Antibacterial Effects of Hydrogen Peroxide and Methods for Its Detection and Quantitation†

BENJAMIN J. JUVEN* and MERLE D. PIERSON

Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

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ABSTRACT

Hydrogen peroxide is responsible for certain bactericidal effects observed in biological systems, such as growth inhibition of one bacterial species by another and killing of invading microorganisms by activated phagocytic cells. \( \text{H}_2\text{O}_2 \) might be generated in bacteriological media by their exposure to light and/or oxygen and become an important mediator of toxic effects. \( \text{H}_2\text{O}_2 \) cytotoxicity is apparently due to its capacity—generally mediated by transition metal ions—to generate more reactive and cytotoxic oxygen species such as the hydroxyl radical, which is a powerful oxidant, and which can initiate oxidation of biomolecules. The conversion of \( \text{H}_2\text{O}_2 \) into more cytotoxic compounds may be potentiated by reducing agents and by peroxidases. Cells may protect themselves against \( \text{H}_2\text{O}_2 \) toxicity either by the action of catalases or, in the case of DNA damage, by repairing the damage after it has taken place. Assays for the detection and quantitation of \( \text{H}_2\text{O}_2 \) in cell cultures include those based on (i) catalase-dependent oxidation of formate to \( \text{CO}_2 \), (ii) generation of fluorescent products due to a \( \text{H}_2\text{O}_2 \)-mediated oxidative reaction, (iii) the loss of fluorescence upon the oxidation of scopoletin, (iv) change in absorbance upon oxidation of phenol red, or (v) formation of complexes with peroxidases. Some possible antimicrobial uses of \( \text{H}_2\text{O}_2 \) in the food industry are presented.

Key words: Hydrogen peroxide, mechanism of antibacterial action, bacterial resistance, methodology

Hydrogen peroxide is generated by almost all bacteria growing aerobically, but it is usually detected in aerobic cultures of catalase-negative bacteria (5, 154). The best-understood mechanism by which activated phagocytic cells (macrophages, monocytes, neutrophils) act to kill invading microorganisms is via a respiratory burst characterized by enhanced generation of toxic oxygen species and in particular \( \text{H}_2\text{O}_2 \) (90). The antimicrobial properties of \( \text{H}_2\text{O}_2 \) have been recognized for years and a variety of applications have been suggested and developed. Dilute solutions of \( \text{H}_2\text{O}_2 \) are used as antiseptics (57). Certain types of honey were found to exhibit antagonistic effects against bacteria connected with skin infections (e.g., \textit{Staphylococcus aureus}) attributable to their \( \text{H}_2\text{O}_2 \) content (2, 155).

This article reviews the production of \( \text{H}_2\text{O}_2 \) by bacterial cells, its antibacterial effects and mode of action, mechanisms of bacterial resistance to \( \text{H}_2\text{O}_2 \), and the methodology developed for its detection and quantitation. Antimicrobial applications of \( \text{H}_2\text{O}_2 \) in the food industry are also briefly described.

PRODUCTION OF \( \text{H}_2\text{O}_2 \) BY BACTERIAL CELLS

Growth inhibition of one bacterial species by \( \text{H}_2\text{O}_2 \) generated by another species is a well-recognized mechanism of bacterial antagonism (35, 118, 153) and has, in some cases, been referred to as one of the possible factors responsible for the predominance of a certain species (66). Raccach and Baker (120) examined the production of \( \text{H}_2\text{O}_2 \) in vitro by two commercial strains of meat starter cultures of \textit{Pediococcus cerevisiae} and \textit{Lactobacillus plantarum}. They found a maximal level of 0.85 \( \mu \text{g} \) (25 nmoles) of \( \text{H}_2\text{O}_2 \) per ml of suspension containing \( 10^9 \) cells, which was not considered high enough for antimicrobial activity.

\( \text{H}_2\text{O}_2 \) might be also generated in bacteriological media by their exposure to light and/or oxygen and become an important mediator of the toxic effects observed (4, 77), those effects being reversed by the addition of catalase to the culture media (67, 77). Accumulation of metabolic \( \text{H}_2\text{O}_2 \), attributed to low catalase activity, has been implicated as a major factor for the decreased colony-forming ability of thermally stressed cells of \textit{S. aureus} (24, 47). Cells of \textit{Campylobacter jejuni} surviving mild heating or freezing treatments were unable to grow in nutrient agar, apparently due to their increased sensitivity to \( \text{H}_2\text{O}_2 \); the addition of catalase reversed medium toxicity (67).

