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Inhibition of *Streptococcus mutans* by the Lactoperoxidase Antimicrobial System

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Inhibition of bacterial metabolism by the lactoperoxidase (LP)-hydrogen peroxide (H_2O_2)-thiocyanate system was studied with representatives of serotypes *a* through *g* of *Streptococcus mutans*. The aims were to determine whether the amount of H_2O_2 released from these catalase-negative bacteria is sufficient to activate the LP system and whether these oral bacteria are resistant to inhibition by the LP system, which is active in human saliva. When the washed, stationary-phase cells were incubated aerobically with LP, thiocyanate, and glucose (Glc), greater than 90% inhibition of Glc utilization and lactate production was obtained with strains that released large amounts of H_2O_2 (BHT, FA-1, OMZ-176); 20 to 50% inhibition was obtained with strains that released about half as much H_2O_2 (B-13, Ingbritt); and no inhibition was obtained with strains that released only small amounts of H_2O_2 (AHT, HS-6, GS-5, LM-7, OMZ-175, 6715-15). Inhibition was most effective at pH 5, whereas release of H_2O_2 and accumulation of the inhibitor (hypothiocyanite ion) were highest at pH 8. With H_2O_2-releasing cells from early stationary phase, preincubation with Glc abolished inhibition, though it did not influence H_2O_2 release. Cells harvested 24 h later were depleted of sulfhydryl compounds. Inhibition of these cells was abolished by preincubation with Glc and certain sulfhydryl or disulfide compounds (reduced or oxidized glutathione, cysteine or cystine). This preincubation increased cell sulfhydryl content but had no effect on H_2O_2 release. All strains were inhibited when incubated with LP, thiocyanate, and added (exogenous) H_2O_2. Smaller amounts of H_2O_2 were required to inhibit at pH 5, and larger amounts were required to inhibit cells preincubated with Glc or with Glc and the sulfhydryl or disulfide compounds. The results indicate that pH, amount of H_2O_2, cell sulfhydryl content, and stored-carbohydrate content determine susceptibility to inhibition.

The combination of lactoperoxidase (LP), hydrogen peroxide (H_2O_2), and thiocyanate ion (SCN^-) forms a bacteriostatic system in milk, tears, and saliva (14, 19, 24, 30, 38). LP catalyzes the oxidation of SCN^- by H_2O_2 to yield the antimicrobial oxidizing agents hypothiocyanous acid (HOSCN) or hypothiocyanite ion ($OSCN^-$) (2, 18). These two agents are in acid-base equilibrium (34), and $OSCN^-$ is the predominant form at pH values above the pK_a of 5.3. The inhibitor is referred to as $OSCN^-$, although assays for the inhibitor determine the sum of HOSCN and $OSCN^-$ ($HOSCN\text{-}OSCN^-$) and HOSCN may be the form with greater antimicrobial activity. Antibacterial action is greater at low pH (16), which may be owing to the ability of the uncharged HOSCN to penetrate microbial membranes and thus to attack functional groups of essential intracellular enzymes (34). Inhibition of metabolism is correlated with oxidation of cell sulfhydryls to the sulfenyl or disulfide oxidation states (3, 21–23, 36).

Whereas LP and SCN^- are secreted into saliva (26, 39), the major source of H_2O_2 in the oral environment is oral bacteria (20, 38). Production and release of H_2O_2 to the extracellular medium is characteristic of certain catalase-negative bacteria (7), which are the predominant microorganisms of the oral environment (15, 20). Carbohydrate metabolism is the source of reducing equivalents for the reduction of oxygen (O_2) to H_2O_2 (7). Therefore, bacterial metabolism is required to produce the inhibitor of bacterial metabolism.

No H_2O_2 is produced under anaerobic conditions, so that oral bacteria may escape inhibition within environments such as dental plaque. Bacterial O_2 metabolism, together with the low rate of O_2 diffusion into plaque, result in O_2 depletion (12). However, oral bacteria must have at least a limited resistance to inhibition under aerobic

conditions to grow to the cell density required to establish the anaerobic environment and to account for the levels of $OSCN^-$ in human saliva (33, 37, 38).

Several studies have shown that certain streptococci exhibit limited resistance (11, 17, 28, 32). It has been reported that a certain minimum (threshold) concentration of $OSCN^-$ is required for inhibition (17, 32). Resistance varies with the metabolic state (17), although the aspects of metabolism that contribute to resistance have not been identified. In another study (28), resistance was attributed to one or more enzymes that could reduce the inhibitor back to SCN^-.

Bacteria classified as *Streptococcus mutans* are of special interest as targets for the LP system in that these organisms are oral pathogens (13). All *S. mutans* strains are catalase negative and release detectable amounts of H_2O_2, although only a few release large amounts (E. L. Thomas and K. A. Pera, submitted for publication). The aims of this study were to determine whether the amount of H_2O_2 released is sufficient to have an antimicrobial effect in combination with LP and SCN^- and whether all strains can be inhibited when H_2O_2 is supplied exogenously. Also, we sought to identify factors that contribute to resistance.

MATERIALS AND METHODS

LP purified from bovine milk (25) was provided by M. Morrison, St. Jude Hospital, Memphis, Tenn., and its concentration was calculated from the molar extinction coefficient of 11,400 at 412 nm. Horseradish peroxidase and reagents for determination of L-lactate were from Sigma Chemical Co. Catalase and reagents for determination of acetate were obtained from Boehringer Mannheim Corp. Catalase crystals were washed by centrifugation in water and then dissolved (2.4 mg/ml) in 20 mM KCl–4 mM phosphate, pH 8. Reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid (Nbs) with sodium borohydride was done as described previously (2, 37). H_2O_2 (30%; Fisher Chemical Co.) was diluted into water, and its concentration was determined by the method based on horseradish peroxidase-catalyzed oxidation of leucocrystal violet (27). D-[U-^{14}C]glucose ([^{14}C]Glc; 283 Ci/mol) was obtained from Amersham Corp.

