A PEROXIDASE-MEDIATED, *STREPTOCOCCUS MITIS*-DEPENDENT ANTIMICROBIAL SYSTEM IN SALIVA*

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An antimicrobial system that inhibited the growth of lactobacilli and certain other organisms in human saliva was described over 30 years ago (1, 2). This system could be distinguished from lysozyme (3–7) and IgA (8). Antilactobacillus activity was present in sterile, cell-free parotid or submaxillary saliva (6, 9), suggesting that the source of the antimicrobial system was the salivary glands and that the phagocytes and microorganisms of saliva were not essential under the conditions employed. Attempts to define the nature of this antimicrobial system were unsuccessful until it was found to contain at least two components: a heat-stable, dialyzable component and a heat-labile, nondialyzable component (7, 10, 11). The heat-stable, dialyzable component could be replaced by thiocyanate ions in the concentrations present in saliva (11, 12). Iodide ions also were effective; however, the concentration required was outside the physiological range (11, 12). The heat-labile, nondialyzable component was found to be the salivary peroxidase; it could be replaced by bovine milk lactoperoxidase (LPO)[1] (13) (an enzyme immunologically and chemically indistinguishable from bovine salivary gland peroxidase [14]) or by the peroxidase of human parotid saliva (8) in amounts equivalent to those present in saliva.

The addition of reagent H_2O_2 was not required when lactobacilli were the test organisms; a requirement for H_2O_2 was suggested, however, by the inhibitory effect of catalase (13). Lactobacilli and the other organisms designated by Orla-Jensen as lactic acid bacteria (e.g. streptococci, pneumococci) form and secrete H_2O_2 into the medium (15). These organisms, in general, do not contain heme and thus utilize flavoproteins for terminal oxidations with the reduction of oxygen to H_2O_2. The spectrum of organisms whose growth is inhibited by the peroxidase-mediated antimicrobial system of saliva is extended to non-H_2O_2-generating organisms (e.g. *Escherichia coli*, *Staphylococcus aureus*) when H_2O_2 or an H_2O_2-generating system is added to the reaction mixture (16).

The studies outlined above suggest that saliva may contain an antimicrobial system that consists of three components, the salivary peroxidase (LPO), thiocyanate ions, and H_2O_2, and that microbial metabolism can serve as a source of H_2O_2 for this system. The importance of viridans streptococci in the control of the oral microbial

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[1] *Abbreviations used in this paper:* KRP, Ca^{++}-free Krebs-Ringer phosphate; LPO, lactoperoxidase; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole.

flora has recently been emphasized (17). Patients treated with high doses of penicillin parenterally are subject to overgrowth and suprainfection with enteric bacilli, leading in some instances to gram-negative pneumonia. A study of the resident flora during antibiotic therapy has shown a strong correlation between the disappearance from the oropharynx of viridans streptococci and the appearance of potential pathogens in significant numbers. Previous prophylaxis with oral penicillin in patients scheduled to receive large doses of penicillin parenterally may prevent oropharyngeal overgrowth by the selection of penicillin-resistant viridans streptococci.

This paper will report on the formation of H_2O_2 by *Streptococcus mitis*, a viridans streptococcus and normal resident of the oral cavity, and the utilization of the H_2O_2 so formed to inhibit or kill potential pathogens in the presence of LPO and either thiocyanate or iodide ions in the concentrations present in saliva. Human mixed saliva has antimicrobial properties when combined with *S. mitis*, and the involvement of the salivary peroxidase and thiocyanate ions is suggested. The role of the LPO-mediated, *S. mitis*-dependent antimicrobial system in the control of the oral microbial flora is considered. A preliminary report on these studies has appeared elsewhere (18).

Materials and Methods

Growth and Collection of Microorganisms.—*E. coli* (ATCC no. 11775), *S. aureus* 502A, *S. mitis* (ATCC no. 6249), and *Candida tropicalis* (hospital strain) were maintained on blood agar plates. *E. coli*, *S. aureus*, and *C. tropicalis* were transferred daily to Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.). An overnight culture was washed twice and suspended in water to the required absorbancy at 540 nm in a Cary M15 spectrophotometer (Cary Instruments, Monrovia, Calif.).

Growth of *S. mitis* in the Trypticase soy broth resulted in a fall in pH. If the pH was allowed to fall below 5.6, there was a loss of metabolic activity, indicated by a decrease in formate oxidation. A number of procedures were employed to prevent a fall in pH below 5.8. These included an increase in the initial pH of the Trypticase soy broth, a decrease in the inoculum size, a decrease in the growth period, and variations in the mode of inoculum transfer. A procedure found to be convenient and effective was as follows. An inoculum from the blood agar plate was transferred with a loop to 14 ml of Trypticase soy broth and grown overnight at 37°C. A 2-ml sample was transferred to 12 ml of fresh broth and grown for approximately 4 h. The pH was monitored and the growth terminated when the pH fell to 5.8–6.0. The broth culture of *S. mitis* was washed twice and suspended in Ca^{++}-free Krebs-Ringer phosphate buffer (KRP), pH 6.5, to the required absorbancy at 540 nm in a Cary M15 spectrophotometer. Where indicated, the *S. mitis* was heated at 100°C for 15 min.

