

Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action

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Hydrogen peroxide is extensively used as a biocide, particularly in applications where its decomposition into non-toxic by-products is important. Although increasing information on the biocidal efficacy of hydrogen peroxide is available, there is still little understanding of its biocidal mechanisms of action. This review aims to combine past and novel evidence of interactions between hydrogen peroxide and the microbial cell and its components, while reflecting on alternative applications that make use of gaseous hydrogen peroxide. It is currently believed that the Fenton reaction leading to the production of free hydroxyl radicals is the basis of hydrogen peroxide action and evidence exists for this reaction leading to oxidation of DNA, proteins and membrane lipids *in vivo*. Investigations of DNA oxidation suggest that the oxidizing radical is the ferryl radical formed from DNA-associated iron, not hydroxyl. Investigations of protein oxidation suggest that selective oxidation of certain proteins might occur, and that vapour-phase hydrogen peroxide is a more potent oxidizer of protein than liquid-phase hydrogen peroxide. Few studies have investigated membrane damage by hydrogen peroxide, though it is suggested that this is important for the biocidal mechanism. No studies have investigated damage to microbial cell components under conditions commonly used for sterilization. Despite extensive studies of hydrogen peroxide toxicity, the mechanism of its action as a biocide requires further investigation.

Keywords: oxidizing agents, disinfection, toxicity, mechanism of action

Introduction

The interest in environmentally friendly, non-toxic and degradable yet potent biocides has never been so high. Oxidizing agents, notably hydrogen peroxide (H₂O₂), are increasingly used in a number of medical, food and industrial applications but also in environmental ones such as water treatment. In the medical arena, oxidizing agents are particularly useful for hard surface disinfection and the high-level disinfection of medical devices. Their main advantages are their broad-spectrum activity, which includes efficacy against bacterial endospores, their lack of environmental toxicity following their complete degradation, and the fact that, with imaginative formulation, their surface corrosiveness and smell (for peracetic acid-based products) have been greatly reduced. H₂O₂ is particularly interesting for its application in liquid but also vaporized form for antisepsis and for the disinfection of surfaces and medical devices and for room fumigation (the so-called deep clean).

H₂O₂ was discovered by Louis Thénard in 1818¹ and its use as a disinfectant first proposed by B. W. Richardson in 1891.² It is now in widespread use as a biocide, particularly in applications where its decomposition into non-toxic by-products (water and oxygen) is important. For example, 3%–6% (v/v) peroxide in water is widely used as an antiseptic (in particular

on wounds) and general surface disinfectant. Commercial dental disinfectant formulations such as Dentasept[®] (Muller Dental) and Oxigenal (Kavo) use 1% (294 mM) and 0.4% (118 mM) H₂O₂ as an active ingredient, respectively.³ Many contact lens disinfectant solutions use 3% (882 mM) H₂O₂ as an active ingredient or preservative, including Concerto (Essilor), Oxysept[®] 1 Step (Abbott), Multi[™] (Sauflon) and AOSep[®] 1-step (Ciba Vision).⁴

The relative safety of H₂O₂ solutions has meant that it had also found extensive use in the food industry. Sapers and Sites⁵ discuss a commercial post-harvest wash (Biosafe[®]) that uses H₂O₂ as an active ingredient at an in-use concentration of between 0.27% (79 mM) and 0.54% (159 mM) and a surface disinfectant (Sanosil-25) with an in-use concentration of 0.24% (71 mM) H₂O₂. They also demonstrated the efficacy of 1% (294 mM) H₂O₂ as a wash to decontaminate apples.

Nikkhah *et al.*⁶ state that 'hydrogen peroxide is the most commonly used packing sterilant in aseptic processing systems'. Typically it is used at very high concentrations (35%, 10.3 M) and often in combination with heat.⁷ In contrast, H₂O₂ gas (often referred to as vaporized or gaseous H₂O₂) is typically used at much lower concentrations and temperatures; indeed it has been suggested that the biocidal activity of gaseous peroxide is distinct from that of liquid peroxide.^{8,9}

There is remarkably little literature discussing the exact mechanism(s) of the biocidal action of H₂O₂. As suggested in general reviews on the mechanisms of action of biocides, H₂O₂ is considered an oxidizing agent reactive with the biomolecules (proteins, lipids, nucleic acids, etc.) that make up cellular and viral structure/function.^{10,11} This situation is complicated by the importance of H₂O₂ as a physiological source of reactive oxygen species (ROS) in respiring cells and as a component of the human innate immune system. The majority of studies investigating the toxic mechanism of H₂O₂ therefore consider it as a source of oxidative stress in the cell to model chronic oxidative damage to cells or to investigate the various killing mechanisms of leucocytes.¹²

This review aims to provide a critical overview of the body of knowledge on the toxic mechanisms of H₂O₂ and, importantly, to examine the relevance of various frequently cited studies to the understanding of the biocidal mechanism of H₂O₂ at typical in-use concentrations, in both the liquid and gaseous phases.