Growth medium. Todd-Hewitt broth (Difco Laboratories) at 3 g/liter was filtered through an acid-washed, 4- to 5.5-μ sintered glass funnel, and 100-ml portions were sterilized by filtration through 0.22 μ filters (Nalge Co.).

Bacteria. *S. mutans* strains obtained from T. Kral, Temple University, Philadelphia, Pa., were grown aerobically to stationary phase (24 h), and portions of the culture were frozen in liquid N_2 and stored at −80°C. Portions were thawed at 37°C, and 1 ml was used to inoculate 100 ml of medium in 250-ml flasks with a foam plug for aerobic growth ($+O_2$) or interconnected to a flask with 100 ml of 0.1 M phosphate (pH 7) to which 1 g of sodium hydrosulfite (dithionite) was added immediately before closing the system for anaerobic growth ($-O_2$). Results presented below were obtained with cells from aerobic cultures, except for strains AHT and HS-6, which are less aerotolerant (6; Thomas and Pera, submitted for publication). Flasks were shaken at 200 rpm at 37°C. Cells were harvested in the early stationary phase (16 to 24 h) or the late stationary phase (40 to 48 h). To obtain cells in the logarithmic growth phase, 10 ml of a 16-h culture was transferred to 100 ml of medium, and the cells were harvested about 3 h later, when absorbance at 600 nm was 60 to 70% of that obtained at the stationary phase.

Cultures were centrifuged at 5,000 × *g*, suspended in 2 volumes of cold 0.12 M NaCl–1 mM $MgSO_4$, centrifuged again, and resuspended with a Teflon-glass homogenizer to an absorbance at 600 nm of 4.5, corresponding to about 2.3 mg/ml (dry weight) or two to four times the cell density obtained at stationary phase. Suspensions were held at 4°C under N_2 and used within 1 h. Anaerobic preincubations (under N_2) were carried out in a total volume of 20 to 40 ml at 37°C in 0.12 M NaCl–1 mM $MgSO_4$–50 mM potassium phosphate, pH 7. Cells were washed twice by centrifugation in NaCl-Mg. All centrifugations were carried out at 20,000 × *g* for 15 min at 4°C.

Aerobic incubations. Unless otherwise indicated, cells at one-fifth the cell density indicated above (relative cell density, 0.2) were incubated with 10 mM Glc in 2 ml of 0.12 M NaCl–1 mM $MgSO_4$–20 mM potassium phosphate buffer in closed 50-ml Erlenmeyer flasks on a reciprocal shaker at 100 cycles per min. H_2O_2 accumulation was measured after incubation without other additions. Accumulation of $OSCN^-$ was measured after incubation with 2 nM LP and 1 mM SCN^-. Glc, lactate, and acetate were measured after incubation with either LP and SCN^- or with 50 μg of catalase per ml.

Anaerobic incubations. Unless otherwise indicated, cells at the density indicated above (relative cell density, 1) in 2 ml of 0.12 M NaCl–1 mM $MgSO_4$–50 mM potassium phosphate buffer at 4°C were supplemented with 10 mM Glc or [^{14}C]Glc, 2 nM LP, 1 mM SCN^-, and H_2O_2 added in 15-, 30-, or 60-μM increments at 1-min intervals. Suspensions were warmed to 37°C and incubated for 15 min under N_2, and then Glc, lactate, acetate, and cell-associated ^{14}C label were measured.

H_2O_2 accumulation. After centrifugation, H_2O_2 concentration in the supernatant was determined with horseradish peroxidase and leucocrystal violet.

HOSCN-$OSCN^-$ accumulation. Reaction mixtures were cooled to 4°C, and then 10 μg of catalase and excess Nbs in 0.1 M Tris-chloride (pH 7) were added. After centrifugation, absorbance at 412 nm was measured, and the sum of HOSCN and $OSCN^-$ (HOSCN-$OSCN^-$) was calculated as one-half the difference between the amount of Nbs added and the amount of Nbs remaining (2).

Glc metabolism. Glc, lactate, and acetate concentrations were determined in portions of supernatants supplemented with 10 μg of catalase. Glc was determined by the phenol-sulfuric acid method (1). Lactate was determined by L-lactic dehydrogenase-catalyzed reduction of NAD^+ measured at 340 nm (Bulletin 826-UV; Sigma). Acetate was determined by reduction of NAD^+, using the coupled enzyme assay with acetyl coenzyme A synthetase, citrate synthase, and malate dehydrogenase (Boehringer Mannheim). At the con-

TABLE 1. Effect of culture conditions and pH of incubation on H_2O_2 or HOSCN-$OSCN^-$ accumulation and on inhibition of lactate production[a]

Strain (class)	Culture age (h)	pH	H_2O_2 (μM)	HOSCN-$OSCN^-$ (μM)	Lactate (mM)		% Inhibition
					LP, SCN^-	Catalase	
OMZ-176 (I)	3	5	10	0	0.87	0.86	−1
		7	15	3	1.34	1.29	−4
	24	5	31	3	0.25	0.57	56
		6	62	6	0.53	1.17	55
		7	70	7	0.67	1.16	42
		8	81	12	0.92	1.15	20
	40	5	9	5	0.04	0.23	83
		6	29	7	0.10	0.54	81
		7	109	22	0.23	1.02	77
		8	129	20	0.85	1.53	44
Ingbritt (II)	24	5	12	3	0.84	1.17	28
		7	15	16	1.47	1.69	13
	40	5	15	4	0.20	0.41	51
		7	32	10	0.97	1.23	21
GS-5 (III)	24	5	0	1	0.60	0.73	18
		7	0	0	1.18	1.17	−1
	40	5	0	1	0.35	0.38	8
		7	0	3	0.97	0.95	−2

[a] Cells harvested at the indicated times were incubated aerobically for 1 h at the indicated pH with 10 mM Glc to measure H_2O_2 accumulation; with Glc, LP, and SCN^- to measure HOSCN-$OSCN^-$ accumulation; and with either Glc, LP and SCN^- or Glc and catalase to measure lactate production. Inhibition of metabolism was calculated from the decrease in lactate production in the presence of LP and SCN^-.

centrations present in diluted portions of supernatants, LP, SCN^-, or $OSCN^-$ did not interfere. Conversion of [^{14}C]Glc to macromolecular, cell-associated forms was measured after centrifugation by resuspending cells in 5% (wt/vol) cold trichloroacetic acid, filtering through 0.45-μ filters (Millipore Corp.), dissolving the filters in scintillation fluid (4), and determining radioactivity in a liquid scintillation spectrometer.

