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Hydrogen Peroxide Excretion by Oral Streptococci and Effect of Lactoperoxidase-Thiocyanate-Hydrogen Peroxide

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Approved type strains of *Streptococcus sanguis, S. mitis, S. mutans*, and *S. salivarius* were grown under aerobic and anaerobic conditions. The rate of hydrogen peroxide excretion, oxygen uptake, and acid production from glucose by washed-cell suspensions of these strains were studied, and the levels of enzymes in cell-free extracts which reduced oxygen, hydrogen peroxide, or hypothiocyanite ($OSCN^-$) in the presence of NADH or NADPH were assayed. The effects of lactoperoxidase-thiocyanate-hydrogen peroxide on the rate of acid production and oxygen uptake by intact cells, the activity of glycolytic enzymes in cell-free extracts, and the levels of intracellular glycolytic intermediates were also studied. All strains consumed oxygen in the presence of glucose. *S. sanguis, S. mitis*, and anaerobically grown *S. mutans* excreted hydrogen peroxide. There was higher NADH oxidase and NADH peroxidase activity in aerobically grown cells than in anaerobically grown cells. NADPH oxidase activity was low in all species. Acid production, oxygen uptake, and, consequently, hydrogen peroxide excretion were inhibited in all the strains by lactoperoxidase-thiocyanate-hydrogen peroxide. *S. sanguis* and *S. mitis* had a higher capacity than *S. mutans* and *S. salivarius* to recover from this inhibition. Higher activity in the former strains of an NADH-OSCN oxidoreductase, which converted $OSCN^-$ into thiocyanate, explained this difference. The change in levels of intracellular glycolytic intermediates after inhibition of glycolysis by $OSCN^-$ and the actual activity of glycolytic enzymes in cell-free extracts in the presence of $OSCN^-$ indicated that the primary target of $OSCN^-$ in the glycolytic pathway was glyceraldehyde 3-phosphate dehydrogenase.

Hydrogen peroxide can be highly toxic to mammalian cells (5, 31, 44, 46), and the excretion of hydrogen peroxide by some species of *Mycoplasma* has been implicated in the pathogenesis of lesions in the mucous membranes of the respiratory tract (9, 34). Although hydrogen peroxide is produced by oral bacteria, especially streptococci (15, 22, 43), there is very little evidence that hydrogen peroxide actually induces any lesions in the mucous membranes of the human oral cavity. The only oral disorder that has been ascribed to hydrogen peroxide is the gangrene of the soft oral tissues in patients with acatalasemia (40).

Lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide (4, 17). The products of this reaction are much less toxic than hydrogen peroxide to bacteria (1, 8). It is conceivable that these products also are less deleterious than hydrogen peroxide to mammalian cells, and it has been suggested that the mucous membranes of the oral cavity are protected from the toxic effects of hydrogen peroxide from oral bacteria by lactoperoxidase and thiocyanate of the salivary secretions (1).

Very little is known about the hydrogen peroxide-producing bacteria of the oral cavity. In the present study it is demonstrated that some oral streptococci excrete large amounts of hydrogen peroxide in the presence of an energy source, glucose, in a reaction dependent on oxygen and NADH.

The products of the lactoperoxidase reaction inhibited glycolysis, oxygen uptake, and, consequently, hydrogen peroxide excretion by the oral streptococci. The primary site of inhibition of the glycolytic pathway by products of the lactoperoxidase reaction was glyceraldehyde 3-phosphate dehydrogenase.

MATERIALS AND METHODS

Microorganisms. The following type strains (39) were used: *Streptococcus mutans* NCTC 10449 (ATCC 25175), *S. sanguis* ATCC 10556, *S. mitis* NCTC 3165, and *S. salivarius* NCTC 8618 (ATCC

7073). They were grown on blood agar plates (13) aerobically and anaerobically. The anaerobic atmosphere was 10% hydrogen and 5% carbon dioxide in nitrogen in a glove box (48).

Media. The strains were grown in a broth containing the following (per liter): 17 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 3 g of phytone (B-D Merieux, Mary-L-Etoile, France), 2.5 g of NaCl, 10 g of glucose, 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.1 mol of potassium phosphate (pH 7.0), 0.01 mol of sodium pyruvate, and 0.01 mol of ammonium bicarbonate. Sodium pyruvate and ammonium bicarbonate solutions were sterilized by filtration. The rest of the ingredients of the medium were autoclaved. Phosphate and glucose were autoclaved separately. The medium used in anaerobic experiments was prepared aerobically and then stored for a week in the anaerobic box. Pyruvate was included in the medium to overcome the growth-inhibiting effect of the hydrogen peroxide produced aerobically by some of the strains. Ammonium bicarbonate provided bicarbonate to the capnophilic *S. mutans*.

Growth conditions. Anaerobic cultures (500 ml) were incubated at 37°C in the anaerobic box. Aerobic cultures (500 ml) were incubated at 37°C in the air in 1,000-ml Erlenmeyer flasks on a shaker having a circular orbital motion (100 rpm). The turbidity of the cultures was followed at 600 nm, and the cells were harvested in the exponential phase of growth.

Fractionation of cells. For preparation of cell-free extract for the assay of NADH- and NADPH-oxidizing enzymes the cells were harvested by centrifugation from 500-ml cultures and washed four times with 0.04 M potassium phosphate buffer (pH 6.8). They were then suspended in 3 ml of 0.04 M potassium phosphate buffer (pH 7.4) in the anaerobic box, and 2.5 ml of glass beads (0.10 to 0.11 mm; B. Braun, Melsungen, West Germany) was added. The cells were disintegrated in a homogenizer (type MSK; B. Braun) for 1 min under carbon dioxide cooling as previously described (48). A cell-free extract was obtained after unbroken cells and cell debris had been removed by centrifugation at 40,000 × *g* for 30 min in a refrigerated centrifuge under anaerobic conditions. The protein concentration of cell-free extract was measured by the method of Lowry et al. (25).