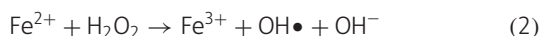
Mechanism of cytotoxicity of H₂O₂

Radical formation and Fenton reaction

The mechanism of cytotoxic activity is generally reported to be based on the production of highly reactive hydroxyl radicals from the interaction of the superoxide (O₂•⁻) radical and H₂O₂, a reaction first proposed by Haber and Weiss¹³ (eq. 1):



Further, it is believed that the production of extremely short-lived hydroxyl radicals within the cell by the Haber–Weiss cycle is catalysed *in vivo* by the presence of transition metal ions (particularly iron-II) according to Fenton chemistry¹⁴ (eq. 2):



It is known that, *in vitro*, the hydroxyl radical and other oxygenated species can act as potent oxidizing agents, reacting with lipids, proteins and nucleic acids.¹⁵ It is easy to propose that such reactions can account for the antimicrobial effects of H₂O₂ and a number of scientific publications have used such an explanation to describe the action of the oxidizing agents.

Evidence for Fenton-like chemistry in biocidal activity of H₂O₂

Evidence for this mechanism in the action of H₂O₂ on bacterial cells was provided by Repine *et al.*¹⁶ They demonstrated that growing *Staphylococcus aureus* overnight in Bacto nutrient broth containing increasing concentrations of iron resulted in an increase in intracellular iron content. Incubation of harvested cells at 37°C in Hanks' balanced salt solution containing a range of H₂O₂ concentrations showed that those cells with an elevated iron concentration had greater susceptibility to H₂O₂, as measured by a decrease in the H₂O₂ concentration required to kill 50% of cells after a 60 min exposure. An increase in iron concentration in the growth medium was shown to have no effect on the growth rate or viability of *S. aureus* or on catalase or

peroxidase activity. Repine *et al.*¹⁶ also showed that the addition of thiourea, dimethyl thiourea, sodium benzoate and dimethyl sulphoxide inhibited the toxic effects of H₂O₂ in proportion to the effectiveness of the substances as hydroxyl scavengers. Again, the addition of these substances had no effect on viability, nor were they found to directly react with H₂O₂. Further evidence was found by Mello Filho *et al.*,¹⁷ who showed that the potent iron chelator 1,10-phenanthroline (1,10-phen) could penetrate cultured mouse cells and protect them against killing by H₂O₂. The chelator alone had no effect on cell viability.

Indirect evidence of DNA damage

Imlay and Linn¹⁸ exposed *Escherichia coli* K12 to varying concentrations of H₂O₂ for 15 min at 37°C in K medium. They observed that cells were more susceptible to low (<3 mM) concentrations of H₂O₂ than to intermediate (5–20 mM) concentrations. At >20 mM H₂O₂, survival was inversely proportional to concentration. This response is shown in Figure 1. A slight dip in the surviving fraction of the culture can be seen at concentrations <3 mM. This effect was found to be reproducible and was greatly magnified in DNA repair-deficient and anoxically grown strains; these were particularly sensitive to H₂O₂ at low concentrations but not especially susceptible to higher concentrations when compared with aerobically grown wild-type cells. Cells starved by incubation in M90 salts for 80 min before exposure to H₂O₂ were not killed by low concentrations of H₂O₂. Comparisons of the kinetics of killing of an exonuclease-II-deficient strain at various H₂O₂ concentrations were also made. Total kill by both lower and higher H₂O₂ concentrations was found to be time-dependent (Figure 2). They postulated that killing of *E. coli* cells by H₂O₂ occurs according to two distinct modes, with mode-1 killing occurring at low concentrations due to DNA damage and mode-2 killing occurring at higher concentrations due to damage to other target(s).

Similar results were obtained by Brandi *et al.*,¹⁹ who also observed the bimodal killing pattern seen by Imlay and Linn¹⁸ after challenging *E. coli* in M9 salts with various H₂O₂ concentrations for 15 min. In addition, it was found that mode-2 killing was markedly reduced in anoxic conditions, whereas no effect was seen on mode-1 killing.

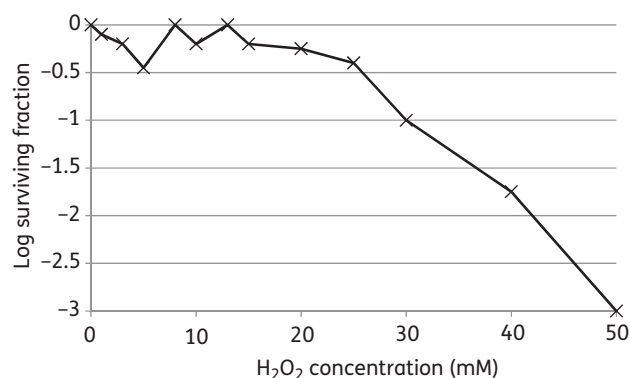


Figure 1. Chart showing the log₁₀ surviving fraction of wild-type *E. coli* K12 culture after 15 min of exposure to various H₂O₂ concentrations. Adapted from Imlay and Linn¹⁸ with permission from the American Society for Microbiology.