Sulfhydryl content. Bacterial sulfhydryls were measured by reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to Nbs (8) in cells permeabilized with detergent (35). Portions (2 ml) of washed cells or reaction mixtures were diluted with 6 ml of cold NaCl-Mg, centrifuged, and suspended in 2 ml of water. Additions of 20 μl of 1 M potassium EDTA (pH 7), 1 ml of 0.1 M Tris-chloride (pH 8), 0.1 ml of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid), and 0.5 ml of 20% (wt/vol) sodium dodecyl sulfate were made with mixing after each addition. Mixtures were incubated for 1 h at 37°C under N_2, cooled on ice for 15 min, and centrifuged, and absorbance of the supernatants was measured at 412 nm.

RESULTS

Inhibition dependent on bacterial H_2O_2 production (autoinhibition). Table 1 shows the results obtained with strains that were representative of the 11 strains examined. Similar results were obtained with strains OMZ-176, FA-1, and BHT (class I). When cells of class I strains were harvested from growing cultures (3 h), washed, and incubated aerobically with Glc at pH 5 or 7, only a small amount of H_2O_2 accumulated in the medium. When these cells were incubated with Glc, LP, and SCN^-, a small amount of $OSCN^-$ was detected at pH 7. There was no inhibition of lactate production in that the amount of lactate in the medium after 1 h was the same in the presence of Glc, LP, and SCN^- or in the presence of Glc and catalase.

However, inhibition was obtained with class I strains from stationary phase (24 or 40 h). H_2O_2 and $OSCN^-$ accumulation increased with culture age. Lactate production and H_2O_2 or $OSCN^-$ accumulation were highest at pH 8, but inhibition of lactate production was highest at pH 5.

Class II consisted of strains Ingbritt and B-13. H_2O_2 accumulated to lower levels, although $OSCN^-$ accumulation was about the same as that seen in class I strains. Inhibition was most effective at pH 5 with cells from 40-h cultures.

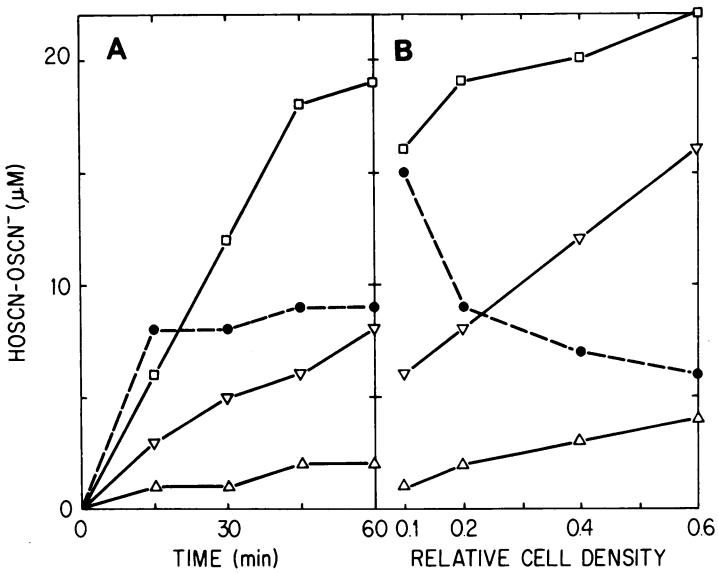

FIG. 1. Accumulation of $OSCN^-$. Cells from 24-h cultures of OMZ-176 (●), Ingbritt (□), HS-6 (▽), and GS-5 (△) were incubated aerobically at pH 8 with Glc, LP, and SCN^- for the indicated periods of time with a relative cell density of 0.2 (A) or for 1 h at the indicated cell density (B).

Class III consisted of strains GS-5, HS-6, AHT, LM-7, OMZ-175, and 6715-15. Although cells of these strains release detectable amounts of H_2O_2, there is no H_2O_2 accumulation in the medium, owing to their high levels of H_2O_2-reducing enzymes (Thomas and Pera, submitted for publication). Table 1 shows that no H_2O_2 accumulated regardless of culture age or pH of incubation. Little or no $OSCN^-$ was detected, and there was no inhibition.

Figure 1 shows the effect of incubation time and cell density on the accumulation of $OSCN^-$ at pH 8. Under these conditions, metabolism of the OMZ-176 cells (class I) was partially inhibited (Table 1). With these cells, $OSCN^-$ reached a maximum level quickly and did not increase with longer incubations. Also, the amount of $OSCN^-$ decreased with increasing cell density. In contrast, there was no inhibition of Ingbritt (class II) or GS-5 and HS-6 (class III) cells under these conditions, and $OSCN^-$ increased with time and cell density. $OSCN^-$ levels observed with HS-6 cells were higher than those observed with GS-5 cells. HS-6 and AHT cells release small amounts of superoxide (O_2^-), which can dismutate to yield H_2O_2 in the medium (Thomas and Pera, submitted for publication).

Table 2 shows that the early stationary-phase OMZ-176 cells (class I) could be converted to a resistant state. When these cells were preincubated with Glc, washed, and then incubated with Glc, LP, and SCN^-, there was a decrease in the percent inhibition related to the time of preincubation with Glc. The effect of preincubation was not due to an effect on H_2O_2 release; in other experiments, Glc-preincubated cells accumulated as much or slightly more H_2O_2 than did control cells. Also, preincubation with Glc slightly increased accumulation of $OSCN^-$ at pH 8.