To prepare a particle fraction, the cells of *S. sanguis* were disintegrated as described above. Unbroken cells were removed by centrifugation at 10,000 × *g* for 40 min, and the supernatant was then centrifuged at 40,000 × *g* for 40 min. The resultant particle fraction was washed with 0.04 M potassium phosphate buffer (pH 7.4) at 40,000 × *g*.

For preparation of cell-free extract for the assay of glycolytic enzymes, the cells were harvested from the culture and washed twice with 0.04 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 3 mM L-cysteine, and the cells were suspended in the anaerobic box in 3 volumes of the same buffer. The cells were disintegrated by sonic oscillation for 15 min at 0°C (200 W, 2 A). A cell-free extract was obtained after the unbroken cells and the cell debris had been removed by centrifugation at 19,000 × *g* for 30 min at 4°C. The cell-free extract was then dialyzed at 4°C against 0.04 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The protein concentration of the cell-free extract was measured by the biuret method (23).

Preparation of $OSCN^-$. A stirred 10-ml ultrafiltration cell (Amicon Corp., Lexington, Mass.) fitted with a Diaflo membrane (PM 30) contained 9 ml of 33 mM potassium phosphate buffer (pH 7.4), 1 or 2 mM KSCN, and lactoperoxidase (25 μg ml^{-1}). Hydrogen peroxide was added to the reaction mixture to give a final concentration of 0.2 or 1.3 mM; 5 min later, the solution was filtered. The concentration of hypothiocyanite ($OSCN^-$) in the filtered solution was about 150 and 550 μM, respectively, as determined by reaction with 2-nitro-5-thiobenzoic acid (42). All products of the lactoperoxidase reaction, which oxidized 2-nitro-5-benzoic acid, were considered to be $OSCN^-$ even if other reaction products such as cyanosulfurous acid and cyanosulfuric acid might have contributed to the oxidation (13, 36). The reagent was prepared by reducing 1 mM solution of 5,5'-dithio-bis(2-nitrobenzoic acid) into 2-nitro-5-thiobenzoic acid with sodium borohydride in the anaerobic box. This reagent was stable in the box. The concentration of 2-nitro-5-benzoic acid was calculated assuming an extinction coefficient of 14 130 M^{-1} cm^{-1} at 412 nm (38). When the effect of $OSCN^-$ on the streptococcal acid production from glucose was studied, 2 mM potassium phosphate buffer (pH 7.0) was used when $OSCN^-$ was prepared.

Assay of enzyme activities. NAD(P)H oxidase was measured spectrophotometrically by following the oxidation of NAD(P)H at 340 nm in 33 mM potassium phosphate buffer (pH 7.4) containing 0.17 mM NAD(P)H and cell-free extract. NADH oxidase was also estimated by measuring oxygen consumption. The assay conditions are described below.

NAD(P)H peroxidase was measured in 33 mM potassium phosphate buffer (pH 7.4) containing 0.17 mM NAD(P)H, 29 mM hydrogen peroxide, and cell-free extract. The reaction mixture was prepared in the anaerobic box in a quartz cuvette fitted with a Thunberg-type side bulb. The reaction was initiated by the addition of hydrogen peroxide, and the change in extinction at 340 nm was followed.

The NAD(P)H-OSCN oxidoreductase activity was assayed in 33 mM potassium phosphate buffer (pH 7.4) containing 1 mM KSCN, 0.17 mM NAD(P)H, catalase (5 μg ml^{-1}), 0.1 or 0.05 mM $OSCN^-$, and cell-free extract. The reaction mixture was prepared in the anaerobic box in a quartz cuvette fitted with a Thunberg-tube side bulb. The reaction was initiated by the addition of cell-free extract, and the change in extinction at 340 nm was followed.

For assay of oxygen consumption and hydrogen peroxide production by cell-free extract, the reaction mixture (37°C) contained 33 mM potassium phosphate buffer (pH 7.4), 0.17 mM NADH with or without 0.12 mM flavin mononucleotide (FMN), and cell-free extract. The reaction was started by the addition of a cell-free extract. A subdued light was used to avoid photochemical reactions of FMN. The oxygen consumption by the cell-free extract was followed polarographically with an oxygen monitor (model 53; Yellow Springs Instruments Co., Yellow Springs, Ohio). To determine the amount of hydrogen peroxide accumulated in the reaction mixture, 20 μl of 1% catalase solution was added to the reaction mixture (3 ml) when the oxygen concentration had decreased to about 50%

saturation. The amount of hydrogen peroxide was estimated from the increase of oxygen concentration after the addition of catalase. The electrode was calibrated by adding 30 μl of 0.1 M potassium ferricyanide and 10 μl of 0.02 M phenylhydrazine-hydrochloride to the reaction mixture (3 ml) and recording the consumption of oxygen (30).

The inhibition of NADH oxidase by $OSCN^-$ was evaluated by following the oxygen consumption by the cell-free extract in a reaction mixture (37°C) containing 33 mM potassium phosphate buffer (pH 7.4), 0.34 mM NADH, catalase (5 μg ml^{-1}), 1 mM KSCN, 0.05 or 0.1 mM $OSCN^-$, and cell-free extract. The reaction was initiated by the addition of cell-free extract.

The activity of NAD-linked glyceraldehyde 3-phosphate dehydrogenase in cell-free extract was measured spectrophotometrically by following the oxidation of NADH at 340 nm in 100 mM triethanolamine-hydrochloride buffer (pH 7.4) containing 1 mM ATP, 1 mM EDTA, 2 mM $MgCl_2$, phosphoglycerate kinase (14 U ml^{-1}), 0.2 mM NADH, 6 mM 3-phosphoglycerate, and cell-free extract.