Brandi *et al.*¹⁹ also compared the effect of the hydroxyl scavenger thiourea on killing by 2.5 or 25 mM H₂O₂. Thiourea at 35 mM was found to markedly reduce killing by 25 mM H₂O₂ (hypothesized to be due to the mode-2 mechanism) whilst the same concentration had no effect on killing by 2.5 mM H₂O₂ (hypothesized to be due to the mode-1 mechanism). An obvious criticism of this work is that thiourea, a potent reducing agent, is capable of reacting directly with H₂O₂. This is a first-order reaction with respect to H₂O₂ concentration, so it is possible that the thiourea simply reduces the higher concentration of H₂O₂ without scavenging hydroxyl radicals. Unlike the earlier work of Repine *et al.*,¹⁶ it was not checked that this direct reaction did not occur in their test system, nor were alternative OH• scavengers tested.

Brandi *et al.*¹⁹ concluded that mode-2 killing was dependent on the presence of oxygen and hydroxyl radicals, and suggested that this mechanism is indeed due to the Fenton chemistry previously outlined, whilst the mode-1 killing was not dependent on oxygen and hydroxyl radicals.

A study by Macomber *et al.*²⁰ using copper export-deficient strains of *E. coli* grown in a copper-supplemented medium showed that these strains accumulated copper within the cell, but that this increase actually inhibited both killing and mutagenesis in a DNA repair-deficient strain by millimolar concentrations of H₂O₂. Though they could find no definitive explanation for the inhibitory effect of the copper, their work shows that mode-1 and mode-2 killing due to DNA damage is not mediated by copper.

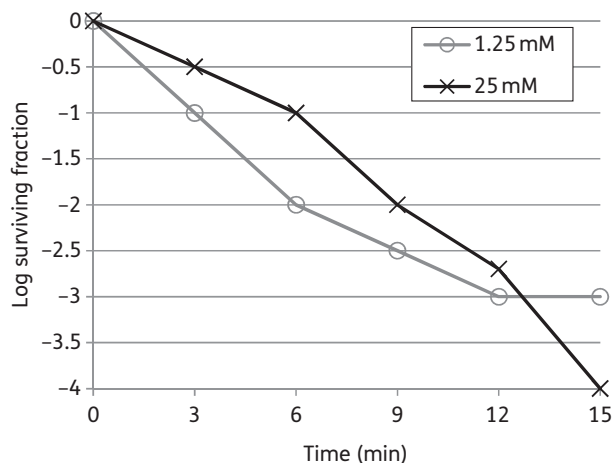


Figure 2. Chart showing change in the surviving fraction with time of exposure to 1.25 mM H₂O₂ (mode-1 killing) and 25 mM H₂O₂ (mode-2 killing). Adapted from Imlay and Linn¹⁸ with permission from the American Society for Microbiology.

In vitro investigations of DNA damage

Due to the relevance of mode-1 killing as a general model of oxidative DNA damage under aerobic conditions and its usefulness in elucidating the cellular mechanisms of prevention and repair of such damage, much subsequent work has focused on the mode-1 mechanisms of DNA damage. Imlay *et al.*²¹ developed an *in vitro* model capable of producing the same pattern of damage to purified DNA as that observed in the process of killing of bacteria. This model consisted of phage PM2 DNA incubated with ferrous sulphate and various concentrations of ethanol and H₂O₂. This system produced single-strand breaks in the purified DNA. The production of breaks was reduced by approximately half by the addition of micromolar concentrations of ethanol, but was not further decreased by the addition of up to 10 mM ethanol. Addition of a range of H₂O₂ concentrations to the system containing 10 mM ethanol produced a DNA nick dose response similar to that seen in mode-1 killing of *E. coli*; the highest number of nicks was produced by 50 μM H₂O₂, and this was reduced by half and remained approximately constant on addition of 1–10 mM H₂O₂. As ethanol is a potent scavenger of hydroxyl radicals, it was concluded that the mode-1 killing of bacterial cells due to DNA damage by H₂O₂ is not dependent on the production of free hydroxyl radicals, and is more likely to be due to the production of ferryl radical intermediates from DNA complexation.