Species of the genus \textit{Propionibacterium} produce intracellular porphyrins (85, 93) that may lead to porphyrin-mediated photosensitizing reactions in the presence of light and oxygen, with production of toxic species of oxygen, \( \text{H}_2\text{O}_2 \) and \( \text{HO}^- \) (9) and damage to microbial cells (56, 84, 97).

\( \text{H}_2\text{O}_2 \) is probably generated in small amounts by almost all microorganisms growing aerobically. In aerobic cultures,
TABLE 1. Substrates and reactions that might lead to production of H_{2}O_{2} by lactobacilli

<table>
<thead>
<tr>
<th>Substrates, reaction</th>
<th>Catalyzed by</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate + O_{2} + phosphate —— acetyl-phosphate + CO_{2} + H_{2}O_{2}</td>
<td>Pyruvate oxidase</td>
<td>(54, 95, 108)</td>
</tr>
<tr>
<td>Lactate + O_{2} —— pyruvate + H_{2}O_{2}</td>
<td>l-Lactate oxidase</td>
<td>(81)</td>
</tr>
<tr>
<td>NADH + H^{+} + O_{2} ——</td>
<td>NADH oxidase</td>
<td>(33, 54)</td>
</tr>
<tr>
<td>NAD + H_{2}O_{2}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O_{2}^{-} + 2 H^{+} —— H_{2}O_{2}</td>
<td>Mn^{2+} complexes or SOD*</td>
<td>(7)</td>
</tr>
<tr>
<td>Saturated fatty acids + O_{2} —— H_{2}O_{2}</td>
<td>Fatty acyl-Co A</td>
<td>(36)</td>
</tr>
<tr>
<td>α-Glycerophosphate + O_{2} —— dihydroxyacetone phosphate + H_{2}O_{2}</td>
<td>α-Glycerophosphate oxidase</td>
<td>(33)</td>
</tr>
</tbody>
</table>

* The replacement of superoxide dismutase (SOD) by Mn^{2+} as a superoxide anion (O_{2}^{-}) scavenger is a unique feature of lactic acid bacteria and the very high manganese requirement and content of lactobacilli may reflect this function (7).

Oxygen is used as an alternative electron acceptor and is reduced to H_{2}O_{2} or water (33). Not possessing heme, lactobacilli, as well as other lactic acid-producing bacteria, do not utilize the cytochrome system (which reduces oxygen to water) for terminal oxidation during their respiratory processes. Whittenbury (154) demonstrated that there is no correlation between H_{2}O_{2} production by lactic acid bacteria and a preference for anaerobic or aerobic growth conditions. He suggested that these organisms contain flavoprotein oxidases which catalyze the production of H_{2}O_{2}, but that certain organisms also possess enzymes which reduce the concentration of H_{2}O_{2} to undetectable levels. Lactobacilli may convert atmospheric oxygen into H_{2}O_{2} via one of the reactions summarized in Table 1.

ANTIBACTERIAL ACTIVITY

Inhibitory and bactericidal effects

Depending on a number of factors, but mainly on its concentration, H_{2}O_{2} may exert either bacteriostatic or bactericidal effects. Reports may differ in their findings on the concentrations of H_{2}O_{2} needed for the inhibition of bacterial growth or for bactericidal action, probably due to differences in bacterial strains used and in other environmental factors such as pH and temperature (34). Acid production by lactic streptococci was slightly inhibited by H_{2}O_{2} at concentrations as low as 5 μg/ml (ca. 0.15 mM) H_{2}O_{2} (138). H_{2}O_{2} was involved in the inhibition of S. aureus by lactic streptococci in associative culture (59). Certain lactobacilli produced sufficient H_{2}O_{2} to inhibit the growth of S. aureus at 35°C. In concentrations of about 0.18 mM H_{2}O_{2} was bacteriostatic, and it became bactericidal in concentrations as high as 0.6 to 1.0 mM (35). Pseudomonas species were inhibited by strains of lactobacilli that produced 0.15 to 0.3 mM H_{2}O_{2} (118). At the concentration commonly used in oral rinses (ca. 0.3 M), H_{2}O_{2} causes transient inhibition of carbohydrate metabolism and of lactic acid production by oral streptococci, but continuous exposure for several minutes is required for a significant reduction in the numbers of viable bacteria (139). Significant bactericidal activity was observed when 0.88 M H_{2}O_{2} was applied to Micrococcus spp. or S. epidermidis (152). The D-values obtained by exposure to ca. 7.6 M of H_{2}O_{2} at 24°C were 0.8 to 7.3 min for spores of Bacillus subtilis, B. coagulans, B. stearothermophilus and Clostridium sporogenes and 0.2 min for Staph. aureus (141). Baldry (10), on the other hand, found H_{2}O_{2} to be more effective as a sporicide than a bactericide, with sporicidal action (within 3 h at pH 5.0 and 6 h at pH 6.5) being obtained using a solution of 0.88 M.