The activity of NADP-linked glyceraldehyde 3-phosphate dehydrogenase was measured by the method of Yamada and Carlsson (48). The reaction mixture contained 1.35 mM glyceraldehyde 3-phosphate, 1 mM NADP, 100 mM Tris-hydrochloride buffer (pH 8.5), and cell-free extract.

The activity of phosphoglycerate mutase was measured by the method of Grisolia and Carreras (12). The increase in extinction at 240 nm due to the formation of phosphoenol pyruvate was followed. The reaction mixture contained 50 mM Tris-hydrochloride buffer (pH 7.0), 5 mM $MgCl_2$, enolase (3 U ml^{-1}), 17 mM 3-phosphoglycerate, and cell-free extract.

The activity of lactate dehydrogenase was measured by the method of Yamada and Carlsson (48). The reaction mixture contained 100 mM Tris-hydrochloride buffer (pH 7.0), 0.2 mM NADH, 10 mM fructose 1,6-bisphosphate, 40 mM pyruvate, and cell-free extract.

The activity of phosphoglycerate kinase was measured by a modification of the method of Rao and Oesper (37). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM 3-phosphoglycerate (free from 2,3-diphosphoglycerate), 10 mM $MgCl_2$, 1 mM NaF, 100 mM ATP, and cell-free extract. A 1-ml sample of the mixture was incubated for 5 min at 37°C, and 1 ml of hydroxylamine solution (mixture of equal volume of 4.0 M NH_2OH-HCl and 3.5 M NaOH) was added. The mixture was then allowed to stand for 10 min at room temperature, and 3 ml of $FeCl_3$ solution (a mixture of equal volume of 12% trichloroacetic acid, 3 N HCl, and 5% $FeCl_3$ in 0.1 N HCl) was added. The extinction at 540 nm of the centrifuged supernatant was then determined.

Unless otherwise stated, all enzyme activities were determined at 25°C.

Assay of oxygen consumption, hydrogen peroxide excretion, and acid production by intact cells. The cells harvested in the exponential phase of growth were washed three times with 0.04 M potassium phosphate buffer (pH 6.8) by centrifugation and finally suspended in a salt solution supplemented with 10 mM potassium phosphate buffer (pH 6.8). The salt solution contained 4.3 g of NaCl, 0.42 g of KCl, 0.24 g of $CaCl_2$, and 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter.

The oxygen consumption and hydrogen peroxide excretion by intact cells in the presence of 5 mM D-glucose were estimated polarographically at 37°C as described above. The bacteria were suspended in 3 ml of the salt solution supplemented with 0.1 M potassium phosphate buffer (pH 6.8).

Acid production by intact cells in the presence of 5 mM D-glucose was estimated at 37°C by recording the titration volume of 0.1 M KOH with an automatic titration device (Radiometer A/S, Copenhagen, Denmark). The bacteria were suspended in 9 ml of salt solution supplemented with 1 mM potassium phosphate buffer (pH 6.8).

Determination of intracellular level of glycolytic intermediates before and after the addition of $OSCN^-$. The cells were washed twice with 0.15 M KCl (pH 7.0) and suspended in this solution. The reaction mixture contained 1 mM potassium phosphate buffer (pH 6.8), 150 mM KCl, 20 mM D-glucose, and the cells. The reaction was started by the addition of glucose. Acid production by the cells was monitored at 37°C by a recording device (models TSC-10A and TSB 10A; TOA Electronics Ltd, Tokyo, Japan). Samples from the reaction mixture were filtered through a glass filter (GA-200; Toyo Roshi Co., Tokyo, Japan) and a membrane filter (pore size, 3 μm; Millipore Corp., Bedford, Mass.) to remove the cells within 3 s. The cells on the filters were then immediately subjected to extraction with 2.0 ml of cold 0.6 N perchloric acid. The glycolytic intermediates in the cells were determined by the method of Minakami et al. (29). 3-Phosphoglycerate was measured by the method of Czok (10).

Chemicals. Lactoperoxidase, catalase, FMN, flavin adenine dinucleotide, and phenylhydrazine-hydrochloride were from Sigma Chemical Co., St. Louis, Mo. Phosphoglycerate kinase, enolase, glyceraldehyde 3-phosphate dehydrogenase, triose phosphate isomerase, glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, aldolase, lactate dehydrogenase, pyruvate kinase, 3-phosphoglycerate, fructose 1,6-bisphosphate, glyceraldehyde 3-phosphate diethylacetal, ADP, NAD, NADH, NADP, and NADPH were from Boehringer Mannheim GmbH, Mannheim, West Germany. ATP was from Yamasa Shoyu Co., Choshi, Japan. Potassium thiocyanate was from Riedel-de Haen AG, Seelze-Hannover, West Germany, or from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Hydrogen peroxide was from E. Merck AG, Darmstadt, West Germany, or from Santoku Chemical Industries Co., Miyagi, Japan. The concentration of hydrogen peroxide was calculated assuming an extinction coefficient in water of 43.2 M^{-1} cm^{-1} at 240 nm. This extinction coefficient of hydrogen peroxide was confirmed by titration with permanganate (21).

RESULTS

Washed suspensions of all species consumed oxygen in the presence of an energy source, glucose (Table 1). Without glucose, no detectable amount of oxygen was consumed. The rate of oxygen uptake was unexpectedly high. In aerobically grown cells of *S. sanguis*, the rate of oxygen uptake was almost 30% of the rate of acid production (Table 1). Cells of *S. sanguis*

TABLE 1. Hydrogen peroxide excretion, oxygen uptake, and acid production by intact cells of four streptococcal species in washed-cell suspension in the presence of glucose[a]

Species	Growth condition	Hydrogen peroxide excretion	Oxygen uptake	Acid production
S. sanguis	Aerobic	66 ± 9	91 ± 16	334 ± 19
	Anaerobic	9 ± 3	16 ± 3	359 ± 31
S. mitis	Aerobic	25 ± 9	44 ± 9	365 ± 53
	Anaerobic	12 ± 3	22 ± 6	350 ± 28
S. mutans	Aerobic	0	50 ± 3	544 ± 47
	Anaerobic	9 ± 3	13 ± 3	518 ± 28
S. salivarius	Aerobic	0	56 ± 6	875 ± 69
	Anaerobic	0	50 ± 3	862 ± 56

[a] Results are given as means ± standard deviations of three experiments; units are nanomoles per milligram (dry weight) per minute.

and *S. mitis* and anaerobically grown cells of *S. mutans* excreted hydrogen peroxide (Table 1). No hydrogen peroxide was excreted by *S. salivarius* and aerobically grown cells of *S. mutans*.

