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Antibacterial Activity of Hydrogen Peroxide and the Lactoperoxidase-Hydrogen Peroxide-Thiocyanate System against Oral Streptococci

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In secreted fluids, the enzyme lactoperoxidase (LP) catalyzes the oxidation of thiocyanate ion (SCN^-) by hydrogen peroxide (H_2O_2), producing the weak oxidizing agent hypothiocyanite ($OSCN^-$), which has bacteriostatic activity. However, H_2O_2 has antibacterial activity in the absence of LP and thiocyanate (SCN^-). Therefore, LP may increase antibacterial activity by using H_2O_2 to produce a more effective inhibitor of bacterial metabolism and growth, or LP may protect bacteria against the toxicity of H_2O_2 by converting H_2O_2 to a less-potent oxidizing agent. To clarify the role of LP, the antibacterial activities of H_2O_2 and the LP-H_2O_2-SCN^- system were compared by measuring loss of viability and inhibition of bacterial metabolism and growth. The relative toxicity of H_2O_2 and the LP system to oral streptococci was found to depend on the length of time that the bacteria were exposed to the agents. During incubations of up to 4 h, the LP system was from 10 to 500 times more effective than H_2O_2 as an inhibitor of glucose metabolism, lactic acid production, and growth. However, if no more H_2O_2 was added, the concentration of the inhibitor $OSCN^-$ fell because of slow decomposition of $OSCN^-$, and when $OSCN^-$ fell below 0.01 mM, the bacteria resumed metabolism and growth. In contrast, the activity of H_2O_2 increased with time. H_2O_2 persisted in the medium for long periods of time because H_2O_2 reacted slowly with the bacteria and streptococci lack the enzyme catalase, which converts H_2O_2 to oxygen and water. After 24 h of exposure, H_2O_2 was as effective as the LP system as an inhibitor of metabolism. H_2O_2 also caused a time-dependent loss of viability, whereas the LP system had little bactericidal activity. The concentration of H_2O_2 required to kill half the bacteria within 15 s was 1.8 M (6%) but fell to 0.3 M (1%) at 2 min, to 10 mM (0.03%) at 1 h, and to 0.2 mM (0.0007%) with a 24-h exposure. The results indicate that if high levels of H_2O_2 can be sustained for long periods of time, H_2O_2 is an effective bactericidal agent, and the presence of LP and SCN^- protects streptococci against killing by H_2O_2. Nevertheless, the combination of LP, H_2O_2, and SCN^- is much more effective than H_2O_2 alone as an inhibitor of bacterial metabolism and growth.

Lactoperoxidase (LP) and its substrate thiocyanate (SCN^-) are secreted into milk, tears, and saliva (15, 22, 31). LP catalyzes the oxidation of SCN^- by hydrogen peroxide (H_2O_2), producing the weak oxidizing agent hypothiocyanite ($OSCN^-$) (2, 24, 26). There are two major sources of H_2O_2 in the oral environment. First, secretions collected directly from the salivary glands contain $OSCN^-$, indicating that there is a source of H_2O_2 in the glands and that SCN^- is oxidized prior to secretion into the oral cavity (12, 13, 18). Second, when whole saliva samples are incubated in vitro, the $OSCN^-$ concentration increases, because of H_2O_2 production by oral bacteria, primarily streptococci (29, 30).

Not all streptococci produce H_2O_2. Among strains that do, abundant H_2O_2 is released into the medium under aerobic conditions by stationary-phase cells that have a supply of metabolizable carbohydrate (27, 36). Streptococci lack the enzyme catalase, which is found in higher organisms and most other bacteria and which detoxifies H_2O_2.

Oral streptococci are facultative anaerobes and do not require oxygen (O_2). They depend on anaerobic glycolysis for their energy supply and convert sugars to lactic acid, which is excreted into the medium. However, when O_2 is available, these lactic-acid bacteria take up O_2 at rates similar to those of aerobic organisms. Part of the NADH produced in glycolysis is utilized by soluble cytoplasmic NADH-oxidase enzymes that reduce O_2 to superoxide ($\cdot O_2^-$), H_2O_2, or water (4, 5, 10, 21, 27, 36). If superoxide is produced, it is converted to O_2 and H_2O_2 by the enzyme superoxide dismutase. Strains that release H_2O_2 have higher levels of the H_2O_2-producing oxidase activity and/or lower levels of a cytoplasmic NADH-peroxidase enzyme that reduces H_2O_2 to water.

O_2 uptake is not linked to ATP production in these bacteria and has no useful role in their metabolism. The significance of O_2 metabolism is in microbial ecology. By using up O_2, the bacteria create anaerobic environments, such as in dental plaque, that inhibit or prevent colonization by aerobic bacteria (7). Moreover, H_2O_2 itself or substances produced from H_2O_2 can inhibit metabolism and growth of competing microorganisms.