Preincubation with Glc was not sufficient to abolish autoinhibition of late-stationary-phase cells. However, these cells could be converted to a resistant state by preincubating with Glc and the sulfhydryl compounds, cysteine or reduced glutathione (GSH), or with Glc and the corresponding disulfide compounds, cystine or glutathione disulfide (GSSG). Table 2 shows that when the 40-h cells were preincubated with Glc and various concentrations of GSSG, there was a dramatic increase in bacterial sulfhydryl content, and autoinhibition decreased in proportion to the increase in sulfhydryls.

In other experiments, preincubation with these sulfhydryl or disulfide compounds in the absence of Glc had no effect on sulfhydryl content or autoinhibition. Also, dithiothreitol, 2-mercaptoethanol, D-cysteine, or D-cystine at 0.25 mM had no effect with or without Glc.

When the excess sulfhydryl or disulfide compounds were removed by washing before incubation with Glc, LP, and SCN^- (Table 2), preincubation had no effect on H_2O_2 accumulation at a pH of 5 to 8 and no effect on $OSCN^-$ accumulation at pH 8. However, the sulfhydryl or disulfide compounds did interfere with $OSCN^-$ accumulation when they were present during the incubation with Glc, LP, and SCN^-.

TABLE 2. Effect of culture and preincubation conditions on inhibition of lactate production[a]

Culture age (h)	Preincubation conditions			Sulfhydryl (μM)	Lactate (mM)		% Inhibition
	Time (min)	Glc (mM)	GSSG (μM)		LP, SCN^-	Catalase	
24	0	10	0	20	0.30	0.66	55
	2				0.47	0.67	30
	5				0.35	0.55	36
	15				0.42	0.60	30
	30				0.53	0.66	20
	60			20	0.60	0.69	13
40	0	10	0	12	0.06	0.41	85
	5				0.11	0.58	81
	30				0.08	0.46	83
	60			10	0.06	0.42	86
	60	10	10	19	0.14	0.52	73
			25	34	0.26	0.58	55
			50	43	0.38	0.46	17
			100	46	0.52	0.56	7

[a] OMZ-176 (class I) cells harvested from 24- or 40-h cultures were preincubated for the indicated periods of time with Glc or Glc and GSSG. After washing by centrifugation, sulfhydryl content was measured with a relative cell density of 1. Also, cells at a relative cell density of 0.2 were incubated aerobically for 1 h with Glc, LP and SCN^-, or Glc and catalase, and then lactate was measured.

Figure 2 shows the time course of lactate production by 40 h OMZ-176 cells in the presence of Glc and catalase or in the presence of Glc, LP, and SCN^-. With catalase, lactate production was nearly linear with time, and similar results were obtained with control cells or with cells that were preincubated with Glc or Glc plus GSSG. With LP and SCN^-, lactate production by control cells decreased with time and stopped after 1 to 2 h. Cells preincubated with Glc or Glc plus GSSG continued to produce lactate for longer periods of time. In other experiments, the time required to detect inhibition was also influenced by cell density. When

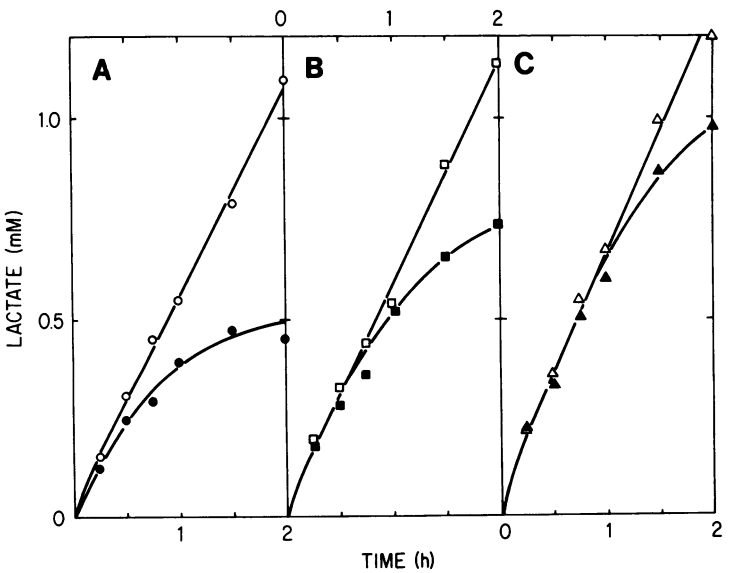

FIG. 2. Time course of autoinhibition. OMZ-176 cells (40 h) were incubated aerobically at pH 7 with Glc and catalase (open symbols) or with Glc, LP, and SCN^- (closed symbols) either without preincubation (A; ○, ●) or after preincubation for 1 h with 10 mM Glc (B; □, ■) or with 10 mM Glc and 0.25 mM GSSG (C; △, ▲).

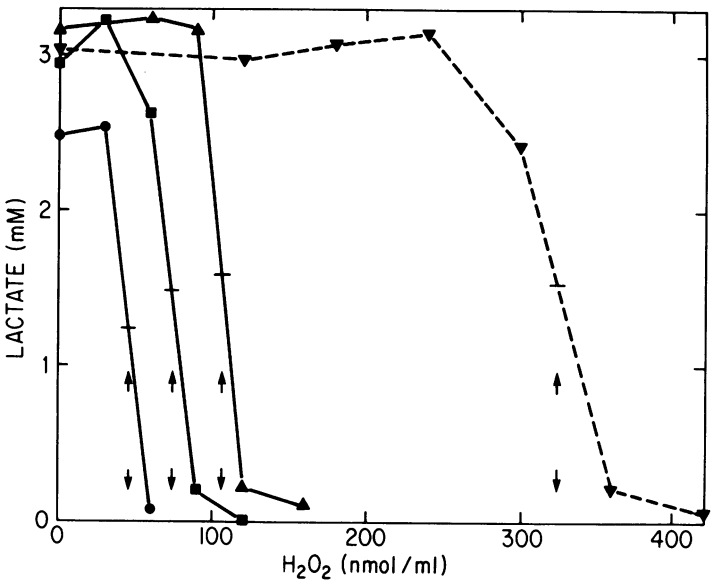

FIG. 3. Inhibition with exogenous H_2O_2. GS-5 cells (24 h) were preincubated for 1 h with 10 mM Glc, washed, exposed to LP, SCN^-, and various amounts of H_2O_2 at pH 5 (●), 6 (■), 7 (▲), or 8 (▼), and then incubated for 15 min at 37°C with Glc. Arrows indicate ED_{50}s.

the number of cells was increased five-fold (relative cell density, 1), significant inhibition was not observed until after 2 to 3 h of incubation.