There was higher NADH oxidase activity in cell-free extracts of aerobically grown cells than in cell-free extracts of anaerobically grown cells (Table 2). NADPH oxidase activity was low in all species. NADH peroxidase activity was also higher in aerobically grown cells than in anaerobically grown cells (Table 2). NADPH peroxidase activity could only be detected in aerobically grown cells of *S. sanguis* (Table 2). Oxygen was reduced into hydrogen peroxide and water by the NADH oxidase activities. The amounts of hydrogen peroxide formed were inconsistent if no FMN was added to the reaction mixtures. Flavin adenine dinucleotide could not substitute for this effect of FMN. Although aerobically grown intact cells of the various species had a different capacity in excreting hydrogen peroxide, cell-free extracts of these species had a similar efficiency in converting oxygen into hydrogen peroxide in the presence of FMN (Table 2). Cell-free extracts of anaerobically grown cells converted a higher percentage of the oxygen consumed into hydrogen peroxide than cell-free extracts of aerobically grown cells (Table 2). The addition of 1.7 mM EDTA, 3.3 mM $MgCl_2$, 1.7 mM $MnCl_2$, or 1.7 mM $CaCl_2$ to the reaction mixture did not have any effect on oxygen consumption or hydrogen peroxide production by the cell-free extracts. Most of the NADH-dependent oxygen consumption was lost when the cell-free extracts were dialyzed or stored at 4°C for more than 1 day. The remaining activity, however, converted almost all of the oxygen consumed into hydrogen peroxide.

The particle fraction of *S. sanguis* neither consumed oxygen nor produced hydrogen peroxide in the presence of NADH or NADPH.

In the presence of lactoperoxidase and thiocyanate, hydrogen peroxide inhibited oxygen uptake and acid production by washed-cell suspensions of all of the species fermenting glucose (Fig. 1). Oxygen uptake or acid production was not inhibited by 0.5 mM hydrogen peroxide alone or in combination with thiocyanate or lactoperoxidase. Anaerobically grown cells of *S. salivarius* were the most sensitive to the products of the lactoperoxidase reaction. In *S. mutans* and *S. salivarius* there was a very narrow range between the concentration of hydrogen peroxide that decreased the rate of oxygen uptake and acid production and the concentration of hydrogen peroxide that completely stopped these activities for more than 30 min (Fig. 1). *S. sanguis* and *S. mitis* had the capacity to recover even after an exposure to 0.5 mM hydrogen peroxide (Fig. 1). In *S. sanguis*, the oxygen uptake recovered earlier than the acid production (Fig. 2). In aerobically grown cells of *S. sanguis*, inhibition of acid production required a lower concentration of hydrogen peroxide in the presence of lactoperoxidase-thiocyanate than the inhibition of oxygen uptake (Fig. 1). In *S. salivarius* oxygen uptake was inhibited at lower concentrations of hydrogen peroxide than the acid production (Fig. 1). A surprising finding in *S. sanguis* was that after the initial recovery from the effect of lactoperoxidase-thiocyanate-hydrogen peroxide the acid production stopped and started several times until it finally stopped after about 1 h (Fig. 3). The high sensitivity of oxygen uptake to lactoperoxidase-thiocyanate-hydrogen peroxide in *S. salivarius* compared with the other species was explained by the finding that NADH oxidase of *S. salivarius* was inhibited by $OSCN^-$ at significantly lower concentrations of $OSCN^-$ than was the NADH oxidase activity of the other species (Fig. 4).

The high capacity of *S. sanguis* and *S. mitis* in

TABLE 2. Enzyme activities[a] in cell-free extracts of four streptococcal species

Species	Growth condition	NADH oxidase		% O_2 converted to H_2O_2[d]	NADPH oxidase	NADH peroxidase	NADPH peroxidase	NADH-OSCN oxidoreductase	NADPH-OSCN oxidoreductase
		$NADH_2$[b]	O_2[c]						
S. sanguis	Aerobic	1.33 ± 0.22	0.66 ± 0.12	15 ± 5	0.013 ± 0.012	0.065 ± 0.007	0.015 ± 0.015	1.28 ± 0.07	0.47 ± 0.03
	Anaerobic	0.31 ± 0.05	0.15 ± 0.01	42 ± 3	0	0.043 ± 0.030	0	0.31 ± 0.12	0.064 ± 0.025
S. mitis	Aerobic	0.67 ± 0.07	0.28 ± 0.06	16 ± 10	0.007 ± 0.001	0.15 ± 0.07	0	5.00 ± 0.34	0.83 ± 0.07
	Anaerobic	0.13 ± 0.03	0.08 ± 0.02	40 ± 3	0	0.017 ± 0.014	0	3.63 ± 0.48	0.90 ± 0.20
S. mutans	Aerobic	0.96 ± 0.12	0.46 ± 0.03	15 ± 10	0	0.15 ± 0.06	0	0	0
	Anaerobic	0.025 ± 0.002	0.015 ± 0.001	84 ± 8	0	0.007 ± 0.004	0	0	0
S. salivarius	Aerobic	0.74 ± 0.20	0.49 ± 0.08	16 ± 2	0	0.10 ± 0.04	0	0.36 ± 0.03	0.20 ± 0.02
	Anaerobic	0.36 ± 0.03	0.21 ± 0.01	26 ± 3	0	0.032 ± 0.012	0	0.022 ± 0.012	0.010 ± 0.010

[a] Enzyme activities are given as micromoles of substrate converted per milligram of protein per minute.
[b] Measured as decrease of NADH; no FMN added.
[c] Measured as O_2 consumption; no FMN added.
[d] FMN (0.12 mM) added.

recovering from the inhibition by lactoperoxidase-thiocyanate-hydrogen peroxide could be explained by an enzyme activity which oxidized NADH and NADPH in the presence of $OSCN^-$ (Table 2). Low activity of NAD(P)H-OSCN oxidoreductase was found in *S. salivarius*. No activity of this enzyme was detected in *S. mutans* (Table 2).