Further work on Imlay and Linn's *in vitro* model by Luo *et al.*²² found that DNA nicking was maximal at 50 μM H₂O₂, dropping to one-third maximal at 3 mM and remaining roughly constant between 3 and 50 mM. Addition of 17 μM ethanol to the model reduced nicking by 30%–50% at all H₂O₂ concentrations. Increasing the ethanol concentration to 10 mM reduced nicking by a further 50% at H₂O₂ concentrations <100 μM, but had no effect at higher peroxide concentrations. Increasing the ethanol concentration to 100 mM caused further inhibition at H₂O₂ concentrations <3 mM, but again had no effect at higher concentrations. These findings are summarized in Table 1. Luo *et al.*²² therefore concluded that there are at least three chemically distinct classes of oxidant species produced by Fenton-type reactions in the presence of iron: type I are sensitive to H₂O₂ but moderately resistant to ethanol; type II are resistant to both H₂O₂ and ethanol; and type III are sensitive to H₂O₂ and ethanol.

Evidence for different types of DNA oxidation reactions

Luo *et al.*²² went on to investigate the effects of the iron chelators 1,10-phen and 2,2'-dipyridyl (2,2'-dipy) both on *E. coli* killing and on DNA nicking in the *in vitro* model. Both chelators blocked mode-1 killing, whilst 2,2'-dipy had no effect on mode-2 killing, and 1,10-phen substantially enhanced it. Both chelators also

Table 1. Effects of increasing H₂O₂ and ethanol concentration on DNA nicking *in vitro*; summarized from Luo *et al.*²²

Ethanol concentration	<100 μM H ₂ O ₂	0.1–3 mM H ₂ O ₂	3–50 mM H ₂ O ₂
No ethanol	maximal nicking	1/3× maximal nicking	1/3× maximal nicking
17 μM	reduced nicking	reduced nicking	reduced nicking
10 mM	further reduced nicking	further reduced nicking	no further reduction in nicking
100 mM	further reduced nicking	no further reduction in nicking	no further reduction in nicking

blocked DNA nicking at low H₂O₂ concentrations but not at higher concentrations; the peak of DNA nicking activity at 50 μM H₂O₂ was completely eliminated by the addition of either chelating agent plus 100 mM ethanol, while nicking was constant at around 50% of the maximum between 50 μM and 50 mM H₂O₂ with 2,2'-dipy and 100 mM ethanol, and was enhanced 6-fold by the addition of 1,10-phen between 50 μM and 50 mM H₂O₂. As 2,2'-dipy chelates unbound Fe²⁺ and remains in solution, whilst 1,10-phen chelates unbound Fe²⁺ and then intercalates into the DNA backbone, they proposed the following model of oxidant damage. Type I oxidants are formed by Fe²⁺ ions associated but not bound to DNA—suggesting that these could exist in a ‘cationic cloud surrounding the polyanionic DNA helix’ (Luo *et al.*²²). Such oxidants are accessible to H₂O₂ quenching, but would require higher concentrations of ethanol to effectively quench them due to high localized concentrations within the ‘cationic cloud’. These oxidants are responsible for mode-1 killing. Type II oxidants are formed by Fe²⁺ more tightly associated with DNA, and once formed they are not accessible to H₂O₂ or ethanol quenching, and are responsible for mode-2 killing. Type III oxidants are produced by free Fe²⁺ ions in solution, they are easily available to H₂O₂ and ethanol to quench, and due to their short half-life are unlikely to be involved in killing due to DNA damage *in vivo*. The action of the chelating agents can be explained thus: 2,2'-dipy removes the ‘cationic cloud’ and causes the formation of type III rather than type I oxidants, inhibiting mode-1 killing; 1,10-phen removes the ‘cationic cloud’ and possibly free Fe²⁺ ions and intercalates them into the DNA backbone and causes the formation of type II rather than type I or type II oxidants, enhancing mode-2 killing and inhibiting mode-1 killing. These findings are summarized in Table 2.

Studies by Henle and Linn²³ suggest that the type I and II oxidants have different preferred cleavage sequences on the DNA molecule, with type I preferentially cleaving the sequences RTGR, TATTY and CTTR and type II preferentially cleaving the sequence NGGG (where the underlined bases show the cleavage site). They suggested that this selectivity could be due to sites of iron localization, or that such sequences act as sinks for radical electrons formed elsewhere on the DNA chain.

Direct evidence of DNA damage *in vivo*

None of the studies described above directly measured DNA damage *in vivo*. DNA repair-deficient strains were found to be more susceptible to killing, and so it was concluded that DNA

Table 2. Properties of three types of DNA oxidant formed by H₂O₂ and Fe²⁺; summarized from Luo *et al.*²²

Property	Type I	Type II	Type III
Resistant to H ₂ O ₂ ?	no	yes	no
Resistant to ethanol?	moderately	yes	no
Effect of 1,10-phen	decreased	increased	possibly decreased
Effect of 2,2'-dipy	decreased	none	increased
Position	cationic cloud	DNA backbone	free in solution
Killing mode	mode-1	mode-2	none

damage was the cause of death, but it could be suggested that this does not logically follow. One can equally easily imagine that lethal DNA damage in fact only occurs in repair-deficient strains and this acts in addition to some other damage to increase the bactericidal effect seen with wild-type strains. Whilst the *in vitro* studies performed using Imlay and Linn's model add support to the hypothesis that DNA damage is the main cause of the bactericidal effect of low concentrations of H₂O₂, they do not provide proof that such damage also occurs *in vivo*. Studies that directly measure DNA damage caused by H₂O₂ have been performed using several different methods to estimate different types of DNA damage.