In other experiments, similar results were obtained with cells from anaerobic or aerobic cultures. Also, similar percentages of inhibition were obtained when lactate production, acetate production, or Glc consumption was used as the measure of bacterial metabolism. Inhibition of class I strains was blocked by catalase, and LP or SCN^- alone did not inhibit these strains.

Similar percentages of inhibition were calculated when lactate production in the presence or absence of catalase was used as the noninhibited control value. Adding catalase increased the rate of lactate production by 0 to 15%, depending on the strain. Because the LP system eliminates H_2O_2, lactate production in the presence of catalase was considered to be the appropriate control.

Inhibition with exogenous H_2O_2. Cells of all classes were inhibited by the LP system when H_2O_2 was supplied exogenously. GS-5 cells (class III) were preincubated with Glc, washed, and incubated wih LP, SCN^-, and various amounts of H_2O_2 at pH 5, 6, 7, or 8 under nonmetabolizing conditions (4°C) (Fig. 3). Glc was added, the reaction mixtures were warmed to 37°C for 15 min, and then lactate was measured. This last incubation was performed in the absence of O_2 (under N_2) to prevent H_2O_2 production by the bacteria.

A smaller amount of H_2O_2 was required for inhibition at the lower pH values. At all pH values, a minimum (threshold) amount of H_2O_2 was required for inhibition. For example, at pH 8 with LP, SCN^-, and increasing amounts of H_2O_2 to about 250 μM, there was no inhibition. With slightly higher amounts of H_2O_2, lactate production decreased sharply, so that the cells went from a completely resistant to a completely inhibited state over a narrow range of H_2O_2 additions. The amount of H_2O_2 required for 50% inhibition (50% effective dose [ED_{50}]) was calculated as the amount required to lower lactate production to one-half of the control value. ED_{50}s were 46, 74, 106, and 324 μM at pH 5, 6, 7, and 8, respectively (Fig. 3). In other experiments, ED_{50}s were proportional to cell density.

LP, SCN^-, or H_2O_2 alone or the combination of any two of these did not inhibit under these conditions. Inhibition was obtained with H_2O_2 alone at higher concentrations, with an ED_{50} of 2 to 3 mM. Therefore, the LP-H_2O_2-SCN^- system was at least 20 to 30 times more effective than H_2O_2 alone. In experiments with cells that were not preincubated with Glc, lower ED_{50}s were obtained with the LP system, whereas the amount of H_2O_2 required for inhibition in the absence of LP and SCN^- was about the same. Under these conditions, the LP system was up to 300 times more effective than H_2O_2 alone.

A similar ED_{50} was obtained when lactate or acetate production or Glc utilization was used as the measure of bacterial metabolism (Fig. 4). With H_2O_2 below the threshold level, lactate

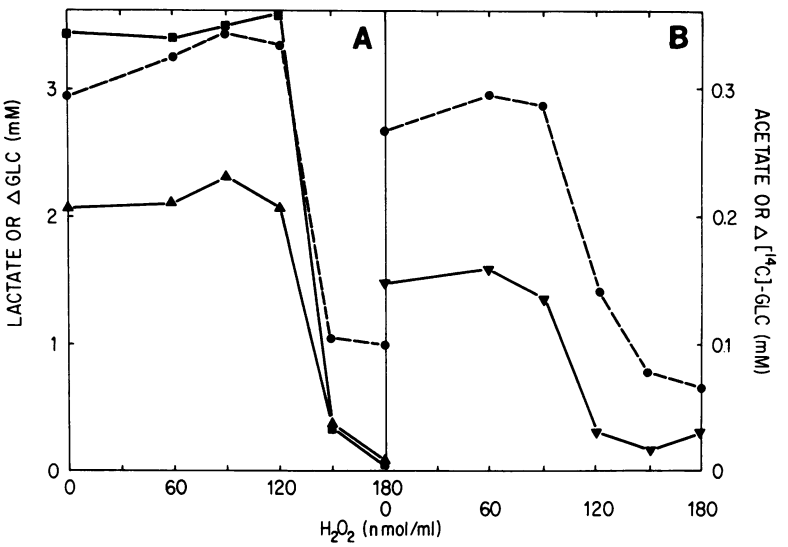

FIG. 4. Inhibition with and without Glc. GS-5 cells (16 h) were preincubated for 1 h with either 10 mM Glc or [^{14}C]Glc, washed, exposed to LP, SCN^-, and various amounts of H_2O_2 at pH 7, and incubated for 15 min at 37°C with Glc (A) or without Glc (B). Lactate (■), the change in Glc concentration (ΔGlc; ▲), acetate (●), and the decrease in cell-associated label from [^{14}C]Glc (Δ[^{14}C]Glc; ▼) were measured.

increased from 0 to 3.5 mM in 15 min, acetate increased from 0 to 0.35 mM, and Glc decreased from 10 to 8 mM, so that ΔGlc = 2 mM. Therefore, lactate production provided the most sensitive measure of metabolism.

In other experiments, GS-5 cells were incubated with 10 mM [^{14}C]Glc. About 2 to 3% of the ^{14}C label was converted to cell-associated (acid-precipitable) forms within 15 min. The threshold and ED_{50} obtained for inhibition of this process was similar to that obtained for inhibition of other forms of Glc utilization.