Because H_2O_2 production is part of the life-style of these bacteria, and they lack the protection of catalase, a number of studies have examined the susceptibility of streptococci to H_2O_2 toxicity. The classic studies by Dolin on O_2 metabolism of *Streptococcus faecalis* (4) reported that these bacteria were highly resistant to killing or inhibition of metabolism by H_2O_2. Resistance was attributed to the absence of hemoproteins and other metalloenzymes. Although H_2O_2 is a powerful oxidizing agent, it reacts slowly with biological materials in the absence

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of catalysts such as the transition metal cations copper (Cu^{2+}) and iron (Fe^{2+} or Fe^{3+}). Similar results were reported in our studies of oral mutans streptococci (27, 36). For example, H_2O_2 accumulated in the medium and reached concentrations of up to 2 mM when stationary-phase cells were incubated with glucose, indicating that glucose uptake, glycolysis, and NADH-oxidase activity were not blocked by H_2O_2 concentrations below 2 mM.

Nevertheless, several studies reported that oral streptococci were highly susceptible to H_2O_2 toxicity (1, 3, 6). Complete killing or inhibition of growth was reported at H_2O_2 concentrations as low as 0.1 mM.

These results raise questions about the biological role of LP. If H_2O_2 is more toxic than $OSCN^-$, then LP protects bacteria by consuming H_2O_2 and producing a less-toxic oxidizing agent. Protection by LP might enable catalase-negative, H_2O_2-positive bacteria to survive and grow in the oral environment. Under some conditions, it might be advantageous to block LP activity and allow H_2O_2 to kill pathogenic oral streptococci.

On the other hand, many studies have reported that the combination of LP, H_2O_2, and SCN^- has a much stronger antibacterial effect than H_2O_2 alone (28, 37). LP is a member of the class of mammalian peroxidase enzymes, including the leukocyte enzymes myeloperoxidase and eosinophil peroxidase that participate in host-defense against infection. In general, these enzymes use H_2O_2 to produce more-effective antimicrobial agents (8, 11, 34). The greater activity of these agents relative to H_2O_2 is due to their ability to react faster with microbial cell components and to enter into a wider range of chemical reactions. In addition, the microorganisms may have no specific defense against these agents, whereas most cells have enzymes that detoxify H_2O_2.

If LP uses H_2O_2 to produce a more effective antibacterial agent, colonization of the oral environment by streptococci could be explained by the limited resistance of these bacteria to inhibition by $OSCN^-$. A certain minimum (threshold) concentration of $OSCN^-$ is required to inhibit metabolism and growth (9, 19, 37). It might be advantageous to promote the activity of the LP-H_2O_2-SCN^- system, in order to overcome the resistance of oral streptococci and suppress bacterial growth and lactic acid production.

The aim of this study was to compare the antibacterial activities of H_2O_2 and the LP-H_2O_2-SCN^- system against oral streptococci, so as to resolve conflicting observations on H_2O_2 toxicity and to clarify the role of LP in oral microbial ecology and host defense.

MATERIALS AND METHODS

Bovine milk LP, horseradish peroxidase, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2, or DTNB), the chelator diethylenetriaminepentaacetic acid (DETAPAC), crystal violet, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and reagents for determination of L-lactate were from Sigma Chemical Co., St. Louis, Mo. The LP concentration was calculated from the molar extinction coefficient of 112,000 at 412 nm. To prepare 5-thio-2-nitrobenzoic acid (Nbs, or TNB), a 1 mM solution of Nbs_2 in 0.14 M NaCl with 0.1 mM DETAPAC and 15 mM phosphate buffer, pH 7.2, was reduced with 0.6 mM 2-mercaptoethanol (33). Reduced (leuco) crystal violet (0.05%) was prepared by adding excess sodium borohydride to 0.05 g of crystal violet in 100 ml of water, adding 0.5 ml of concentrated HCl after 30 min at 25°C, filtering the mixture through a paper funnel, and storing the filtrate in the dark under nitrogen. Catalase crystals (Boehringer Mannheim, Indianapolis, Ind.) were washed twice by centrifugation in water and dissolved in buffer. H_2O_2 (30%; Fisher Chemical Co., Pittsburgh, Pa.) was diluted in autoclaved 0.154 M NaCl, and the concentration was determined from the molar extinction coefficient of 70 at 230 nm.

Bacteria were grown aerobically to stationary phase (24 h) in filter-sterilized Todd-Hewitt broth (Difco Laboratories, Detroit Mich.), harvested by centrifugation at 26,000 × *g* for 15 min at 4°C, washed by centrifugation, and suspended with a Teflon glass homogenizer in 0.154 M NaCl with 1 mM $MgSO_4$ to an A_{600} of 7.1, or about 4.3×10^9 CFU/ml. Incubation mixtures (2 ml, total volume) contained bacteria, 0.154 M NaCl, 1 mM $MgSO_4$, 20 mM potassium phosphate buffer (pH 6, 7, or 8), and 20 mM sodium citrate buffer (pH 6) or 50 mM sodium HEPES buffer (pH 7 or 8). Where indicated, LP was 0.1 μM, SCN^- was 1 mM, and glucose was 10 mM.