Ananthaswamy and Eisenstark²⁴ exposed *E. coli* strain W3110 to 10 mM H₂O₂ in phosphate buffer for 10 min at 25°C then measured the number of single-strand breaks formed using alkaline sucrose gradient sedimentation. They found that the treatment produced 153 single-strand breaks per genome, of which all but 74 could be repaired by further incubating the culture for 15 min in phosphate buffer at 25°C, and all but 14 could be repaired by incubating the culture for 40 min in M9 medium at 37°C.

Hagensee and Moses²⁵ exposed *E. coli* strain W3110 to 117 mM H₂O₂ in phosphate buffer, pH 7.4, for 10 min at 37°C then measured the number of single-strand breaks formed using alkaline gradient centrifugation. They found that the treatment produced 482 single-strand breaks per genome, and all but 18 could be repaired by incubating the culture for 4 h in M9 medium at 37°C.

Rohwer and Azam²⁶ exposed exponential-phase cultures of *E. coli* strain K37 and the archaeon *Haloferax volcanii* to 0.2% (59 mM) H₂O₂ in Luria-Bertani (LB) broth or *H. volcanii* medium, respectively, for 30 min at room temperature. The treated cultures were then analysed using the terminal deoxyribonucleotide transferase-mediated dUTP nick end labelling (TUNEL) method to label 3'-OH DNA ends and flow cytometry. They found that 97.4% of the H₂O₂-treated *E. coli* cells were TUNEL positive (i.e. had 3'-OH ends) compared with <1% of control cells. Similarly, 84.3% of treated *H. volcanii* cells were TUNEL positive compared with 9.6% of control cells. This effect could be reduced by pre-treating the cultures with protein synthesis inhibitors: chloramphenicol pre-treatment of *E. coli* reduced the percentage of TUNEL-positive cells after H₂O₂ exposure to 7.8%, whilst diphtheria toxin pre-treatment reduced the percentage of TUNEL-positive *H. volcanii* cells to 31.4%. Exposing the *E. coli* culture to H₂O₂ for a longer time (60 min, compared with 30 min) increased fluorescence in TUNEL-positive cells, as did treatment with an increased concentration of H₂O₂ [0.4% (118 mM), compared with 0.2%]. Stationary-phase *E. coli* cultures exposed to 0.4% H₂O₂ for 30 min did not demonstrate any detectable breaks. The authors therefore concluded that H₂O₂ exposure results in oxidation of DNA bases and that these oxidized bases are recognized and excised by DNA repair mechanisms, resulting in single-strand breaks in the DNA molecule. The amount of breaks thus produced increases with exposure time and concentration of H₂O₂.

Fernández *et al.*²⁷ exposed both exponential- and stationary-phase *E. coli* strain TG1 cultures to 10 mM H₂O₂ in LB broth for 10 min at room temperature. DNA damage was then assessed using a diffusion assay, and 100% of nucleoids in both stationary and exponential phases were found to show ‘extensively

fragmented DNA', compared with 0.4% and 37.6% of untreated exponential- and stationary-phase cultures, respectively. They therefore concluded that exposure to H₂O₂ causes substantially more damage to DNA than can be assessed using the TUNEL method—i.e. the TUNEL method can detect only the formation of 3'-OH ends in single-strand breaks, and these are not the only lesions that occur.

Summary of evidence of DNA damage by H₂O₂

Whilst the studies described here give a very complete model of the genotoxic action of low concentrations of H₂O₂ on bacterial cells, one should use caution in applying these findings to the bactericidal mechanism of H₂O₂ as a disinfectant. None of these studies simultaneously measured reduction in cell count and formation of DNA damage for a range of H₂O₂ concentrations and/or exposure times in order for a correlation between cells killed and DNA damage to be calculated. Variations in strains and species used, treatment conditions and exposure times also make comparison of the studies difficult. In particular, the media used to treat cultures varied greatly between the studies, from simple phosphate buffer to complex media such as LB broth. It has been shown that the presence of other substances can have a significant effect on the efficacy of bactericidal action of H₂O₂, both positive and negative. For example, Berglin *et al.*²⁸ showed that the presence of 0.1 mM cysteine in the growth medium increases sensitivity of *E. coli* strain K12 100-fold to 0.1 mM H₂O₂, so conclusions about the bactericidal mechanism need to take media composition into account.