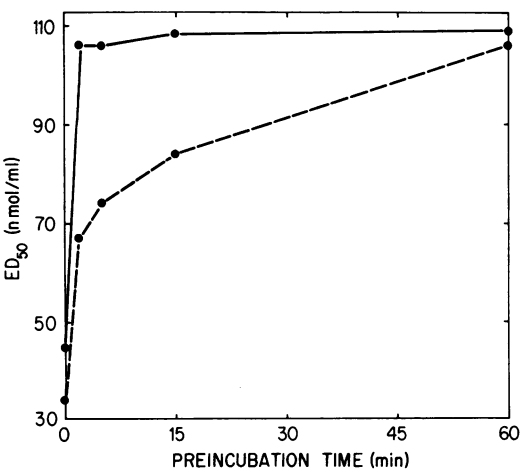

FIG. 5. Effect of preincubation time on ED_{50}. GS-5 cells (24 h) were preincubated with 10 mM Glc for the indicated periods of time, washed, then either held at 4°C (●——●) or incubated for 1 h without Glc (●——●), and then washed again. Cells were exposed to LP, SCN^-, and various amounts of H_2O_2 and incubated wth Glc for 15 min at 37°C, and lactate was measured. ED_{50}s were calculated as described in the text.

GS-5 cells were also preincubated with [^{14}C]Glc, washed, exposed to the LP system, and then incubated at 37°C without Glc. Stored forms of carbohydrate were metabolized, as indicated by release of ^{14}C label in soluble forms and by acetate production (Fig. 4B). However, no lactate was produced in the absence of Glc. Only 15% of cell-associated ^{14}C label was released within 15 min, corresponding to metabolism of 0.15 mM Glc units, calculated as the amount of label per milliliter of cell suspension divided by the specific radioactivity of the [^{14}C]Glc. About 2 h at 37°C were required for release of 50% of the cell-associated label. However, acetate production without Glc was about the same as that with Glc. Therefore, acetate production provided the most sensitive measure of metabolism of carbohydrate reserves. A similar threshold was observed for inhibition of metabolism of extracellular Glc (Fig. 4A) or carbohydrate reserves (Fig. 4B).

When GS-5 cells were preincubated with 10 mM [^{14}C]Glc, the amount of cell-associated label increased linearly with time up to at least 1 h, at which time about 10% of the label was acid precipitable. However, resistance to inhibition by the LP system did not increase linearly (Fig. 5). As little as 2 min of preincubation with Glc

TABLE 3. Effect of culture and preincubation conditions on sulfhydryl content and on amount of H_2O_2 required for inhibition[a]

Strain (class)	Culture age (h)	Preincubation	Sulfhydryl (μM)	ED_{50} (nmol/ml)
OMZ-176 (I)	3		22	90
	24		18	23
		Glc	17	82
		Glc + GSH	50	105
	40		12	9
		Glc	11	61
		Glc + GSH	29	73
Ingbritt (II)	3		24	90
	24		21	50
		Glc	23	108
		Glc + GSH	48	133
	40		16	30
GS-5 (III)	3		28	82
		No additions	21	33
		Glc	23	103
		Glc + GSH	51	133
		Glc + cysteine	48	133
	24		26	45
		Glc	24	108
		Glc + GSH	62	136
		Glc + cysteine	59	138
		Glc + GSSG	50	137
		Glc + cystine	32	130
		Glc + 2-mercaptoethanol	21	110
		Glc + dithiothreitol	27	110
		GSH	18	45
		cysteine	22	45
	40		15	15
		Glc	15	48
		Glc + GSH	32	68

[a] Cells were harvested at the indicated times and either held at 4°C for 1 h or preincubated for 1 h at 37°C with or without 10 mM Glc or the sulfhydryl or disulfide compounds at 0.25 mM. Cells were washed, and the sulfhydryl content was measured. Cells were exposed to LP, SCN^-, and increasing amounts of H_2O_2 at pH 7 and incubated with Glc for 15 min at 37°C, and lactate was measured. ED_{50}s were calculated as described in the text.

was sufficient to raise the ED_{50} 2.5-fold, but the ED_{50} was not raised above this level in longer incubations. Therefore, the level of resistance was not directly proportional to the amount of stored carbohydrate.

A relation between the level of resistance and the amount of stored carbohydrate could be demonstrated in a different way (Fig. 5). After preincubation with Glc for differing periods of time, the cells were washed, incubated for 1 h at 37°C without Glc to deplete their carbohydrate reserves, washed again, exposed to LP, SCN^-, and varying amounts of H_2O_2, and then warmed to 37°C for 15 min with Glc. Under these conditions, the ED_{50} was related to the time of the first incubation with Glc. Therefore, the length of time that the cells could remain resistant was related to the amount of stored carbohydrate.

Table 3 shows the effect of varying experimental conditions on cell sulfhydryl content and ED_{50}. High sulfhydryl content and high resistance to inhibition (high ED_{50}s) were observed with cells from growing cultures (3 h). Preincubating 3 h GS-5 cells with Glc had little effect on the ED_{50}, suggesting that the cells were

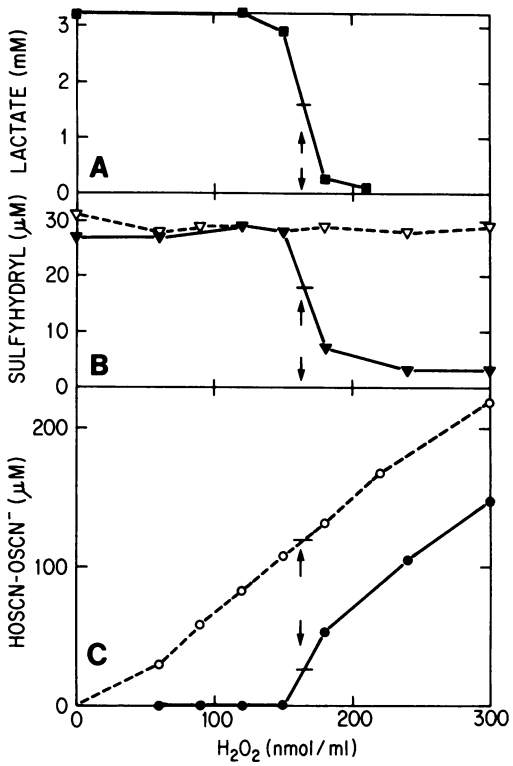

FIG. 6. Correlation of inhibition, sulfhydryl oxidation, and appearance of unreacted $OSCN^-$. GS-5 cells (16 h) were preincubated for 1 h with 10 mM Glc, washed, and exposed to LP, SCN^-, and increasing amounts of H_2O_2 at 4°C. (A) Cells were incubated with Glc for 15 min at 37°C, and then lactate was measured. (B) Reaction mixtures were centrifuged without incubation at 37°C (▽) or incubated with Glc for 15 min at 37°C and then centrifuged (▼). After washing, cell sulfhydryl content was measured. (C) $OSCN^-$ was measured without incubation at 37°C (○) or after 15 min at 37°C with Glc (●). Arrows indicate ED_{50}s.