One mole of NADH reduced 1.1 ± 0.1 mol of $OSCN^-$. This suggested the following reaction:

$$NADH_2 + OSCN^- \xrightarrow{\text{NADH-OSCN oxidoreductase}} NAD + SCN^- + H_2O.$$

The intracellular levels of 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate decreased remarkably in all the strains when acid production was stopped by the addition of $OSCN^-$ to the reaction mixtures (Table 3). These results suggested that the activity of glyc-

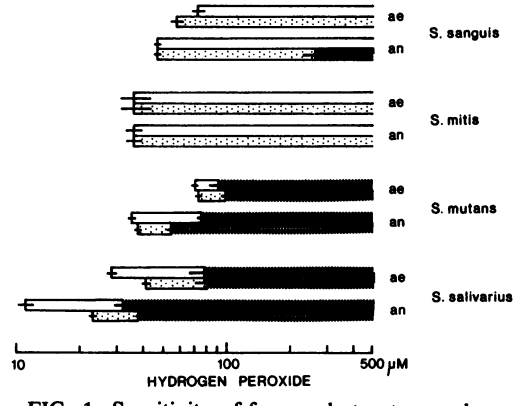

FIG. 1. Sensitivity of four oral streptococcal species to lactoperoxidase-thiocyanate-hydrogen peroxide. The strains were grown under aerobic (ae) and anaerobic (an) conditions. Washed-cell suspensions were incubated at 37°C in salt solution containing lactoperoxidase (25 μg min^{-1}) and 1 mM KSCN. The glycolysis of the cells was initiated by the addition of 5 mM D-glucose; after 2 min, hydrogen peroxide was added. Acid production and oxygen uptake were followed in separate reaction vials. The striped bars show those concentrations of hydrogen peroxide that completely stopped acid production and oxygen uptake for more than 30 min. The stippled bars show the latitude of hydrogen peroxide concentrations that decreased the rate of acid production or stopped it for less than 30 min. The blank bars show this latitude of hydrogen peroxide concentrations for oxygen uptake. The hydrogen peroxide concentrations given for the hydrogen peroxide-excreting strains include both the hydrogen peroxide added to the reaction mixture and that excreted by these strains before the addition of hydrogen peroxide. Means and standard deviations for three experiments are given.

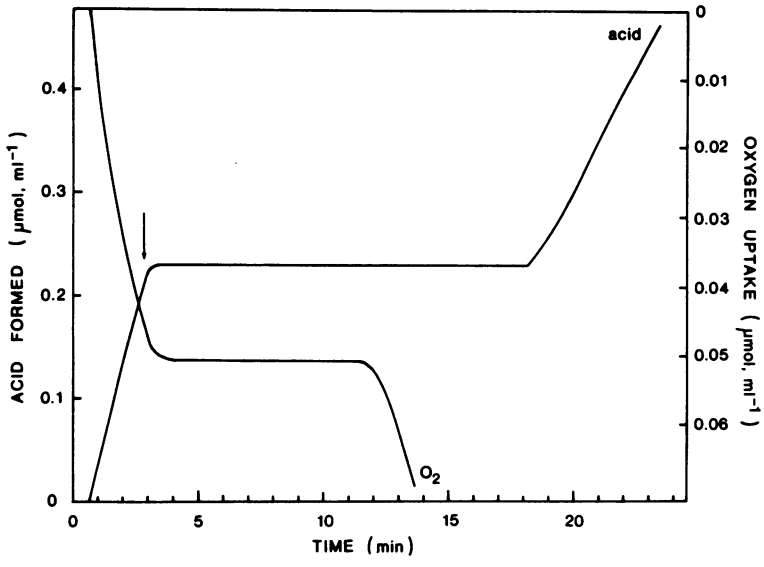

FIG. 2. Effect of lactoperoxidase-thiocyanate-hydrogen peroxide on acid production and oxygen consumption by *S. sanguis*. Glycolysis of a washed-cell suspension (37°C) in salt solution containing lactoperoxidase (25 μg ml^{-1}) and 1 mM KSCN was initiated by the addition of 5 mM D-glucose. After 2 min, 0.15 mM hydrogen peroxide was added (arrow).

eraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase was inhibited by $OSCN^-$.

The level of glucose 6-phosphate in *S. sanguis* and *S. mitis* decreased just after the addition of $OSCN^-$ (Table 3, sample B). This implied that the transport of glucose was inhibited by the high concentrations of $OSCN^-$ added to these two strains or that glucose 6-phosphate was metabolized through the hexose monophosphate shunt in *S. sanguis* and *S. mitis*, but not in *S. mutans* and *S. salivarius*.