Viability was determined by making serial 10-fold dilutions in 0.154 M NaCl with 1 mM $MgSO_4$ and plating 1 ml on solid medium containing Todd-Hewitt broth and 2% agar (Difco). Lactate was measured by the assay based on reduction of NAD^+ to NADH catalyzed by L-lactate dehydrogenase. Catalase (1 μg/ml) was added, incubation mixtures were centrifuged at 12,000 × *g* for 10 min at 4°C to remove bacteria, portions of the supernatants were incubated 45 min at 37°C with 5 U of L-lactic dehydrogenase per ml–2.1 mM NAD^+ in glycine buffer with hydrazine at pH 9.2, A_{340} of NADH was measured, and lactate concentrations were calculated from standard curves prepared with L-lactate. H_2O_2 was measured by the assay based on oxidation of reduced crystal violet (16). Portions of supernatants were incubated 5 min at 37°C with 20 μg of horseradish peroxidase per ml–0.005% reduced crystal violet in 1.0 M acetate buffer (pH 4.3). A_{596} of crystal violet was measured, and H_2O_2 concentrations were calculated from standard curves. Measurements of $OSCN^-$ were done by the assay based on oxidation of 2 mol of the sulfhydryl compound Nbs to the disulfide Nbs_2 (2, 33). Incubation mixtures were placed on ice, and 0.5 ml of the 0.6 mM Nbs solution with 3 μg of catalase per ml was added, followed by 2 ml of cold 0.14 M NaCl with 15 mM phosphate buffer (pH 7.2). The bacteria were removed by centrifugation at 4°C for 10 min at 12,000 × *g*, and A_{409} of the supernatants was measured. The $OSCN^-$ concentration (micromolar) was calculated from the difference in absorbance between the control and sample multiplied by the ratio of the final and starting volumes (4.5/2), divided by the micromolar extinction coefficient for Nbs (0.01405), and all divided by 2.

RESULTS

H_2O_2-negative and H_2O_2-positive bacteria. Two strains of mutans streptococci were used. The GS-5 strain (*Streptococcus mutans*, serotype c) produces little or no H_2O_2 (36). Glucose metabolism is not inhibited when these bacteria are incubated with LP and SCN^- unless H_2O_2 is added (37). Cells of this strain were used for most experiments because bacterial H_2O_2 production would interfere in experiments intended to measure effects of known amounts of added H_2O_2. The OMZ-176 strain (*Streptococcus sobrinus*, serotype d) was used for experiments in which the effects of bacterial H_2O_2 production were examined (36).

Effect of cell density. Various amounts of H_2O_2 were added to washed cells of the GS-5 strain in pH 7 buffer with or without LP and SCN^-. Glucose was added, the mixtures were incubated for 15 min at 37°C, and then lactate in the medium was measured to determine the rate of glucose metabolism. Lactic acid production as a percent of the control versus the

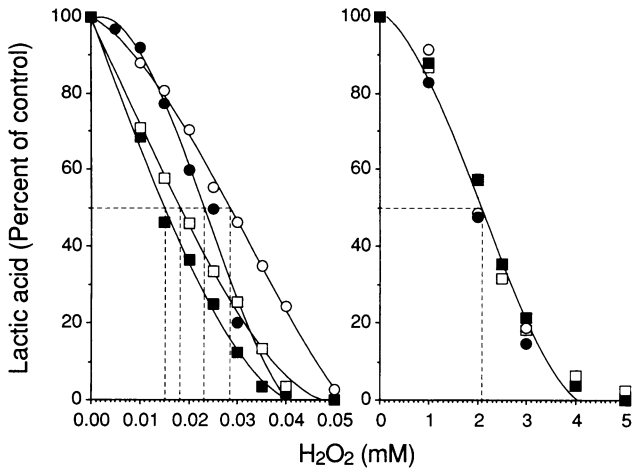

FIG. 1. Effect of the number of cells. (Left) H_2O_2 with LP and SCN^-. (Right) H_2O_2 alone. GS-5 cells were suspended to 3×10^7 CFU/ml (■), 1×10^8 CFU/ml (□), 3×10^8 CFU/ml (●), or 1×10^9 CFU/ml (○) in pH 7 buffer without glucose at 37°C. Glucose (10 mM) was added immediately after the H_2O_2 additions, the mixtures were incubated 15 min at 37°C, and lactate in the medium was measured. Dotted lines show the H_2O_2 concentrations required for 50% inhibition.

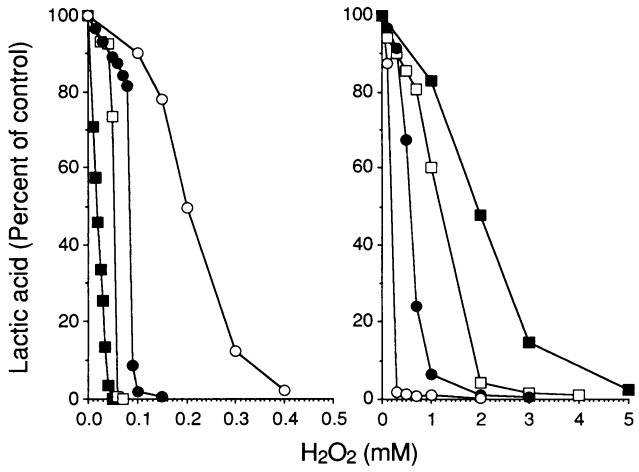

FIG. 2. Effect of time. (Left) H_2O_2 with LP and SCN^-. (Right) H_2O_2 alone. GS-5 cells were suspended to 3×10^8 CFU/ml in pH 7 buffer without glucose at 37°C. After H_2O_2 was added, the mixtures were preincubated for 0 h (■), 1 h (□), 4 h (●), or 24 h (○). Glucose (10 mM) was added, the mixtures were incubated for 15 min at 37°C, and lactate was measured. Note that the curves for the LP system shift from left to right with increasing time, whereas the curves for H_2O_2 shift from right to left.