replete with carbohydrate reserves. Preincubating for 1 h without Glc to deplete these reserves substantially lowered the ED_{50}. In other experiments, cells from 3-h cultures produced acetate in the absence of Glc, indicating that they contained stored carbohydrate.

Cells from 24- or 40-h cultures had a lower sulfhydryl content and lower resistance to inhibition. These cells produced no acetate in the absence of Glc, indicating that their carbohydrate reserves were depleted. Preincubation with Glc or with Glc plus the sulfhydryl or disulfide compounds increased resistance up to four-fold. Higher levels of cell sulfhydryls were obtained with GSH or GSSG than with cysteine or cystine.

Preincubating with sulfhydryl or disulfide compounds in the absence of Glc had no effect. Also, 2-mercaptoethanol or dithiothreitol had no effect with or without Glc, and no effect was obtained with Glc and D-cysteine or D-cystine (data not shown).

Figure 6 shows the relation between the threshold phenomenon, oxidation of bacterial sulfhydryls, and $OSCN^-$ levels. The cells were exposed to LP, SCN^-, and various amounts of H_2O_2 at 4°C. When the cells were washed by centrifugation at 4°C and then incubated with Glc, there was no inhibition of lactate production (data not shown). However, if the cells were warmed to 37°C without washing, lactate production was inhibited.

When the cells were removed from the reaction mixture without incubation at 37°C, there was no sulfhydryl oxidation. However, sulfhydryls of the inhibited cells were oxidized, and the loss of sulfhydryls showed the same threshold and ED_{50} as did the inhibition of lactate production.

After the cells were removed by centrifugation, the amount of unreacted $OSCN^-$ in the supernatants was measured. When the cells were removed without incubation at 37°C, the amount of $OSCN^-$ was only slightly less than that detected when LP, SCN^-, and H_2O_2 were mixed together in the absence of cells. Therefore, very little of the $OSCN^-$ reacted with bacterial components during the brief incubation at 4°C. However, when the mixtures were warmed to 37°C, the $OSCN^-$ disappeared at H_2O_2 concentrations below the threshold level. At H_2O_2 concentrations above the threshold level, excess unreacted $OSCN^-$ was observed. Therefore, the cells were able to eliminate up to about 150 μM $OSCN^-$ within 15 min, and this elimination was accomplished without inhibition of metabolism or net loss of sulfhydryls. However, when the initial $OSCN^-$ concentration was above the threshold level, metabolism and the ability to eliminate $OSCN^-$ were inhibited, and there was a net loss of cell sulfhydryls.

DISCUSSION

Autoinhibition. The diagram in Fig. 7 illustrates the design of experiments in which inhibition dependent on bacterial H_2O_2 production was studied. Glc was the substrate for metabolism, which yielded lactic acid and other acids. When O_2 was present, some of the *S. mutans* strains released large amounts of H_2O_2. The H_2O_2 interacted with LP and SCN^- to produce $OSCN^-$. At pH 8, the rate of production of $OSCN^-$ exceeded the rate of reaction of $OSCN^-$ with bacterial components, so that excess unreacted $OSCN^-$ was detected in the medium at the end of the incubation. At pH 5, HOSCN reacted with bacterial components, resulting in inhibition of metabolism. Because the inhibitor was consumed in these reactions, there

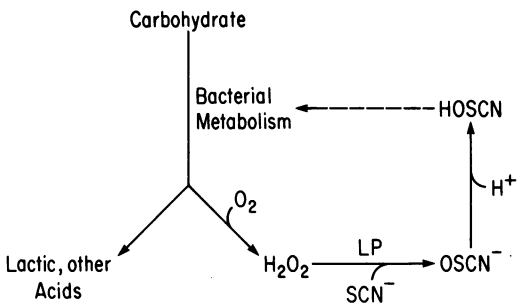

FIG. 7. Autoinhibition. Solid arrows represent metabolic steps that convert carbohydrate to lactate and other products and reduce O_2 to H_2O_2, LP-catalyzed oxidation of SCN^- by H_2O_2 to yield $OSCN^-$, and conversion of $OSCN^-$ to HOSCN at low pH. The dashed arrow represents reactions of HOSCN with bacterial components that result in inhibition of metabolism.

was effective inhibition but little accumulation of HOSCN or $OSCN^-$. Lactate production was lower in the presence of LP and SCN^- than in their absence, and this difference provided a measure of inhibition of metabolism by the LP system.

The amount of $OSCN^-$ observed at pH 7 to 8 was correlated with the degree of inhibition at pH 5; $OSCN^-$ levels of 10 to 20 μM at pH 7 to 8 were associated with significant inhibition at pH 5. The more effective inhibition at low pH was probably due in part to the greater lipid solubility of HOSCN as compared with $OSCN^-$ (34). If the rate of reaction of HOSCN-$OSCN^-$ mixtures with bacterial components is proportional to HOSCN concentration, then the rate will be over 300 times faster at pH 5 than at pH 8 (34).