Most crucially, the majority of the studies described used low concentrations of H₂O₂ and long exposure times—typically 50 μM to 2.5 mM H₂O₂ with >15 min of exposure. In contrast, the typical concentration of surface sterilant solution of H₂O₂ is 3%, equivalent to 882 mM. The literature on DNA oxidation by low concentrations of H₂O₂ is therefore of questionable significance to the use of H₂O₂ as a surface disinfectant. As mode-2 killing due to DNA damage is hypothesized to be reliant on Fe²⁺ ions closely associated with the DNA molecule and the presence of cellular reductants to drive the Fenton cycle, it is possible that the DNA damage dose-response to H₂O₂ will reach a maximum at some concentration of H₂O₂, and that damage to other cellular components will start to become more important at higher concentrations. The mechanisms of H₂O₂ damage to the other major macromolecular targets within the bacterial cell, namely protein and lipids, are not so well studied as DNA damage.

Interactions of H₂O₂ with proteins and amino acids

Unlike DNA damage, where *in vitro* work shows that H₂O₂ alone is unreactive with DNA, a mechanism exists for the non-radical-based reaction of H₂O₂ with proteins even in the absence of metal ions. Luo *et al.*²⁹ and Ashby and Nagy³⁰ discussed in detail the kinetics and mechanism of the reaction of H₂O₂ with cysteine in the absence of metal ions. Kim *et al.*³¹ developed a method based on the selective and competitive reaction of H₂O₂ and biotin-conjugated iodoacetamide with cysteine residues exhibiting low pK_a to allow labelling of proteins containing such residues. Using this method, they identified

several proteins present in various mammalian cells lines that are preferentially oxidized by H₂O₂. Finnegan *et al.*⁹ have also demonstrated oxidation of cysteine, methionine, lysine, histidine and glycine on exposure of 100 mM amino acid to 100 mM H₂O₂ in the absence of added metal ions.

Returning to the metal-ion-catalysed production of hydroxyl radicals, oxidation of several amino acid residues has been shown to occur by this reaction; Dean *et al.*³² summarized the various products of radical-mediated oxidation of amino acids. The oxidation of protein amino acids can result in a range of modifications, from total cleavage of the protein backbone to subtle side-chain modification of individual residues. The production of carbonyl residues is often used as an indicator of protein oxidation, as it is the outcome of many oxidative modifications and is easily quantified. The effect of H₂O₂ treatment on the amount of protein carbonyls has been assessed by several groups, though, as for DNA damage, H₂O₂ treatment was generally considered as a source of oxidative stress rather than as a biocide.

Tamarit *et al.*³³ grew *E. coli* K12 strain ECL1 anaerobically and challenged these cultures with 2 mM H₂O₂ for up to 45 min, following which 1 mL samples of the cultures were taken and crude protein extracts prepared. The protein extracts were derivatized with dinitrophenyl hydrazine (DNPH) and separated by one-dimensional SDS-PAGE. Western blot immunoassay was used to detect DNPH-derivatized carbonyl groups on the protein bands, and bands of interest were identified using Edman degradation. It was found that H₂O₂ stress caused a 30% reduction in cell viability (as previously discussed, mortality under these conditions has been suggested to be mostly due to mode-1 killing) and a 3-fold increase in protein carbonyl content of crude extract. Protein bands exhibited widely varying increases in carbonyl content—several proteins, such as alcohol dehydrogenase E, enolase, DNA K, EF-G and an outer membrane protein A, showed a substantial increase in carbonyl content, whilst two major protein bands (EF-Tu and outer membrane protein C) were not oxidized at all. This same pattern was seen with cells grown under aerobic conditions, though the increase in carbonyl content here was not so dramatic (50% compared with 300%) due to the activation of oxidative stress response systems.

Cabiscol *et al.*³⁴ went on to repeat this work with the yeast *Saccharomyces cerevisiae* treated with 5 mM H₂O₂ for 45 min, and again a selective pattern of protein oxidation was observed.

Whilst the work of Tamarit *et al.*³³ and Cabiscol *et al.*³⁴ was performed using low concentrations of H₂O₂ more usually associated with mode-1 killing, the exposure time of 45 min used was far longer than the normal exposure time tested for a biocide. In other words, it is possible that the selective oxidation of proteins by low concentrations of H₂O₂ over long exposure times could be reproduced by high concentrations of H₂O₂ over short time scales. The action of 3% H₂O₂ as a surface disinfectant might therefore be far more selective, in terms of those proteins most damaged, than often considered.

At this stage it is appropriate to reflect on the different effects that can be observed with H₂O₂ when tested diluted in water, in formulation with other chemicals or, notably, when in gas form. Finnegan *et al.*⁹ observed important differences in the interaction of vaporized (gaseous) and liquid H₂O₂ against amino acids.

Vaporized H₂O₂ (2 mg/L for 10 min, when tested under true gas, non-condensed conditions) was shown not to be able to oxidize amino acids (100 mM) whereas liquid H₂O₂ was shown to oxidize cysteine, methionine, lysine, histidine and glycine at various H₂O₂/amino acid ratios. However, both liquid (12 mg/L) and vaporized H₂O₂ (2 mg/L for 10 min) completely degraded BSA and aldolase. Others have reported potential cross-linking effects on protein exposure to liquid peroxide but protein degradation (into smaller peptides) on exposure to gaseous peroxide.^{35,36} It is clear that liquid and gaseous H₂O₂ interact differently with macromolecules, which may explain their differences in biocidal efficacy.