The sporicidal properties of H_{2}O_{2} have been reviewed by von Bockelmann and von Bockelmann (146), Ito et al. (73) and others. The effects of factors such as pH and temperature on the sporicidal action of H_{2}O_{2} have been reviewed by Block (19). Killing of bacterial spores by H_{2}O_{2} is highly temperature dependent (141); H_{2}O_{2} is only a weak sporicide at room temperature but is very potent at higher temperatures. Industrial processes commonly make use of concentrated peroxide solutions, e.g., 30% by weight, at temperatures of about 85°C. Untreated spores of Clostridium bifermentans were 500 times more resistant to H_{2}O_{2} than spores pretreated with dithiothreitol (11).

Oxidative killing of metabolically active cells by H_{2}O_{2} generally involves metabolic formation of radicals. As spores are completely dormant, radical formation must be nonmetabolic, and might be catalyzed by transition metal ions, mainly Fe^{2+}. Degradation of cortex material has been shown with H_{2}O_{2}, although no attempt has been made to correlate this effect with sporidal activity (150). King and Gould (83) found that at high concentrations H_{2}O_{2} causes lysis of dormant bacterial spores, primary lytic damage being to the spore coat. However, neither lysis nor loss of refractivity is an absolute requirement for killing (6, 83). Findings summarized by Russell (127) suggest that H_{2}O_{2} exerts sporidical activity by removing protein, presumably from the spore coat. Further damage involves oxidative cortex hydrolysis or germination-like changes due to activation of cortex lytic enzymes (6, 49). At lower concentrations (less than 0.3 M) H_{2}O_{2} also kills spores but causes neither germination-like changes nor lysis (83).

Mechanisms of H_{2}O_{2} activation

H_{2}O_{2} cytotoxicity is apparently due to its capacity—as an intermediate in oxygen reduction—to generate more reactive and cytotoxic oxygen species such as the hydroxyl radical (HO_), which is a powerful oxidant (52, 60), and which can initiate oxidation and cause damage to nucleic acids, proteins, and lipids (63, 156). Among the ways by which H_{2}O_{2} can be converted into hydroxyl radicals are (i) by transition metal ions (e.g., iron, copper), which have been implicated in the formation of hydroxyl radicals from H_{2}O_{2} (62, 63); (ii) by superoxide anions interacting with H_{2}O_{2} (44, 55) in the presence of Fe^{2+} to produce hydroxyl radicals, the iron-catalyzed Haber-Weiss reaction, or the superoxide-driven Fenton mechanism; and (iii) by UV irradiation (149).
The production of OH· may involve so-called Fenton chemistry, typically initiated with reduction of Fe^{3+} (or other transition metal ions) to Fe^{2+} by agents such as the superoxide radical (O_2^-). Fe^{2+} then reacts with H_2O_2 to yield hydroxyl anion, hydroxyl radical, and Fe^{3+}. Either the higher-valency ions or hydroxyl radicals formed might be the causative agents of cellular damage.

Particular attention has been paid to low-molecular mass iron ion complexes and to iron proteins as potential promoters of HO· formation in vivo. Intact oxymyoglobin or metmyoglobin molecules do not react with H_2O_2 to form HO·, but eventually H_2O_2 leads to release of iron ions from the proteins. These released iron ions can react to form HO·outside the proteins or close to their surface (119).