Autoinhibition was obtained with 5 of the 11 strains examined, but only with cells from stationary phase in that the amount of H_2O_2 released was related to culture age and because carbohydrate reserves and sulfhydryl content decreased with increasing age. The median $OSCN^-$ concentration is about 10 μM in stimulated human saliva (37) and about 40 μM in resting (unstimulated) saliva (33). These concentrations appear sufficient to inhibit metabolism of *S. mutans* even at high cell densities, provided that the bacteria are depleted of carbohydrate reserves and sulfhydryl compounds.

Inhibition with exogenous H_2O_2. All strains were inhibited when incubated with LP, SCN^-, and exogenous H_2O_2. However, all strains had limited resistance, and a minimum (threshold) concentration of $OSCN^-$ was required for inhibition. Inhibition was more readily obtained at low pH, with cells from stationary-phase cultures.

Preincubating with Glc resulted in conversion of Glc to stored macromolecular forms and increased resistance to inhibition. These bacteria synthesize glycogen-like intracellular carbohydrate polymers that serve as a reserve of metabolizable carbohydrate (13). The LP system inhibited metabolism of these reserves and utilization of Glc from the medium. Therefore, the inhibitory effect was not limited to inhibition of Glc transport. Although transport may be inhibited (22), other essential metabolic processes must also be blocked. It has also been proposed that inhibition of hexokinase activity accounts for inhibition of metabolism (29). However, these bacteria take up sugars by way of the phosphotransferase system (31), so that hexokinase activity is not required for Glc phosphorylation. The mechanism by which intracellular carbohydrate reserves are mobilized is unknown but may involve phosphorylase activity rather than hexokinase. Therefore, it is not clear that these bacteria need or have hexokinase activity. Based on the observation that the LP system can deplete the bacteria of sulfhydryl components, inhibition may be due to a cumulative effect on many enzymes and transport systems that require reduced sulfhydryls for activity.

Resistance to inhibition. Resistance was associated with an ability to eliminate $OSCN^-$, presumably by reducing the inhibitor back to SCN^-. Disappearance of $OSCN^-$ occurred without detectable inhibition or alteration of metabolism or net loss of cell sulfhydryls.

Resistance was not directly proportional to the level of carbohydrate reserves. Instead, the level of reserves determined the length of time that the cells remained resistant. Therefore, resistance may be related to levels of substances derived from carbohydrate metabolism, such as NADH and NADPH. Studies on reduction of O_2 indicated that these bacteria obtain a higher yield of reducing equivalents (NADH) when metabolizing carbohydrate reserves than when taking up and metabolizing Glc from the medium (E. L. Thomas and K. A. Pera, submitted for publication). This change in metabolism is associated with decreased production of the reduced metabolite lactate and increased production of acetate. Therefore, increased resistance may be due to increased production of NADH or NADPH.

Resistance was also increased by preincubating with Glc and certain sulfhydryl or disulfide compounds, which resulted in increased cell sulfhydryl content. In particular, late-stationary-phase cells had a low sulfhydryl content, and the preincubation procedure was required to convert them to a resistant state. Cystine was previously reported to block inhibition of *Streptococcus agalactiae* (5).

The means by which the preincubation procedure increases sulfhydryl content of *S. mutans*

will be described in another report. In summary, these bacteria have energy-dependent transport systems for certain of these compounds, and GSH and cysteine are accumulated to high intracellular levels. The bacteria also have one or more NADPH-dependent enzymes that reduce the disulfide compounds to the sulfhydryl forms. Despite the presence of NADPH-dependent GSSG reductase activity, no GSH peroxidase activity has been detected. Loading the cells with GSH (or cysteine) does not alter the amount of H_2O_2 produced or released to the extracellular medium.

Certain oral streptococci have been reported to lack GSH or GSSG (9), although their ability to take up GSH was not examined. Mammalian cells contain high concentrations of GSH and GSSG reductase and GSH peroxidase activities (10). GSH or GSSG may be secreted into saliva or released when oral tissues are damaged. The ability of *S. mutans* to reduce GSSG and to accumulate GSH may represent an adaptation of these bacteria to their host that results in increased resistance to host defense systems.

A hypothesis about the role of the sulfhydryl (RSH) and disulfide (RSSR) compounds in resistance is that enzymatic reduction of RSSR to RSH is followed by nonenzymatic oxidation of RSH to RSSR by HOSCN or $OSCN^-$.

$$RSSR + NADPH + H^+ \xrightarrow{\text{reductase}} 2\ RSH + NADP^+$$

$$2\ RSH + OSCN^- \xrightarrow{\text{nonenzymatic}} RSSR + H_2O + SCN^-$$

$$\text{Net:}\quad NADPH + OSCN^- + H^+ \rightarrow NADP^+ + H_2O + SCN^-$$

The result is NADPH-dependent reduction of $OSCN^-$ to SCN^- without net loss of cell sulfhydryls. However, when $OSCN^-$ exceeds the threshold level, the rate of reaction with cell sulfhydryls exceeds the rate at which NADPH or RSH can be generated. There is a net loss of sulfhydryls, metabolism and production of NADPH become inhibited, and the ability to eliminate $OSCN^-$ is lost. According to this hypothesis, preincubation with Glc increases cellular levels of NADPH or the rate of production of NADPH, and loading the cells with GSH or cysteine provides a cofactor required for reduction of $OSCN^-$.

Results of this study may have implications in oral microbial ecology and oral disease. Resistance to the action of the LP system could account for the ability of *S. mutans* to survive and grow in the presence of the salivary antimicrobial system. Indeed, the interaction of H_2O_2-releasing bacteria with the LP system may confer a selective advantage, assuming that other microorganisms are not resistant. Nevertheless, resistance can be overcome, particularly at low pH. The LP system may be well adapted to inhibit metabolism of acid-producing bacteria at sites such as dental plaque, where the pH is lower than that of whole saliva. The results also suggest that nonspecific antimicrobial activity could be enhanced by stimulating bacterial H_2O_2 production or by introducing exogenous H_2O_2. Antimicrobial activity might be made specific to *S. mutans* by blocking processes involved in resistance, such as accumulation of carbohydrate reserves, reduction of disulfide compounds, and uptake of sulfhydryl compounds.

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