Interactions of H₂O₂ with bacterial cell membranes and lipids

Studies of the effect of H₂O₂ on bacterial cell membranes are also limited. Whilst much work has been performed using H₂O₂ as a source of ROS to simulate the effects of oxidation during ageing on mammalian cells, a literature search using PubMed found only three studies investigating the effects of H₂O₂ on the membrane of any bacteria. Brandi *et al.*³⁷ exposed *E. coli* cells to mode-1 and mode-2 concentrations of H₂O₂ and studied effects on cell morphology and the cell membrane. They found that low concentrations (1.75 mM), producing mode-1 effects, caused extensive cell filamentation, but that this change in morphology did not occur at higher (17.5 mM) H₂O₂ concentrations; instead a large decrease in cell volume was observed.

Brandi *et al.*³⁷ also found that loss of intracellular contents, as measured by lactate dehydrogenase activity in culture medium, occurred at a low rate initially under mode-1 killing conditions, and ceased after 150 min. In contrast, the higher concentration of H₂O₂ produced a much larger increase in lactate dehydrogenase activity in the culture medium. They hypothesized that the reduction in cell volume seen during mode-2 killing was due to cell membrane damage and loss of intracellular material, and suggested that this was the major component of mode-2 killing.

Baatout *et al.*³⁸ exposed *Ralstonia metallidurans*, *E. coli*, *Shewanella oneidensis* and *Deinococcus radiodurans* cultures to concentrations of H₂O₂ up to 880 mM (the only study to investigate the typical 3% H₂O₂ used in disinfectant solutions) for 1 h then measured various indicators of cell physiology. They found that cell membrane permeability, as measured by propidium iodide uptake, was markedly increased in all strains at H₂O₂ concentrations >13.25 mM.

Finally, Peterson *et al.*³⁹ measured the release of organic compounds from the cyanobacterium *Aphanizomenon flos-aquae* after exposure to various water treatments, including H₂O₂. They found a substantial increase in the release of dissolved organic compounds and the odour compound geosmin with increasing H₂O₂ concentration up to 0.025% (equivalent to 0.73 mM), and that cell membrane damage, as measured by potassium leakage, also increased with peroxide concentration up to 0.01% (equivalent to 0.29 mM).

All three studies showed some degree of cell membrane damage due to exposure to H₂O₂, and Brandi *et al.*³⁷ suggested that such damage may be a major component of mode-2 killing of *E. coli*.

Vaporized H₂O₂ as a sterilant

The use of vaporized (or gaseous) H₂O₂ as a sterilant was pioneered in the packaging industry by Wang and Toledo in the late 1980s.⁴⁰ Its use in preference to a liquid solution of 35% (equivalent to 880 mM) H₂O₂ was initially investigated in order to avoid residual traces being retained on packaging; however, it also offers the advantage over liquid in that large volumes and devices that might be damaged by exposure to water can be easily sterilized. Other advantages over alternative vapour-phase methods (e.g. based on ozone or peracetic acid) are low toxicity and spontaneous breakdown into completely harmless by-products.⁴⁰

Since Wang and Toledo's initial work, commercial vapour-phase H₂O₂ treatments have been developed and their efficacy investigated in several applications, including decontamination of laboratory and medical equipment, hospital wards and pharmaceutical manufacturing facilities. They have been shown to be efficacious against a wide range of organisms, including those producing endospores,^{41,42} Gram-positive and Gram-negative vegetative cells,^{43,44} DNA and RNA viruses^{45–47} and fungi.^{44,48,49} These systems vary in their use of H₂O₂, ranging from pure gas-based processes (often referred to as 'dry'), condensed peroxide (formed from a saturated gas, referred to as 'wet') and liquid misting systems (for liquid peroxide distribution within an area⁵⁰). Antimicrobial efficacy, surface compatibility and safety aspects can vary between these systems.

Despite the increasing use of such decontamination methods and the growing body of literature detailing the validation of these methods for use in various applications, it appears that little work has been done on understanding the mechanism(s) of biocidal activity of the vapour form of H₂O₂. Indeed, one early study of a vapour system to sterilize centrifuges performed by Klapes and Vesley⁵¹ concluded 'The application of VPHP [vapour phase H₂O₂] as a potential sterilant is still clearly in its infancy: definitive knowledge of the mechanism(s) of cidal action, and the factors which influence it, is lacking', whilst a study of the use of H₂O₂ vapour to deactivate *Mycobacterium tuberculosis* performed by Hall *et al.*⁵² stated in the conclusion 'the exact mechanism of action of HPV remains to be fully elucidated'.