The cytotoxic effects of H_2O_2 have been investigated extensively in *Escherichia coli* (23, 69, 71, 125). In these cells, the lethal response elicited by increasing concentrations of the oxidant is characterized by two regions of killing (mode one and mode two killing), which are produced by concentrations of H_2O_2 below 2.5 mM or higher than 12.5 mM, respectively. Mode two killing, unlike the mode one type, does not require active cellular metabolism and is not enhanced in DNA repair-deficient strains (70). Brandi et al. (23) have reported that the toxicity of H_2O_2 in *E. coli* cells is highly dependent on the composition of the extracellular milieu and that, in particular, the amino acid cystine (when applied together with the peroxide) markedly sensitizes bacterial cells to the cytotoxic effect of low concentrations (2.5 mM) of H_2O_2 (26).

The conversion of H_2O_2 into more cytotoxic compounds may be potentiated by reducing agents, e.g., cysteine (14), cystine (26), nitric oxide (113) and by peroxidases (101, 121, 123). Cantoni et al. (26) found that cysteic acid—the most likely oxidation product of cystine—had no bactericidal effects and hypothesized that cystine itself impairs the cellular defense mechanism against H_2O_2.

The microbicidal activity of H_2O_2 is considerably increased by the enzyme peroxidase in the presence of a halide ion (87, 88). Among the peroxidases that can function in this way are those of milk and saliva (lactoperoxidase) (124), neutrophils and monocytes (myeloperoxidase) (87, 89). Lactobacilli and other lactic acid-producing bacteria are among the organisms which can generate the H_2O_2 required for peroxidase-catalyzed microbial antagonism. In phagocytic blood cells, the enzyme myeloperoxidase forms hypo-chlorous acid from chloride ions and H_2O_2, and the HOCl reacts with a second molecule of H_2O_2 to yield an additional singlet oxygen (O_2•). Levels of toxic oxygen are formed sufficient to kill ingested bacterial cells.

Biological fluids contain high levels of chloride and other anions, e.g., bicarbonate, which might form secondary radicals from HO· which are sufficiently long-lived to oxidize vulnerable cellular targets selectively. In this context, carbonate/bicarbonate has been reported to potentiate protein oxidation by HO· radicals and to promote photohemolysis of erythrocytes (105).

While the mechanism by which hydrogen peroxide kills spores is not known, killing of growing vegetative cells involves DNA damage (4), much of which is DNA backbone cleavage mediated by hydroxyl radicals generated from hydrogen peroxide by the Fenton reaction (72), usually with Fe^{3+}.

H_2O_2 itself in aqueous solution does not oxidatively modify nucleic acids, lipids, or proteins in the absence of catalysts for radical formation (58). Presumably, the need for radical formation is a major basis for the very strong temperature dependence of H_2O_2 killing (134, 141). Attempts to enhance radical production for sporcidal action by the addition of transition metal ions, especially Cu^{2+}, to H_2O_2-treated spore suspensions have been successful for some spores but not for others (11, 83, 148).

**Antibacterial systems involving H_2O_2**

Aside from the generation of hydroxyl radicals, the in vivo bactericidal effects of H_2O_2 may be related to the activation of a peroxidase-H_2O_2 system, commonly referred to as the lactoperoxidase system. The lactoperoxidase-thiocyanate-H_2O_2 antimicrobial system (LP system) is a naturally occurring system first discovered in raw milk. It has since been found in tears, saliva, and other biological fluids (121). A wide range of gram-negative (16, 20, 122, 157) and gram-positive (112, 136) bacteria are reported to be inhibited by the LP system. The LP system appears to be more inhibitory towards gram-negative than gram-positive bacteria (102, 121). In this system, the enzyme lactoperoxidase (LP) catalyses the oxidation of the thiocyanate ion (SCN\(^-\)) by H_2O_2, producing a relatively weak oxidizing agent, hypothiocyanite (OSCN\(^-\)), which has bacteriostatic activity and is the major antimicrobial product of the system at physiological pH (8). A similar lactoperoxidase-H_2O_2 system, detected in the upper gastrointestinal tract of calves, was found to be activated in the abomasum fluid and to become bactericidal against *E. coli* without affecting the H_2O_2-producing lactobacilli (123). In the absence of LP and SCN\(^-\), H_2O_2 has an effective bactericidal activity against oral streptococci if they are exposed for relatively long periods of time (e.g., LD_{50} values of 24 h with 0.2 mM; 4 h with 1.5 mM or 1 h with 9.0 mM) (139). During incubations of up to 4 h, the LP system is from 50 to 100 times more inhibitory towards oral streptococci than H_2O_2 alone, whereas after 24 h of exposure, H_2O_2 is as active as the LP system as an inhibitor of bacterial metabolism (139). Studying the effect of two exogenous sources that generate H_2O_2, Dionysius et al. (39) found that the final product of the LP reaction depends both on the method of H_2O_2 generation and the relative proportions of the substrates.