H_2O_2 concentration was plotted, and ED_{50} values (the concentrations that gave 50% inhibition) were read from the plots.

Figure 1 shows that varying the number of bacteria had remarkably little effect on the amount of $OSCN^-$ or H_2O_2 that was required for inhibition. ED_{50} values for the LP system varied only about 2-fold, from 0.015 to 0.028 mM, when the number of bacteria per milliliter was varied 33-fold, from 3×10^7 to 1×10^9 CFU/ml. ED_{50} values for H_2O_2 varied even less and were about 2 mM. Under these conditions, H_2O_2 with LP and SCN^- was about 100 times more effective than H_2O_2 alone as an inhibitor of metabolism.

Effect of pH. Experiments similar to those for which the results are shown in Fig. 1 were carried out in buffer at pH 6, 7, and 8 with bacteria at 3×10^8 CFU/ml. Inhibition by the LP system was more pH dependent than inhibition by H_2O_2. ED_{50} values for H_2O_2 with LP and SCN^- were 0.004, 0.018, and 0.096 mM at pH 6, 7, and 8, respectively, whereas ED_{50} values for H_2O_2 alone were 1.9, 1.9, and 2.7 mM, respectively. Therefore, the LP system was from 30 to 500 times more effective than H_2O_2 in this pH range.

Effect of time. Experiments similar to those in Fig. 1 were carried out, but the mixtures were preincubated for various periods of time before glucose was added and lactic acid production over a 15-min period was determined. Figure 2 (left) shows that inhibition by the LP system became less effective with time. That is, higher concentrations of H_2O_2 were required to maintain the bacteria in an inhibited state when the preincubation periods were increased. ED_{50} values for H_2O_2 with LP and SCN^- were 0.02, 0.05, 0.09, and 0.20 mM for preincubation times of 0, 1, 4, and 24 h, respectively.

In contrast, Fig. 2 (right) shows that H_2O_2 alone became more effective with time. ED_{50} values were 1.9, 1.2, 0.6, and 0.2 mM for preincubation times of 0, 1, 4, and 24 h, respectively. ED_{50} values for the LP system and for H_2O_2 alone were the same after 24 h. Therefore, during 24 h of incubation of the oxidizing agents with the bacteria under nongrowing, nonmetabolizing conditions, inhibition by the LP system decreased and inhibition by H_2O_2 increased to the point that H_2O_2 was as effective as the LP system as an inhibitor of metabolism.

Recovery from inhibition. Exposing GS-5 cells to LP, SCN^-, and 0.3 mM H_2O_2 resulted in complete inhibition of metabolism following a preincubation of 4 h but not after 24 h, indicating that the bacteria recovered from inhibition. To examine the time course of recovery, the cells were incubated with 10 mM glucose, LP, SCN^-, and various amounts of H_2O_2. At intervals to 24 h, portions were removed, and lactate and the inhibitor $OSCN^-$ were measured in the medium.

Figure 3 (top) shows that when no H_2O_2 was added, lactic acid production was linear with time until all the glucose was consumed. Nearly 2 mol of lactic acid were produced per mol of glucose within 6 h. When 0.1 mM H_2O_2 was added, about 0.1 mM $OSCN^-$ was produced (Fig. 3, bottom). The bacteria were completely inhibited for the first 6 h, during which time $OSCN^-$ in the medium slowly fell from 0.1 to 0.008 mM. At that point, the bacteria emerged from inhibition and metabolized glucose at a rate similar to that of control cells.

When more H_2O_2 was added, more $OSCN^-$ was produced and inhibition lasted longer. In all cases, the bacteria recovered from inhibition when $OSCN^-$ in the medium fell below 0.01 mM. Therefore, this level of $OSCN^-$ is the threshold concentration for these bacteria; $OSCN^-$ below about 0.01 mM does not inhibit.

Figure 3 (bottom) also shows results obtained when LP, SCN^-, and 0.3 mM H_2O_2 were incubated without bacteria. Levels of $OSCN^-$ in the medium were only slightly higher than when the bacteria were present, indicating that only a small fraction of the $OSCN^-$ reacted with the bacteria. Most of the loss of $OSCN^-$ with time was due to decomposition of $OSCN^-$ rather than reactions of $OSCN^-$ with bacterial components or detoxification of $OSCN^-$ by bacterial enzymes.