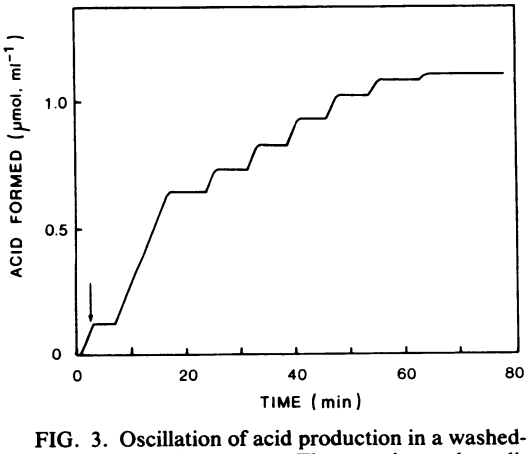

FIG. 3. Oscillation of acid production in a washed-cell suspension of *S. sanguis*. The experimental conditions were similar to those described in the legend to Fig. 2, but 0.05 mM instead of 0.15 mM hydrogen peroxide was added (arrow).

Glyceraldehyde 3-phosphate dehydrogenase of all four species was strongly inhibited by $OSCN^-$, whereas phosphoglycerate kinase, phosphoglycerate mutase, and lactate dehydrogenase were not inhibited by 100 μM $OSCN^-$ (Fig. 5). NAD-linked glyceraldehyde 3-phosphate dehydrogenase was completely inhibited by 20 μM $OSCN^-$. NADP-linked glyceraldehyde 3-phosphate dehydrogenase of *S. sanguis*

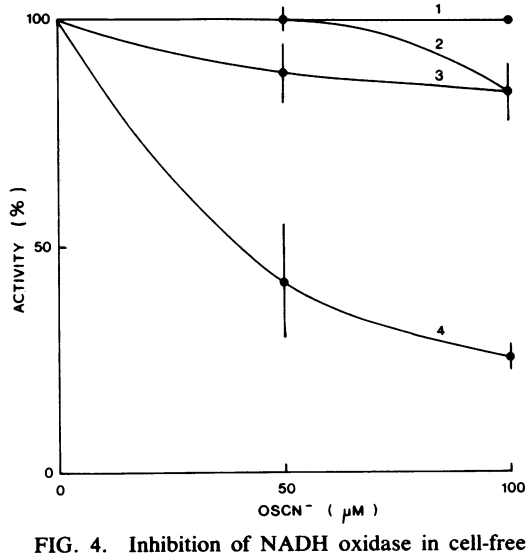

FIG. 4. Inhibition of NADH oxidase in cell-free extracts by $OSCN^-$. 1, *S. mitis*; 2, *S. sanguis*; 3, *S. mutans*; 4, *S. salivarius*.

TABLE 3. Levels of glycolytic intermediates in four streptococcal species during glycolysis and after treatment with $OSCN^-$ [a]

Species	$OSCN^-$ concn (μM)	Sample	Glucose 6-phosphate	Fructose 6-phosphate	Fructose, 1,6-bis-phosphate	DHAP[b]	Glycer-aldehyde 3-phosphate	3-Phospho-glycerate	2-Phospho-glycerate	Phospho-enol-pyruvate	Pyruvate
S. sanguis	210	A	28	4	270	58	28	170	26	77	120
		B	14	5	410	73	11	2	0	6	32
		C	39	12	410	67	13	2	0	2	28
		D	29	6	480	77	21	100	7	41	52
S. mitis	323	A	9	1	190	42	16	69	9	25	16
		B	4	1	200	48	14	0	1	0	7
		C	14	3	270	59	8	1	0	1	2
		D	14	2	370	63	27	49	7	14	18
S. mutans	47	A	27	3	270	42	8	58	6	13	45
		B	69	19	340	68	18	0	1	0	44
		C	82	23	310	46	8	0	3	0	35
		D	26	5	300	44	12	81	16	28	40
S. salivarius	60	A	13	1	120	19	84	140	9	37	210
		B	38	11	160	30	60	0	0	0	120
		C	53	14	200	34	60	0	0	0	88
		D	11	4	97	11	160	130	7	34	210

[a] Glycolysis of washed suspensions of aerobically grown streptococci was initiated by the addition of 20 mM glucose. After about 5 min, glycolysis was inhibited by the addition of $OSCN^-$. A sample for determination of levels of glycolytic intermediates (nanomoles per 20 mg [dry weight] of cells) was taken during glycolysis before the addition of $OSCN^-$ (sample A). Samples were taken 1 min (sample B) and 2 min (sample C) after glycolysis had been stopped by $OSCN^-$. Sample D was taken after the cells had recovered from the inhibition by $OSCN^-$.

[b] DHAP, Dihydroxyacetone phosphate.