UV irradiation of bacterial spores in the presence of H_2O_2 has been shown to produce synergistic kills when compared with UV light and H_2O_2 used sequentially (149). In the presence of H_2O_2, UV irradiation of spores of *Bacillus subtilis* produced a rapid kill that was up to 2,000-fold greater than that produced by irradiation alone (12). These results suggest that the action of UV is not directly on the spore DNA but may be related to the production of free hydroxyl radicals from H_2O_2 (149). UV absorption by H_2O_2 is greater at shorter wavelengths, and at wavelengths of less than ca. 270 nm so much UV energy may be absorbed by H_2O_2 outside the spore that few free HO· radicals are formed.
within the spore, hence reducing the kill at these wavelengths (149). This is supported by evidence from previous work (11, 148) in which the presence of cupric ions markedly increased the lethal effect of H₂O₂ on spore suspensions of *Clostridium bifermantans*, suggesting that the levels of such ions within spores might determine their resistance to H₂O₂.

**MECHANISMS OF BACTERIAL RESISTANCE TO H₂O₂**

Biologically hazardous reactive oxygen species arise from a variety of chemical and metabolic reactions. Bacteria that utilize and reduce O₂ must cope with reactive oxygen species such as the superoxide anion, H₂O₂, and the hydroxyl radical, produced as incomplete reduction products of molecular oxygen and with singlet oxygen formed photochemically (25, 61). The systems that protect the organisms against H₂O₂ cytotoxicity include glutathione and associated enzymes and catalases. For many kinds of bacteria, catalase may be most important in protection against high H₂O₂ concentrations because catalases generally have high turnover rates (29). The absence or low levels of catalase generally result in the formation of H₂O₂ in amounts which are in excess of the capacity of the organism to degrade it.

Furthermore, many bacteria, e.g., *E. coli* lack glutathione peroxidase (115) and cannot use glutathione for enzymatic catalabolism of H₂O₂. Bacteria grown aerobically encounter these oxidants not only inside the cell, but also from outside, as occurs for example when pathogenic bacteria are attacked by phagocytic cells (65). However, it seems that endogenous catalase in *E. coli* cells cannot protect the individual cell against exogenous H₂O₂, apparently due to the high diffusibility of H₂O₂ into the cell (96). On the other hand, in high-density populations catalase-positive *E. coli* cells produce enough catalase to protect the cells against environmental H₂O₂ (96).

Cells protect themselves against oxidant toxicity either by scavenging the oxidants with enzymes such as catalase, or in the case of DNA damage, by repairing the damage after it has taken place (42). Several oxidative defence proteins have been found in bacteria. These enzymes might repair DNA damage caused by H₂O₂ (69). It appears that at low H₂O₂ concentrations, DNA repair systems play a role in the protection of the cells against H₂O₂-induced mode one killing (4, 70).

Procaryotic cells respond to environmental or chemical stresses by inducing specific sets of proteins characteristic to each stress; in some cases proteins associated with one stimulus can be induced during other stresses. For instance, *E. coli* (145) and *Salmonella typhimurium* (106) were shown to synthesize heat-shock proteins when the cells were exposed to H₂O₂. A cross-protective effect has been also demonstrated for a limited number of stimuli that share common proteins. Thus *E. coli* and *S. typhimurium* exposed to an adaptive dose of H₂O₂ exhibit increased thermal resistance (32, 145). Similarly, *E. coli* cells exposed to starvation stresses (to glucose or nitrogen) developed increased resistance to H₂O₂ (76). Cells that exhibit enhanced tolerance to high temperatures appear to be better able to survive in the presence of H₂O₂ (68, 106). Induction of defence proteins by treating cells with nonlethal concentrations of H₂O₂ allows adaptation as well as resistance to this oxidant (92). A pretreatment of *S. typhimurium* with 60 μM H₂O₂ caused the induction of a great number of proteins and protected the cells against the otherwise lethal effects of 10 mM H₂O₂ (32). Bacteria constitutively resistant to H₂O₂ can be obtained, by selection, after exposure to increasing concentrations of the oxidant (64).