Figure 3 (bottom right) shows that much different results were obtained with H_2O_2 alone. When added to the medium without bacteria, H_2O_2 was very stable. There was negligible loss of 2 mM H_2O_2 over a period of 6 h. With bacteria and 2 mM H_2O_2, there was a slight loss of H_2O_2, but about 90% of

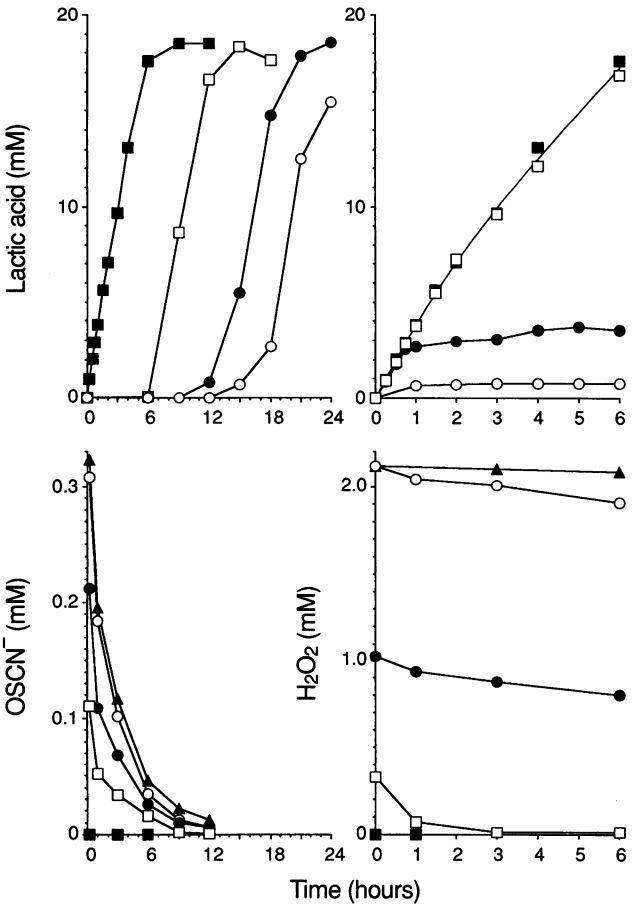

FIG. 3. Effect of time and H_2O_2 concentration. (Left) H_2O_2 with LP and SCN^-. (Right) H_2O_2 alone. GS-5 cells were suspended to 3×10^8 CFU/ml in pH 7 buffer with 10 mM glucose at 37°C, and H_2O_2 was added immediately. (Left) H_2O_2 concentrations were 0 mM (■), 0.1 mM (□), 0.2 mM (●), and 0.3 mM (○ and ▲). (Right) H_2O_2 concentrations were 0 mM (■), 0.3 mM (□), 1.0 mM (●), and 2.0 mM (○ and ▲). After incubation for the indicated periods of time, lactate in the medium was measured (top). In duplicate incubation mixtures, $OSCN^-$ or H_2O_2 was measured (bottom). $OSCN^-$ and H_2O_2 were also measured in incubation mixtures without bacteria (▲).

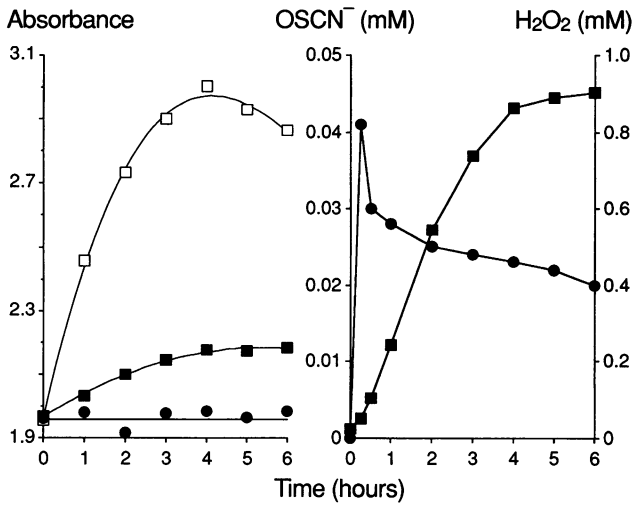

FIG. 4. Inhibition of growth. Stationary-phase cultures of strain OMZ-176 were supplemented with 10 mM glucose and 5 μg of catalase per ml (□), glucose alone (■), or glucose with LP and SCN^- (●). At the indicated times, portions of the cultures were taken and diluted to measure the A_{600} (left). Portions were also taken to measure $OSCN^-$ or H_2O_2 in the medium (right).

the H_2O_2 was still present after 6 h. With 1 mM H_2O_2 the loss was greater, with 74% remaining after 6 h. With 0.3 mM H_2O_2, most of the H_2O_2 was consumed within 1 to 2 h.

Figure 3 (top right) shows that 0.3 mM H_2O_2 did not inhibit metabolism. All of the 10 mM glucose was converted to about 18 mM lactic acid within 6 h. Because the bacteria were metabolically active, NADH was produced and was available for reduction of H_2O_2 by the bacterial NADH-peroxidase enzyme, which could account for the loss of H_2O_2 from the medium. For comparison, the combination of LP, SCN^-, and 0.3 mM H_2O_2 caused complete inhibition that lasted for nearly 16 h, whereas 0.3 mM H_2O_2 alone had no effect. Complete inhibition by H_2O_2 required a 10-fold higher concentration (3 mM).

Figure 3 (top right) also shows that with 1 or 2 mM H_2O_2, some glucose was metabolized and lactic acid was produced before the cells became inhibited. With 1 mM H_2O_2, the bacteria metabolized at the control rate for the first 45 min and then entered an inhibited state.

