fe^{3+}(EDTA)\text{–}[HO\cdot] + NADH \rightarrow H_2O + NAD\cdot + Fe^{3+}(EDTA) \quad (6)$$

$$NAD\cdot + Fe^{3+}(EDTA) \rightarrow NAD^+ + Fe^{2+}(EDTA) \quad (7)$$

$$NADH + H_2O_2 + H^+ \rightarrow NAD^+ + 2H_2O \quad (8)$$

Equation 8, the sum of reactions 5, 6, and 7, would account for the 1:1 stoichiometry of NADH and H_2O_2 utilization observed in Table 2 and the ferryl-EDTA radical, which is an intermediate, would be the DNA-damaging agent. Reactions 5 to 7 might also be initiated by free radicals generated by carcinogens or radiation should such radicals form reduced iron or NAD·.

A Putative Protective Role for Oxygen and SOD

When oxygen is present, it rapidly forms $O_2\cdot^-$ from $Fe^{2+}(EDTA)$ (*26*).

$$Fe^{2+}(EDTA) + O_2 \rightleftharpoons Fe^{3+}(EDTA) + O_2\cdot^- \quad (9)$$

More relevant from a biological perspective, oxygen rapidly reacts with NAD· with a rate constant (k) of 2×10^9 (*41*).

$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2\cdot^- \quad (10)$$

Superoxide can then effectively be removed by SOD:

$$2\ O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (11)$$

These reactions not only provide a mechanism by which SOD would prevent the Fenton reaction and DNA nicking observed in vitro (Table 2), but also suggest a means by which it might protect against mode-one toxicity by H_2O_2 in vivo without $O_2\cdot^-$ being directly involved in the generation of DNA damage. Active radicals would be transformed to the less reactive $O_2\cdot^-$ which would be degraded to oxygen and water by SOD and catalase. Ironically, the cell would utilize molecular oxygen as a protective free-radical scavenger!

Of course, at abnormally high levels, $O_2\cdot^-$ can be toxic. It can donate electrons to the Fenton reaction by the reverse of Eq. 9 and it dismutes to form H_2O_2. Thus, the overproduction of $O_2\cdot^-$ by redox-cycling drugs or hyperbaric oxygen might provide both H_2O_2 and reducing equivalents to support the Fenton reaction. Indeed, the overproduction of SOD sensitizes *E. coli* to paraquat and hyperbaric oxygen (*42*).

Toxicity Not Involving Iron-Mediated Fenton Chemistry

In addition to oxyradicals, a variety of redox-activating drugs and metalchelates mediate toxicity via Fenton reactions. Mitomycin C, adriamycin, streptonigrin, amsacrine, bleomycin, neocarsinostatin, and elliptinium acetate produce DNA damage through redox reactions with transition metals (*12, 15, 43*). Anthraquinone, paraquat, and nitrofurantoin radicals can directly reduce H_2O_2 to generate a free-radical oxidant (*28, 32, 44*), and semiquinone components of cigarette tar appear to mediate oxygen-radical formation from H_2O_2 to produce single-stranded breaks in DNA (*45*). Not only do many of these agents serve as electron donors for Fenton reactions, but they also can bind tightly to DNA to ensure that oxidants are generated on the DNA surface. Indeed, molecules with this property have been used as probes to determine DNA structure (*34*).

Not all oxidative toxicity can be ascribed to Fenton chemistry, however. Mode-two killing of *E. coli* by H_2O_2 appears to require neither metals nor electrons and the rate of killing is unaffected by SOD levels. Although the site (or sites) of toxic lesions in this case has not been identified, DNA damage does occur as evidenced by the electron- and metal-independent mutagenesis that accompanies this killing (*21, 25, 46*).

Superoxide can also exert toxic effects apart from its participation in Fenton reactions. Phorbol-stimulated leukocytes generate $O_2\cdot^-$ and suffer the simultaneous production of single-stranded DNA breaks, but a subfraction of this damage is insensitive to the addition of catalase and is stimulated rather than inhibited by the metal-chelator *o*-phenanthroline (*7*). In *E. coli*, the redox-cycling drug plumbagin is cytotoxic but, unlike the Fenton oxidant, it neither nicks DNA nor acts with greater potency upon an exonuclease III-deficient strain (*47*). Superoxide, unlike H_2O_2, can induce endonuclease IV biosynthesis (*48*); since the induction of DNA repair enzymes is customarily triggered by the accumulation of DNA damage, the implication is that $O_2\cdot^-$ can generate a lesion spectrum distinct from that produced by the Fenton reaction. Other examples exist in which $O_2\cdot^-$ is destructive independently of the presence of H_2O_2 (*49*).

Distinctions have also been drawn between the actions of H_2O_2 and *t*-butylhydroperoxide. The latter compound is more toxic toward an endonuclease IV–deficient strain of *E. coli* than an exonuclease III–deficient strain (*11*); H_2O_2 has the opposite effect. Furthermore, iron chelators are capable of protecting rat hepatocytes against H_2O_2, but not against *t*-butylhydroperoxide (*24*).

Near-UV irradiation of oxygenated *E. coli* generates single-stranded DNA breaks which, like Fenton-generated breaks, are repaired by the actions of exonuclease III and DNA polymerase I (*50*). The ability of exogenous or cell-induced catalase to protect against near-UV damage (*51*) suggests that radiation-excited chromophores contribute to a Fenton reaction by generating either H_2O_2 or reducing equivalents. Indeed, near-UV radiation and H_2O_2 synergistically inactivate bacteriophage (*52*). However, recent work has indicated that lower energies of near-UV radiation can be toxic even in the absence of measurable H_2O_2 (*53*). It seems likely that near-UV radiation, like $O_2\cdot^-$, can damage biomolecules by avenues independent of its contribution to Fenton chemistry.