One of the possible contributors to the H₂O₂ resistance of growing bacterial cells is catalase (72). Among bacterial pathogens, catalase has been proposed as a potential virulence factor, since the ability to detoxify peroxide might protect organisms from the oxidative bursts of neutrophils and other inflammatory cells of the immune response (13).

During incubations of up to 4 h, the lactoperoxidase (LP)-thiocyanate-H₂O₂ system is from 50 to 100 times more inhibitory towards oral streptococci than H₂O₂ alone (139). When high H₂O₂ concentrations exist, the presence of LP and thiocyanate anion might protect streptococci against killing by H₂O₂ by converting H₂O₂ to the less potent oxidizing agent hypothiocyanate (139).

Chelators such as o-phenanthroline protect against oxidative killing by H₂O₂ (99). It is likely that the radicals are formed outside of the protoplast in regions that can be penetrated by o-phenanthroline.

The DNA in dormant spores of *Bacillus* species is covered with a group of small, acid-soluble proteins (SASP) of the α/β type (131) that are synthesized in the developing spore (130). Setlow and Setlow (132) have shown that (i) in wild-type spores of *Bacillus subtilis* there is a relationship between the acquisition of H₂O₂ resistance and the synthesis of α/β-type SASP, and that (ii) in vitro, binding of those proteins to DNA provides strong protection against cleavage of DNA by H₂O₂ by a mechanism not completely clear. Hydroxyl radicals generated from H₂O₂ cause significant DNA strand breakage in growing cells as well as in vitro (72, 143).

For some spores of *Bacillus* spp. spore coats are not involved in the resistance to H₂O₂ (132, 147). This may not be a general rule for all sporeformers, as the coats of *Clostridium bifermantans* spores do provide a significant increment of H₂O₂ resistance (11).

 Destruction of ingested bacteria by phagocytes involves the liberation of H₂O₂, and enhanced tolerance to this compound may facilitate infection. Once inside a phagocytic cell, some pathogenic bacteria can escape its killing mechanisms by a variety of ways. Lipoarabinomannan, a cell wall-associated microbial glycolipid, may play a role in the pathogenicity and virulence of intracellular pathogenic *Mycobacterium* spp. by scavenging potentially cytotoxic oxygen species (28).

**METHODS FOR DETECTION AND QUANTITATION OF H₂O₂**

Once formed, H₂O₂ may react with other chemical constituents. Knowledge of H₂O₂ formation in biological
systems, microbiological media, and food products is of importance to the explanation of antimicrobial effects.

Existing assays for the cellular release of H$_2$O$_2$ include those based on (i) catalase-dependent oxidation of formate to CO$_2$ (74), (ii) generation of fluorescent products due to a H$_2$O$_2$-mediated oxidative reaction (17, 82), (iii) the loss of fluorescence upon the oxidation of scopoletin (21), (iv) change in absorbance upon oxidation of phenol red (116), or (v) formation of enzyme-substrate complexes with peroxidases (22, 80).

Most of the sensitive methods for measuring H$_2$O$_2$ use peroxidases, especially horseradish peroxidase (HRP), and involve measuring the oxidation of substrates in the presence of H$_2$O$_2$. Methods based on peroxidase might be susceptible to interference by reducing cosubstrates that compete with the indicator (9, 37). Some common substrates used in the HRP-coupled oxidation assays of H$_2$O$_2$, benzenedi, o-tolidine, o-toluidine and o-dianisidine, are carcinogenic. One of the assays based on less hazardous substrates is a modified 4-aminoantipyrine/phenol assay (51); in the presence of peroxidase these compounds react with H$_2$O$_2$, yielding a quinone-imine chromogen with maximum absorption at 505 nm. Frew et al. (51) have recommended this method as being highly selective for H$_2$O$_2$, and allowing determination of micromolar concentrations of H$_2$O$_2$ in mixtures of peroxidases.