Effect of bacterial H_2O_2 production on growth. The H_2O_2-producing OMZ-176 strain was used to determine whether the bacteria can produce enough H_2O_2 to inhibit their own growth and whether H_2O_2 production in the presence of LP and SCN^- inhibits growth. The bacteria were allowed to grow to stationary phase in Todd-Hewitt broth, and then 10 mM glucose was added to permit additional growth, which was monitored by measuring A_{600}. To one culture, glucose and catalase were added to obtain the control noninhibited rate of growth. The second culture received only glucose, to determine the rate of growth in the presence of the H_2O_2 produced by the bacteria. At intervals, portions were removed and placed on ice, the bacteria were removed by centrifugation, and H_2O_2 in the medium was measured. The third culture received glucose, LP, and SCN^-, to determine the rate of growth in the presence of the $OSCN^-$ produced from the H_2O_2 that was released by the bacteria. At intervals, portions were removed, and $OSCN^-$ in the medium was measured.

Figure 4 (left) shows that when H_2O_2 was eliminated by catalase, the bacteria grew rapidly and reached a new stationary-phase level within 4 h. At the time that glucose and catalase were added, the H_2O_2 concentration in the growth medium was 0.025 mM and the pH was 5.9. After 6 h, no H_2O_2 was present and the pH had fallen to 4.9.

When no catalase was added, growth was slower and stopped after about 6 h. The pH fell to 5.7. Figure 4 (right) shows that H_2O_2 accumulated in the medium and reached 0.9 mM after 6 h. Therefore, about 6 h of exposure to their own H_2O_2 was sufficient to stop growth of these bacteria.

When LP and SCN^- were added, growth was completely blocked. The pH remained at 5.9. Figure 4 (right) shows that the highest observed level of $OSCN^-$ was 0.04 mM, which was achieved within the first 15 min. The $OSCN^-$ level slowly dropped to 0.02 mM, but growth did not resume.

In control experiments, adding inactivated catalase with glucose had no effect, indicating that the growth-promoting effect of catalase was due to removing H_2O_2. Similarly, when glucose and either LP or SCN^- were added, growth was the

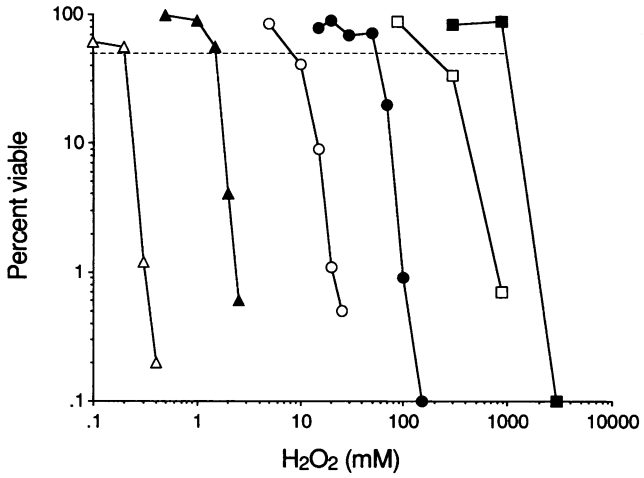

FIG. 5. Effect of time on killing by H_2O_2. GS-5 cells were suspended to 3×10^8 CFU/ml in pH 7 buffer without glucose at 37°C. H_2O_2 was added, and the mixtures were incubated for 15 s (■), 3 min (□), 15 min (●), 1 h (○), 4 h (▲), or 24 h (△). Catalase was added, and the mixtures were diluted and plated to measure CFU per milliliter. The dotted line indicates the 50% viable level, from which LD_{50} values were obtained.

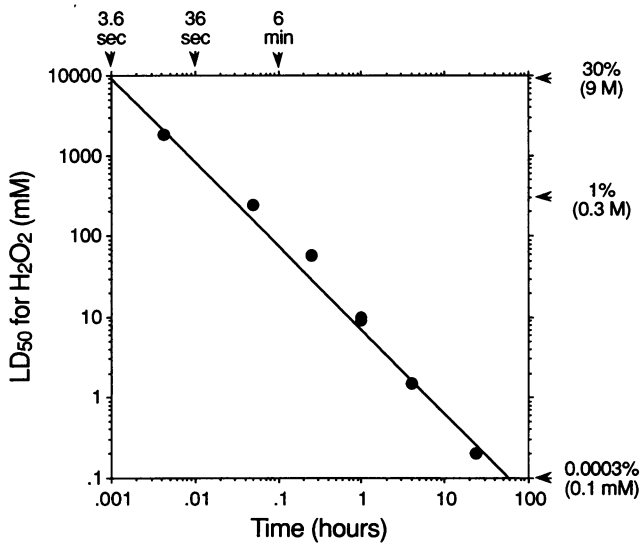

FIG. 6. Effect of time on LD_{50} values for H_2O_2. LD_{50} values obtained from Fig. 6 versus the time of incubation are plotted.

same as that when only glucose was added, indicating that the formation of $OSCN^-$ was required to block growth.

The results indicate that H_2O_2 with LP and SCN^- was much more effective than H_2O_2 alone as an inhibitor of growth. Exposure to increasing H_2O_2 concentrations of up to 0.9 mM over a period of 6 h was required to stop growth, whereas the LP system caused an immediate complete blockage of growth, metabolism, and H_2O_2 production at $OSCN^-$ levels of about 0.04 mM.