The lack of investigation into the vapour-phase H₂O₂ cidal mechanism appears more remarkable in the light of a study performed by Fichet *et al.*,³⁵ which showed that, of several decontamination methods tested, gaseous H₂O₂ prevented the exhibition of spongiform pathologies in hamsters following intercerebral inoculation with steel wires contaminated with infectious brain matter and then treated with the decontamination methods.

Despite this, Yan *et al.*⁵³ reported little effect with an H₂O₂ gas plasma system, but this may be explained by the fact that such a system can be associated with condensed (therefore liquid/gas) H₂O₂ in contrast to a non-condensed gas-based process (as discussed by Fichet *et al.*^{35,36}). A follow-up study performed by Fichet *et al.*³⁶ with a vacuum-based sterilization process using non-condensed gas reproduced this destruction of infectivity with gaseous H₂O₂ but not liquid H₂O₂. *In vitro* studies showed unfolding and degradation of the prion proteins by gaseous but not liquid H₂O₂.

These studies suggest that the ability of H₂O₂ to degrade protein oxidatively is greatly enhanced in the vapour phase

compared with the liquid phase. This had also been observed in studies with the neutralization of bacteria protein toxins, such as those produced by *Clostridium botulinum* and *Bacillus anthracis* (McDonnell⁸). Further evidence for this was provided by Finnegan *et al.*,⁹ who showed that vaporized H₂O₂ could completely degrade BSA and aldolase, whilst liquid H₂O₂ had no effect on either. These results suggest that there are subtle differences in the mechanisms of action of liquid and gaseous H₂O₂, with potential impact on antimicrobial efficacy. This may not only be the case with protein neutralization or prion infectivity reduction. It has been known for some time that the organism most resistant to liquid peroxide appears to be *Bacillus subtilis* (or *Bacillus atrophaeus*), in contrast to that for gaseous peroxide, which is *Geobacillus stearothermophilus*.^{4,8} Similarly, antimicrobial efficacy against viruses can vary between condensed and non-condensed peroxide disinfection processes, with the condensed (high peroxide concentration liquid) systems potentially allowing viruses to be protected from the antimicrobial effects of the liquid/gas.^{44,46}

Conclusions

The two decades since Imlay and Brandi's initial demonstration of the existence of the bimodal effect have seen studies showing that the precise mechanism of genotoxicity of low H₂O₂ concentrations is far subtler than might initially have been thought. Although H₂O₂ is obviously far too simple a substance to exhibit innate selectivity of action, such is the complexity of the cellular environment that it would nevertheless appear that H₂O₂ genotoxicity occurs due to two distinct mechanisms, both of which cleave preferentially at different sets of nucleotide sequences. H₂O₂ might itself act as a sink for the majority of radicals produced by Fenton chemistry in the target cell, with only those radicals produced immediately proximate to the DNA chain able to react and cause damage. Based on this observation, it is possible that, at higher concentrations of H₂O₂, the amount of DNA-associated iron becomes the limiting factor in the reaction between H₂O₂ and DNA, and thus further increases in the H₂O₂ concentration might not necessarily result in an increase in the rate of DNA damage. Oxidation of proteins and lipids could thus be more significant to the biocidal mechanism at higher concentrations of H₂O₂.

Compared with DNA damage, oxidation of other bacterial cell components by H₂O₂ is far less studied, though evidence exists of damage to both proteins and the cell membrane. A number of studies have suggested that oxidation of bacterial proteins is also a more selective process than typically reported, with specific proteins being more or less vulnerable to oxidation.

Whilst the studies described provide clues as to the biocidal mechanism of H₂O₂, there is a limit to how much can be learned about this from studies designed to investigate the effect of oxidative stress on a particular type of macromolecule. For instance, a study designed only to investigate damage to DNA can tell us whether such damage occurs, but will give no information as to how important this damage is to the bactericidal effect; it is impossible to draw conclusions as to the importance of the putative lesions to the lethal mechanism of H₂O₂ without a simultaneous measurement of damage to all bacterial cell components, and a correlation of this damage with a reduction in viable cell count. Such a study has not been performed, and

consequently our knowledge of the bactericidal mechanism of H₂O₂ action at the molecular level must be considered incomplete, especially with the high H₂O₂ concentrations and short contact times representative of H₂O₂ biocidal applications. Certainly, examination of the evidence appears to dispel the model of free hydroxyl radical production as the main mechanism of H₂O₂ action at higher concentrations.

Finally, it is apparent that there is likely a qualitative difference between the biocidal mechanisms of liquid-phase and gas-phase H₂O₂ that cannot be explained by current models. Evidence suggests that H₂O₂ vapour can fragment protein without Fenton-like reactions and the importance of this phenomenon needs to be examined.

Despite extensive studies of H₂O₂ toxicity, the mechanism of its action as a biocide requires further investigation. This may assist in the optimization of its antimicrobial effects for future antimicrobial and neutralization applications.

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