The peroxidase-catalyzed oxidation of scopoletin has been widely used, with a number of modifications, in the measurement of H$_2$O$_2$ production in biological systems (21, 38, 110, 126). Scopoletin (7-hydroxycoumarin) is an organic hydrogen donor that fluoresces (excitation at 350 nm and emission at 460 nm) in aqueous solutions in direct proportion to its concentration. Scopoletin is highly stable nonauto-oxidizing nontoxic fluorophore that can be incubated directly with cells for continuous or intermittent determination of H$_2$O$_2$ with considerable accuracy and sensitivity (21). HRP uses H$_2$O$_2$ to oxidize scopoletin, with the resultant quenching of the scopoletin-associated fluorescence. While the o-dianisidine, formate oxidation, and phenol red oxidation assays can detect concentrations of H$_2$O$_2$ in the range 5 to 60 mM, the scopoletin assay can detect concentrations as low as 0.2 to 2.0 mM H$_2$O$_2$. The scopoletin method has been automated and modified to test very small samples. De la Harpe and Nathan (37) have reported about detection of levels as low as 0.1 nmol produced by 1 µg of cell protein, allowing calculations of specific secretion in nanomoles per milligram of cell protein as well as cumulative H$_2$O$_2$ secretion in individual cells. The readings are nondestructive and permit repetitive monitoring of the time-course of secretion in small cell samples.

Carmine et al. (27) reported that a sensitive peroxidase-dependent luminol-enhanced chemiluminescence assay could detect H$_2$O$_2$ generated by tumor cells at concentrations as low as 50 nM.

Another method based on a nontoxic fluorophore that can be incubated directly with cells for continuous determination of H$_2$O$_2$ concentrations is based on the (cumulative) oxidation of formate through the peroxidative activity of catalase on H$_2$O$_2$ (117, 128). The method enables the accumulation of extracellular H$_2$O$_2$ to be measured. Studies of formate oxidation to CO$_2$ in biological material have demonstrated the involvement of catalase and H$_2$O$_2$ in the reaction (1, 104). The oxidation of added formate by endogenous or exogenous catalase may, therefore, be referred to as intra- or extracellular H$_2$O$_2$ production (104). Juven et al. (79) have used this method to establish the specificity of the formate oxidation assay and to study production of H$_2$O$_2$ by lactic acid starter cultures. The authors found that formate oxidation was inhibited by the addition of sodium azide and did occur in the absence of catalase, indicating that H$_2$O$_2$ is the compound involved in the oxidation of formate, and that the lactic acid cultures do not produce carbon dioxide directly from formate. With a specific culture, increasing the weights of bacterial cells increased the levels of formate oxidation. After 2 h incubation at 25°C, catalase-mediated formate oxidation for several meat starter cultures of Lactobacillus plantarum and Pediococcus pentosaceus produced 260 to 330 nmol per mg (dry weight) of cells (79). At low rates of H$_2$O$_2$ formation (as probably occurs with lactic cultures), the peroxidatic pathway will predominate; when H$_2$O$_2$ is high, the catalatic pathway will predominate (30). It is possible that in a system containing catalase, H$_2$O$_2$, and formate only, an alternative reaction may occur which does not lead to the oxidation of formate and formation of CO$_2$, but to the decomposition of residual peroxide by compound I (catalytic pathway) and the production of O$_2$ and water (128).

Another method based on nontoxic substrates has been described by Pick and Keisari (116). This assay is based on the horseradish peroxidase-mediated oxidation of phenol red by H$_2$O$_2$ which results in the formation of a compound demonstrating increased absorbance at 610 nm. The method makes it possible to follow H$_2$O$_2$ accumulation in cell cultures over long periods of time with a sensitivity in the range of 5 to 60 mM, which is similar to that of the o-dianisidine method, while having the advantage of using nontoxic reagents. Using this method with minor modifications, Juven et al. (78) have found that a chicken intestinal strain of Lactobacillus acidophilus produced 280 nmol of H$_2$O$_2$/h/mg of cell dry weight in a culture medium.

Several methods have been used for the detection of H$_2$O$_2$ in milk and dairy products with apparently satisfactory results. Gilliland (53) used HRP with o-dianisidine as a chromogenic hydrogen donor and measured the amount of color developed (at 400 nm). The author reported detection of an amount as low as 1 µg (ca. 30 nmol) of H$_2$O$_2$. Several authors (3, 15, 18, 45) have reported about a spectrophotometric method that enabled detection of as little as 2 to 3 µg of H$_2$O$_2$ based on the use of a titanium salt (commonly tetrachloride) that forms a yellow-colored complex (absorbing at 415 nm) with H$_2$O$_2$. Toyoda et al. (142) have suggested the use of an oxygen electrode that could detect levels of as little as 0.1 µg of H$_2$O$_2$ per ml of milk. The method involves purging the dissolved oxygen from the milk, adding catalase, and measuring the resultant oxygen with a sensitive oxygen electrode. Problems may exist when this assay is used in viscous high-fat materials (18).