Bactericidal activity. Washed GS-5 cells were incubated with various amounts of H_2O_2 at pH 7 without glucose. After incubation periods of 15 s to 24 h, catalase was added to remove H_2O_2, and viability was measured by diluting, plating, and counting colonies after growth at 37°C. Figure 5 shows that high levels of H_2O_2 killed the bacteria and that less H_2O_2 was required for killing in the longer incubations.

The LP system had no bactericidal activity during a 1-h incubation at pH 7. Loss of viability with an LD_{50} value of 0.4 mM was observed after 24 h. However, viability did not fall below 20% of the control as H_2O_2 was increased, whereas Fig. 5 shows that H_2O_2 alone lowered viability to 0.1% and was probably capable of sterilizing the incubation mixtures.

Concentrations of H_2O_2 required to kill half the bacteria (LD_{50} values) were read from the plots in Fig. 5. When the LD_{50} values were plotted versus time on a log/log plot, a linear relation was obtained (Fig. 6). From this plot it is possible to estimate the H_2O_2 concentration required to kill half the bacteria within a given period of time or the time required for killing by a particular H_2O_2 concentration.

ED_{50} values for H_2O_2 at 1, 4, and 24 h were 1.2, 0.6, and 0.2 mM, respectively, whereas the LD_{50} values were 9.0, 1.5, and 0.2 mM, respectively. After 24 h, the values were the same, but with shorter incubations the ED_{50} values were lower. These results indicate that with incubations of less than 24 h, H_2O_2 caused a reversible inhibition of metabolism that could be partially relieved by adding catalase but that inhibition eventually became irreversible. In other experiments, adding catalase stopped the action of H_2O_2. There was no further decline in metabolic capacity or loss of viability after H_2O_2 was removed.

In other experiments, GS-5 cells were incubated with various amounts of H_2O_2 at pH 7 in the presence of glucose. In contrast to the results obtained without glucose (Fig. 5 and 6), H_2O_2 at concentrations below 0.3 mM did not kill the bacteria, regardless of the length of the incubation. The LD_{50} value was 0.6 mM at 24 h. Measurements of H_2O_2 in the medium showed that when glucose was present, H_2O_2 at concentrations below 0.3 mM was consumed within 4 h. Therefore, when glucose was present and H_2O_2 was less than 0.3 mM, the bacteria were able to eliminate all the H_2O_2 from the medium, and prolonging the incubation after H_2O_2 was gone had no effect on viability.

DISCUSSION

Antibacterial activity in vitro. The antibacterial activity of H_2O_2 against oral streptococci increases with time of exposure to H_2O_2. Provided that no catalase-containing microorganisms or human cells are present, H_2O_2 can persist in the medium for long periods of time when incubated with streptococci, and H_2O_2 has a slow-acting antibacterial activity that becomes strong in prolonged incubations. The large difference in the results of experiments with a time scale of a few minutes to hours versus those carried out for a day or longer can account for conflicting reports on H_2O_2 toxicity.

H_2O_2 has significant bactericidal activity in prolonged incubations, whereas the combination of LP, H_2O_2, and SCN^- is primarily bacteriostatic. Therefore, the presence of LP and SCN^- can protect the bacteria against killing by H_2O_2. If the loss of viability is the only assay used to evaluate antibacterial activity, H_2O_2 will almost always appear to be more effective than the LP system.

Streptococci do have a limited ability to detoxify H_2O_2. For example, when GS-5 cells were incubated with glucose and 0.3 mM H_2O_2, metabolism was not inhibited and all of the H_2O_2 was consumed. Similarly, when glucose was present, 0.3 mM H_2O_2 did not kill the bacteria. Therefore, if a supply of metabolizable carbohydrate is available and the H_2O_2 concentration is not so high as to completely block metabolism, then

NADH can be generated for reduction of H_2O_2 to water by the streptococcal NADH-peroxidase enzyme. Under these conditions, the bacteria escape from the slow-acting toxicity of H_2O_2.

At low levels of H_2O_2, the combination of LP, H_2O_2, and SCN^- is much more effective than H_2O_2 alone as an inhibitor of bacterial metabolism and growth. However, when H_2O_2 is added as a single addition at the start of an experiment, the LP system becomes less effective with time, because of decomposition of the inhibitor $OSCN^-$. Measurement of metabolism and/or growth are required to evaluate the activity of the LP system, and even these measurements may overlook the activity if measurements are made after the bacteria have recovered from inhibition.

Under more physiologic conditions, low levels of H_2O_2 would be supplied continuously to the LP system, and inhibition of growth and metabolism could be sustained indefinitely, if the steady-state level of $OSCN^-$ is higher than the threshold concentration. Below this concentration, $OSCN^-$ has no effect.