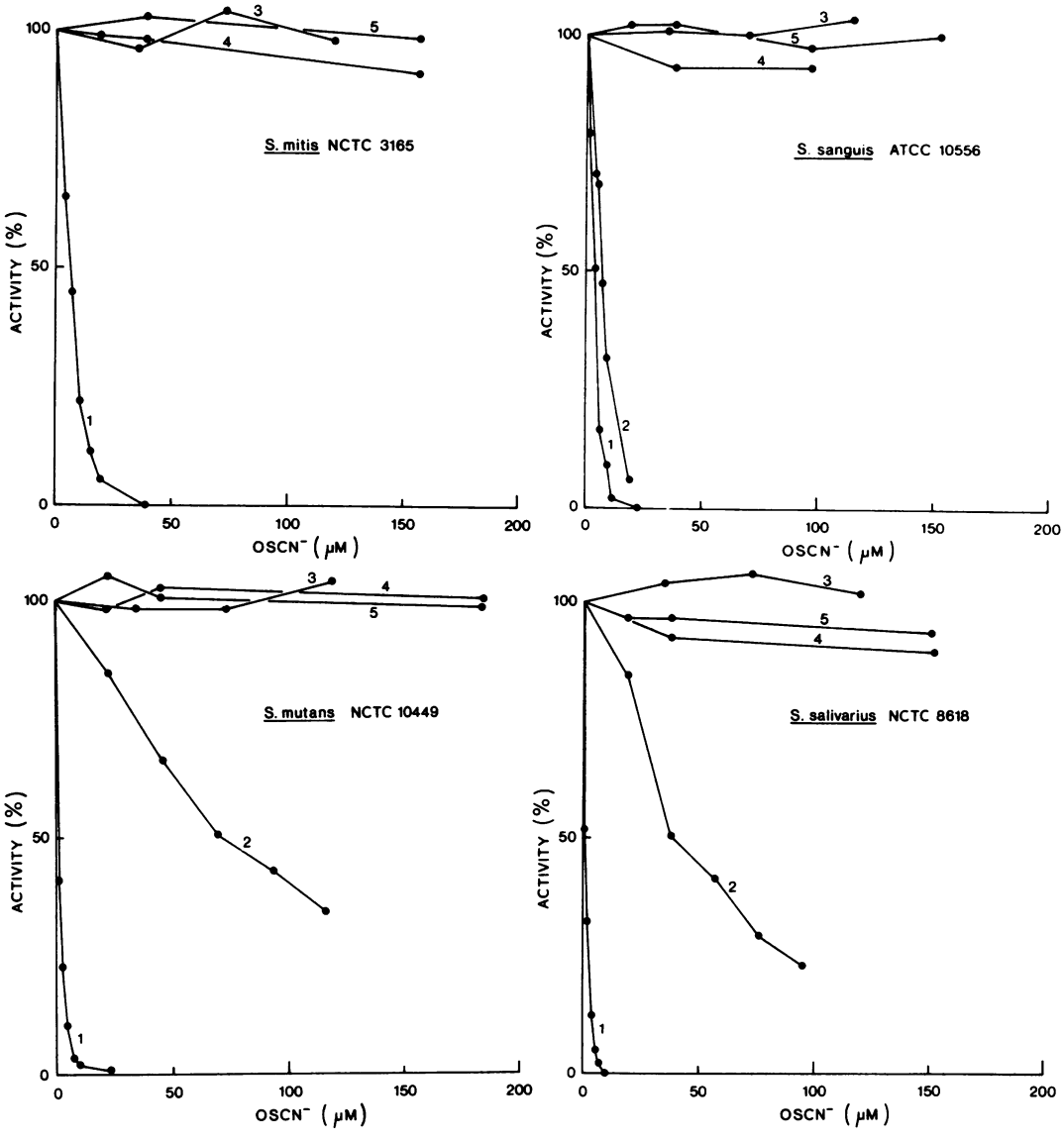

FIG. 5. Inhibition of glycolytic enzymes in cell-free extracts by $OSCN^-$. 1, glyceraldehyde 3-phosphate dehydrogenase (NAD); 2, glyceraldehyde 3-phosphate dehydrogenase (NADP); 3, phosphoglycerate kinase; 4, lactate dehydrogenase; 5, phosphoglyceromutase.

was completely inhibited by 20 μM $OSCN^-$, whereas this enzyme of *S. mutans* and of *S. salivarius* retained 70 to 87% of the activity at the same concentration of $OSCN^-$. NADP-linked glyceraldehyde 3-phosphate dehydrogenase of *S. mitis* was not detected. These results indicated that the inhibition of glyceraldehyde 3-phosphate dehydrogenase activity by $OSCN^-$ stopped acid production, oxygen uptake, and, consequently, hydrogen peroxide excretion by these microorganisms.

DISCUSSION

S. mitis and *S. sanguis* are among the predominant bacteria in the oral cavity (6), and hydrogen peroxide produced by these bacteria (7) may have the potential to damage the oral mucous membranes. It has been suggested that the mucous membranes are protected from the deleterious effects of hydrogen peroxide by lactoperoxidase and thiocyanate of the salivary secretions (1). Lactoperoxidase catalyzes the conversion of

hydrogen peroxide into water and of thiocyanate into hypothiocyanite and other oxidation products (4, 17, 36). The products of this reaction are less toxic than hydrogen peroxide (1), but they are potent inhibitors of glycolysis in streptococci (16, 35). The present study showed that the products of the lactoperoxidase reaction blocked glycolysis of oral streptococci in such a way that not only acid production but also oxygen uptake and, consequently, hydrogen peroxide excretion were inhibited. This suggested that lactoperoxidase and thiocyanate of saliva might have a dual function in protecting the oral mucous membranes against hydrogen peroxide toxicity. It detoxifies hydrogen peroxide, and the product of this reaction, hypothiocyanite, serves as a feedback inhibitor of the hydrogen peroxide excretion by the streptococci.

The unexpectedly high oxygen uptake by the oral streptococci might be of importance in the ecology of dental plaque. The concentration of oxygen in saliva is around 80 μM (20), and after intake of sweets the concentration of sugar could be more than 50 mM (45). When the microbiota of the teeth is exposed to this sugar, the oxygen will readily be consumed. This creates anaerobic conditions for the metabolism of the sugar, but the glycolysis of the bacteria might eventually be inhibited by the $OSCN^-$ formed from the hydrogen peroxide excreted by the oxygen-consuming bacteria.

Similar to *Streptococcus agalactiae* (26), the present species of oral streptococci grown under aerobic conditions consumed significantly more oxygen than those grown under anaerobic conditions. The rate of oxygen uptake was correlated to the level of NADH oxidase activity as in other lactic acid bacteria (19). The hydrogen peroxide excretion by streptococci has been ascribed to the activity of their NADH oxidases (3, 11), but there are also streptococcal NADH oxidases that convert oxygen into water (18). The hydrogen peroxide-producing activity of the NADH oxidase of the present strains was dependent on FMN, similar to the NADH oxidase of *Mycoplasma pneumoniae* (24). Cell-free extract of the species grown aerobically had a similar efficiency in converting oxygen into hydrogen peroxide in the presence of NADH and FMN, whereas intact cells of *S. mutans* and *S. salivarius* formed insignificant amounts of hydrogen peroxide compared with *S. mitis* and *S. sanguis* cells. NADH peroxidase activity was demonstrated in all strains, but there was no significant difference among the strains in the level of this enzyme activity. NADPH peroxidase was only detected in aerobically grown *S. sanguis*. Thus, this study on cell-free extracts did not clarify why various oral streptococci have a different capacity in converting oxygen into hydrogen peroxide. Although various NADH-oxidizing activities of streptococcal cell-free extracts have been known for a long time, these enzymes have not yet been separated and purified. It is thus not clear whether there are separate enzymes for hydrogen peroxide- or water-producing activity or whether these activities can be ascribed to the conformational change of a single protein.