A number of plating media based on the presence of a peroxidase plus a chromogen substrate have been recom-
mended for the detection of H$_2$O$_2$ production and screening of H$_2$O$_2$-producing bacteria. Among the substrates used in those media are o-tolidine (103), o-dianisidine (53), and the less toxic compounds ABTS [2-2' azino-di-(3 ethylbenzthiazoline-6-sulfonic acid)] (31, 100) and TMB (3,3',5,5'-tetramethylbenzidine) (40).

**FOOD INDUSTRY APPLICATIONS**

Awareness of the sporicidal activity of H$_2$O$_2$ (141, 146) has led to its use in the sterilization of packaging materials for aseptic filling (140). While aqueous H$_2$O$_2$ has a long history of use as a sterilant, the concept of vapor-phase hydrogen peroxide as a surface decontaminant and sterilant has been developed only within the past decade. The first published evidence establishing the microbicidal properties of hydrogen peroxide vapor was reported in a patent issued to Fortson and Wardle (48). In this process for cold gas sterilization, articles to be sterilized are placed in an enclosure, H$_2$O$_2$ solution is injected, and a vacuum is drawn to vaporize the H$_2$O$_2$; sterilization is effective with less than 75 mg of H$_2$O$_2$ per liter at temperatures below 80°C. Wang and Toledo (151) have demonstrated that air saturated with H$_2$O$_2$ vapor (produced by bubbling air through a 35% (wt/wt) H$_2$O$_2$ solution at constant temperature) exhibits microbicidal properties, which increase as temperature increases between 20 and 60°C. Being a nontoxic cold gas sterilant, vapor-phase H$_2$O$_2$ has been suggested for surface decontamination, instead of toxic or carcinogenic gaseous sterilants such as ethylene oxide and formaldehyde (86, 151).

H$_2$O$_2$ has been evaluated for possible use in sanitizing hatching eggs (98, 114, 133) and in low-temperature pasteurization of liquid whole egg (129, 144). Alone or in combination with other chemicals it has also been tested as a decontaminant of broiler carcasses (46, 94, 107). At concentrations high enough to cause a significant reduction in bacterial counts, undesirable color and appearance changes in the treated carcasses might occur (75, 94, 107). H$_2$O$_2$ has also been tested as a microaerosol in the eradication of potential foodborne pathogenic bacteria from hatcheries and poultry-houses and equipment (111).

H$_2$O$_2$ has been suggested for use as an antimicrobial agent in water (158) and dairy products (50, 109). It is an approved bactericide in the USA in the processing of milk used in certain varieties of cheese (43, 91). Food grade H$_2$O$_2$ is a lightly stabilized preparation formulated to meet Food Chemicals Codex specifications; it is being recommended for use as an antimicrobial agent in milk intended for use in cheese-making (up to ca. 15 mM), whey intended for use in modified whey (up to ca. 15 mM), corn starch (up to ca. 45 mM) and dried eggs (137). Certain preparations in which H$_2$O$_2$ is the active compound are also being marketed as disinfectants for fruits and vegetables (41). One of these products is “Sanosil,” manufactured by Sanosil Australia Ltd./Sanosil AG, Germany. Simmons et al. (135) have reported about a procedure for mold decontamination in raisins using H$_2$O$_2$, using patented equipment that volatilizes 35% H$_2$O$_2$ into a stream of air to near saturation.

**CONCLUSIONS**

The antibacterial effects of hydrogen peroxide have been extensively investigated due to its possible involvement in a number of important biological events in which bacterial cells are either killed or their growth inhibited. The antagonistic effects of H$_2$O$_2$ are enhanced in the presence of a number of other chemical compounds or when used in combination with certain physical treatments. Reviewing the literature gives us a better understanding of the mechanisms by which H$_2$O$_2$ can become cytotoxic, of the concentrations required for antibacterial effects, and of the mechanisms by which cells can protect themselves against the toxic effects mediated by H$_2$O$_2$. In order to determine that H$_2$O$_2$ is the metabolic product involved in an observed antagonistic effect, sensitive methods based on nontoxic reagents have been developed that allow detection and quantitation of H$_2$O$_2$ in live cell cultures. From the available literature on applicative issues it is apparent that H$_2$O$_2$ is receiving growing attention and that it has the potential to be used in a variety of ways in the food industry.

**REFERENCES**


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