Many factors influence the resistance of streptococci to inhibition by $OSCN^-$, by raising or lowering the threshold concentration (37). Among these factors are pH, intracellular levels of sulfhydryl (thiol) compounds, and intracellular reserves of metabolizable carbohydrate, as well as factors that have yet to be identified. Because $OSCN^-$ reacts almost exclusively with sulfhydryl groups of enzymes, transport carriers, and other proteins, resistance and recovery from inhibition are related to the ability of cells to reduce the oxidized sulfhydryl groups (25, 37). Reduction of $OSCN^-$ to SCN^- by bacterial enzymes also contributes to resistance (3, 17). Both of these processes require the production of reducing equivalents, probably in the form of NADH, and thus may depend on the ability of the bacteria to carry out low levels of metabolism even in the presence of the inhibitor $OSCN^-$.

H_2O_2 and $OSCN^-$ levels in the oral environment. H_2O_2 has a long history of use in oral medicine and hygiene. At the very high concentration of 9 M (30%), H_2O_2 has been used as a bleaching agent for discolored teeth. At the lower level of 0.3 M (1%), H_2O_2 is commonly used as an antiseptic and deodorizing component of oral rinses. The results presented here indicate that this level of H_2O_2 would cause transient inhibition of carbohydrate metabolism and lactic acid production by oral streptococci but that continuous exposure for several minutes would be required for a significant reduction in the number of viable bacteria.

Although 1% H_2O_2 may appear to be a relatively low concentration, a 1% H_2O_2 solution is isotonic in H_2O_2. When 1 ml of 1% H_2O_2 comes into contact with catalase, 3.4 ml of O_2 gas is released, and the heat produced is sufficient to raise the temperature of the solution from 37 to 43°C. Within the confined space of a catalase-containing cell, the toxicity of 1% H_2O_2 may be due not only to oxidizing activity but also to the explosive release of gas and heat. The absence of catalase in streptococci might actually result in greater resistance to H_2O_2 at this high level.

High levels of H_2O_2 also inactivate LP and other peroxidase enzymes and destroy any $OSCN^-$ that might be produced before LP is inactivated (20). Therefore, the LP system is not a significant factor when high levels of H_2O_2 are used. The highest yield of $OSCN^-$ in saliva was obtained with an oral rinse containing additional SCN^- and only 0.7 mM (0.0023%) H_2O_2 (14). The production of H_2O_2 by the salivary glands and oral bacteria is at even lower levels.

An estimate of H_2O_2 production in the oral environment is provided by $OSCN^-$ levels in saliva, because about 1 mol of $OSCN^-$ is produced per mole of H_2O_2 when the SCN^- concentration is greater than that of H_2O_2, the H_2O_2 concentration is lower than about 0.3 mM, and adequate LP or the leukocyte enzyme myeloperoxidase is present (2, 26, 32). Average levels of $OSCN^-$ in stimulated and unstimulated human whole saliva are 0.01 to 0.03 mM and 0.04 to 0.06 mM, respectively (12, 13, 18, 23, 29). In secretions collected directly from the stimulated parotid salivary gland, the average $OSCN^-$ levels are 0.06 mM (18). If the H_2O_2 produced by the salivary glands was not consumed by the LP system or by catalase and other H_2O_2-detoxifying enzymes, the oral tissues and oral bacteria would be subjected continuously to H_2O_2 at levels up to 0.06 mM.

Production of H_2O_2 by oral bacteria in saliva is at even lower levels. When the bacteria present in 1 ml of mixed human saliva were washed free of LP and SCN^- and incubated with glucose and an H_2O_2-trapping system, the average rate of H_2O_2 production was 100 nmol/ml/h (30). If this H_2O_2 was not consumed by the LP system or by catalase and other enzymes, H_2O_2 would reach 0.1 mM in 1 h. Because of the continuous dilution and removal of mixed saliva, steady-state levels of H_2O_2 from this source would probably be well below 0.01 mM. H_2O_2 production by bacteria adhering to oral surfaces may be greater than H_2O_2 production by the bacteria in whole saliva samples. However, the observation that $OSCN^-$ levels in mixed saliva are similar to or less than $OSCN^-$ levels in parotid saliva indicates that the salivary glands are the major source of H_2O_2.

Therefore, results presented here indicate that the H_2O_2 produced by the salivary glands and oral bacteria is unlikely to have significant antibacterial activity against oral streptococci. Even if the LP system was blocked, the amounts of H_2O_2 would be too small and the times of exposure would be too short, because H_2O_2 would be removed by catalase, glutathione peroxidase, NADH-peroxidase, and other H_2O_2-detoxifying enzymes in human cells and oral microorganisms.

On the other hand, the LP system seems to be well designed as an inhibitor of bacterial metabolism and growth in the oral environment. LP uses H_2O_2 to produce a more effective inhibitor and thus amplifies the activity of H_2O_2. Moreover, the LP system is much more effective at low pH. Antimicrobial activity is most needed when the pH falls as a result of bacterial lactic acid production.

Nevertheless, streptococci found in stimulated mixed saliva from most individuals are metabolically active, despite the presence of 0.01 to 0.03 mM $OSCN^-$ (35). Additional H_2O_2 of about 0.09 mM must be added to saliva to raise $OSCN^-$ to levels that completely block metabolism at neutral pH. These results indicate that bacteria in the oral environment have a higher threshold level for inhibition by $OSCN^-$ than they do under the conditions used in this study. The results also suggest that the introduction of low levels of H_2O_2, either directly or by adding H_2O_2-generating systems, would be the most effective approach to inhibiting acid production and growth of pathogenic streptococci.

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