Glycolysis has been reported to be blocked at several sites by the products of the lactoperoxidase-thiocyanate-hydrogen peroxide reaction. Transport of glucose can be inhibited (27), as can the activity of hexokinase (2, 32) and glyceraldehyde 3-phosphate dehydrogenase (26). This inhibition seems to be due to an oxidation of bacterial sulfhydryl groups to yield sulfenic acid and sulfenyl thiocyanate derivatives (28, 41). The present study demonstrated that the primary target of the products of the lactoperoxidase reaction was glyceraldehyde 3-phosphate dehydrogenase of the oral streptococci. At higher levels of these products, other sites of the bacteria might as well be affected. Transport of glucose might be blocked, or the activity of hexokinase might be inhibited. An unexpected finding was that *S. mitis* and *S. sanguis*, but not *S. salivarius* and *S. mutans*, had a high capacity in recovering from this inhibition. Oram and Reiter (32, 33) have described an NADH-oxidizing enzyme activity in streptococci, which reduced $OSCN^-$ into thiocyanate. High activity of such a NAD(P)H-OSCN oxidoreductase was found in the present study in *S. mitis* and *S. sanguis* and might explain their high capacity in recovering from inhibition by $OSCN^-$. In *S. salivarius* and *S. mutans*, which had a low capacity in recovering from the inhibition, the NAD(P)H-OSCN oxidoreductase activity was low or not detected.

The oscillating acid production of aerobically grown *S. sanguis* after exposure to lactoperoxidase and thiocyanate can then also get a reasonable explanation (Fig. 6). *S. sanguis* produced high amounts of hydrogen peroxide from oxygen in the presence of an energy source, glucose (Fig. 6, steps 1 and 2). When lactoperoxidase and thiocyanate were added, thiocyanate was oxidized to $OSCN^-$ (Fig. 6, step 4). This product entered the cell and blocked glycolysis by inhibiting glyceraldehyde 3-phosphate dehydrogenase (Fig. 6, step 1). This also inhibited the excretion of hydrogen peroxide and, consequently, the supply of $OSCN^-$ was limited. The intracellular $OSCN^-$ was converted into thiocyanate by NADH-OSCN or NADPH-OSCN oxidoreductases. The inhibition of glycolysis was released, hydrogen peroxide was excreted, and $OSCN^-$ was formed outside the cell. This $OSCN^-$ entered the cell, and the intracellular level of $OSCN^-$ increased until glycolysis was

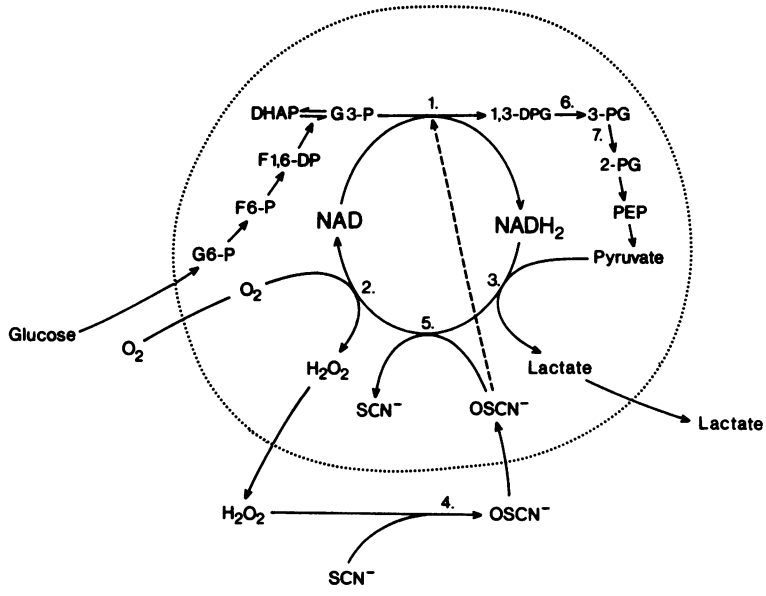

FIG. 6. Suggested scheme for regulation of glycolysis in *S. sanguis* ATCC 10556 in the presence of thiocyanate and lactoperoxidase. 1, glyceraldehyde 3-phosphate dehydrogenase; 2, NADH oxidase; 3, lactate dehydrogenase; 4, lactoperoxidase; 5, NADH-OSCN oxidoreductase; 6, phosphoglycerate kinase; 7, phosphoglyceromutase.

inhibited again. In the actual strain of *S. sanguis* this start and stop of glycolysis was repeated at least six times until glycolysis finally stopped.

In most cases acid production and oxygen uptake were synchronously inhibited by the products of the lactoperoxidase reaction. In *S. salivarius*, oxygen uptake was inhibited, however, at slightly lower levels of the products than the acid production, whereas in aerobically grown *S. sanguis*, acid production was inhibited by lower levels than the oxygen uptake (Fig. 1). The sensitivity of oxygen uptake in *S. salivarius* could be explained by an inhibition of its NADH oxidase by $OSCN^-$ (Fig. 4). There was no obvious explanation why acid production in *S. sanguis* was inhibited at lower levels than oxygen uptake. One possibility could be that the NADH oxidase and NADH-OSCN oxidoreductase had a higher affinity for NADH than the lactate dehydrogenase (Fig. 6).

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