Finally, in vitro investigations by Aust and colleagues (*54*) have established that lipid peroxidation appears to be initiated by a complex of Fe(III), Fe(II), and O_2, and that it can proceed in the complete absence of H_2O_2. Although the details remain to be determined, this system is an encouraging step toward the elucidation of at least one type of Fenton-independent oxidative chemistry.

Cellular Defenses Against Oxygen Radicals

DNA is a weak link in cellular resistance to oxygen radicals induced by Fenton reactions either because it binds the metal which is involved in generating the toxic species or because considerable damage to other biomolecules can be tolerated. Accordingly, bacterial and mammalian cells contain enzymes for excising damaged bases and sugar fragments from DNA and for replacing lost genetic information via genetic recombination. In *E. coli*, we know that the ability to carry out the repair of DNA strand breaks is extremely important, since mutants defective in DNA polymerase I, genetic

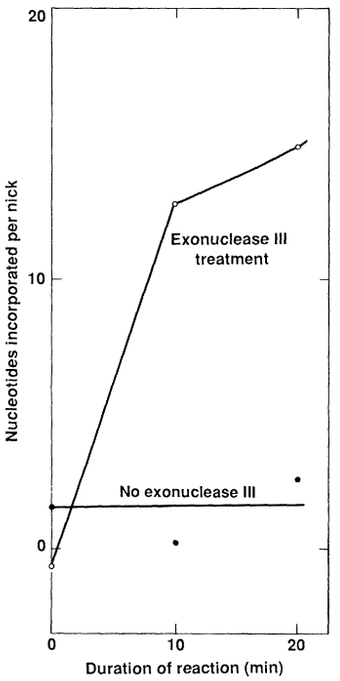

Fig. 6. Single-stranded breaks generated in vitro require treatment with exonuclease III in order to serve as primers for *E. coli* DNA polymerase I. PM2 DNA (20 μM) was mixed with 2.4 μM $FeCl_3$, 3.6 μM EDTA, 200 μM NADH, 1 mM ethanol, and 1 mM H_2O_2. After 9 min at room temperature, the reaction was terminated by the addition of catalase and half of the DNA was exposed to exonuclease III (30 U/ml) in 50 mM tris (*p*H 7.6), 10 mM $MgCl_2$ for 15 min at 37°C. The exonuclease was then inactivated by incubation at 70°C for 5 min and portions were removed for single-stranded break quantitation. The remainder of the DNA was added to a DNA polymerase I reaction containing 7500 cpm/pmol [α-^{32}P]dTTP and 0.3 U/ml DNA polymerase I which was incubated at 37°C with reaction components as described (*66*); the final DNA concentration was 16 μM. Portions were removed at time intervals, and the [α-^{32}P]thymidine incorporated was determined. The Fenton reaction produced 1.2 nicks per DNA molecule, and no additional nicks were generated during the exonuclease III treatment. Identical treatment of DNA which had not been exposed to the Fenton system did not result in significant nucleotide incorporation.

recombination, or sugar fragment excision are most sensitive to oxidative stress (Table 1) (*5, 9, 10*). Under extreme oxidative stress, the SOS operon is induced to further enhance the potential for genetic recombination (*21*).

As a second line of defense against oxidative stress, scavenging enzymes prevent the accumulation of reactive oxygen species. Two catalase enzymes exist in *E. coli*, one of which is induced in response to low levels of H_2O_2. The genetic analysis of this induction has established that catalase is a member of a multigene operon that includes genes encoding alkylhydroperoxide reductase and possibly glutathione reductase (*55*). Induction of this operon contributes resistance to both modes of H_2O_2 toxicity, largely because of the consequent overproduction of catalase (*21*), and catalase-deficient strains are somewhat more sensitive to both modes of killing (*21, 56*).

SOD levels are unresponsive to H_2O_2 exposure; however, they are acutely sensitive to oxygen tension, the presence of $O_2\cdot^-$-generating drugs, and metal concentrations (*57*). Two SODs are present in *E. coli*, and mutation of these enzymes synergistically sensitizes cells to mode-one but not mode-two killing by H_2O_2 (Table 1) (*21, 58*). Glutathione appears to be unimportant to the resistance of *E. coli* to H_2O_2 (Table 1) (*21, 35*); this contrasts to its protective effect in mammalian cells (*59*).

A possible third line of defense might be the deliberate depletion of NADH which could otherwise drive an ongoing Fenton reaction as hypothesized above. In fact, mammalian cells respond to DNA strand breaks by rapidly depleting NAD pools through polymerization to poly(ADP-ribose) (*60*). *Escherichia coli* depletes NAD levels through the NAD-dependent DNA ligase or possibly other mechanisms (*61*).

Conclusions

Studies of the toxicity of H_2O_2 in *E. coli* support the long-held suspicion that the Fenton reaction contributes to mutagenesis and cell death. That reaction is dependent upon the provision of an electron to a metal catalyst. Whereas $O_2\cdot^-$, radiation-excited chromophores, and a variety of cytotoxic drugs may serve as exogenous electron donors, an interesting possibility is that NADH might play this role in vivo, suggesting that even the low level of production of H_2O_2 in routine aerobic metabolism might allow the continuous generation of potent endogenous oxidants. Aerobic organisms synthesize enzymes devoted to the prevention and repair of oxidative damage, and extreme genetic deficiency in either of these two defenses can lead to inviability in aerobic environments (*9, 58, 62*). Oxygen radicals, radiation, and chemicals capable of generating free radicals can also elicit toxic cell damage by mechanisms that are distinct from Fenton chemistry. The explication of these phenomena remains a major challenge for investigators of the toxic effects of oxygen radicals in biology and medicine.

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"Now, of course, comes the awful wait for the other one to drop."