Collection of Saliva.—Parotid and unstimulated mixed saliva were collected from normal adult volunteers. Parotid salivary flow was stimulated with citric acid troches (Sour Lemons, Regal Crown Co., England), and collection was made directly from Stenson's duct with the apparatus described by Curby (19). The mixed or parotid saliva was centrifuged at 1,600 *g* for 5 min, and the supernatant was sterilized either by passage through a membrane filter with a 0.45 μm average pore size (Schleicher & Schuell Co., Keene, N. H.) or by exposure of a thin layer to ultraviolet light (General Electric G8T5 lamp, General Electric Co., Schenectady, N. Y.) at a distance of 5 in for 5 min. Where indicated, the saliva was heated at 100°C for 15 min or dialyzed against 4,000 parts of either distilled water or KRP, pH 6.5, for at least 4 h. The saliva was used on the day of collection.

Other Special Reagents.—LPO was prepared from bovine milk by the method of Morrison and Hultquist (20). Peroxidase activity was determined by the *o*-dianisidine method (21); 1 U of activity is that causing an increase in absorbancy of 0.001/min at 460 nm in a Cary M15 spectrophotometer. Catalase (beef liver, crystalline, 54,500 U/mg) was obtained from Worthington Biochemical Corp., Freehold, N. J. Where indicated, these reagents were heated at 100°C for 15 min.

Formate Oxidation.—[^{14}C]Sodium formate in 70% alcohol (sp act 20–50 mCi/mmol), obtained from New England Nuclear, Boston, Mass., was evaporated to dryness under nitrogen, dissolved in water to an activity of 25 μCi/ml, and frozen in small samples. On the day of the experiment, a tube of the stock solution was thawed, and a drop of 0.01 M HCl was added to drive off dissolved $^{14}CO_2$ (or bicarbonate) present as a contaminant in the preparation. This substantially decreased the background radioactivity usually observed during measurements of the conversion of formate to CO_2.

The reaction mixtures indicated in the legends of the tables and figures were incubated in liquid scintillation vials at 37°C in a water bath oscillating 80 times/min. The vial was stoppered throughout the incubation with a rubber stopper to which was attached a hanging plastic cup containing 0.2 ml of 10% KOH. After incubation for the periods indicated, 0.2 ml of 2 N H_2SO_4 was added through a no. 22 needle already in place in the rubber stopper. The needle was corked when not in use. The reaction mixture was incubated for an additional 45 min. The contents of the plastic cup were transferred to a liquid scintillation vial containing 20 ml of a toluene-ethanol-2,5-diphenyloxazole(PPO)-1,4-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) fluor (22) and were counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The results were expressed as the number of nanomoles of formate oxidized. The radioactivity in the plastic cup after incubation of [^{14}C] formate in buffer alone (background) was subtracted from the experimental values.

Microbicidal Activity.—Microbicidal activity was determined by two procedures:

(*a*) In some of the experiments in which *E. coli* was the target organism, all the components of the reaction mixture (see legends) were incubated in 18 × 75-mm test tubes at 37°C in a water bath oscillating 80 times/min. At intervals, a 0.1-ml sample of the reaction mixture was diluted with water, and the viable cell count was determined by the pour plate method using Trypticase soy agar containing 0.1 U/ml of potassium penicillin G (E. R. Squibb & Sons, New York). This amount of penicillin was found to be sufficient to kill the *S. mitis* while allowing the *E. coli* to form colonies and be counted.

(*b*) TechniLab dialysis cells (1-ml vol; TechniLab Instruments, Division of Bel-Art Products, Pequannock, N. J.) were employed in experiments in which *S. mitis* was separated from the target organism (*S. aureus*, *E. coli*, or *C. tropicalis*) by a dialysis membrane. The components were added to either side of the membrane as indicated in the legends, and the dialysis cells were placed on their faces and rotated 80 times/min on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J.). At intervals, samples were removed from the side containing the target organism, and the viable cell count was determined, as described above, using Trypticase soy agar that did not contain penicillin.

Growth Inhibition.—The components indicated in the legends were placed on either side of a dialysis membrane in TechniLab dialysis cells and were incubated for 60 min at 37°C on a rotary shaker, as described above. At the end of the incubation period, a sample (0.8 ml) from the side containing the target organism was transferred to a 10 × 75-mm Coleman cuvette (Perkin-Elmer Corp., Coleman Instruments Division, Maywood, Ill.) containing 0.2 ml of five-times concentrated Trypticase soy broth. The cuvettes were incubated for the periods indicated at 37°C in a water bath oscillating 80 times/min, and the optical density change at 540 nm was determined with a Coleman Junior Spectrophotometer. Additional phosphate buffer was added to the reaction mixture (see legends) to maintain the pH at 6.5 during the prolonged incubation and growth periods. In its absence the pH fell with a retardation of *E. coli* growth.

Amino Acid Incorporation.—[U-^{14}C]L-valine in 0.01 M HCl (sp act 250 mCi/mmol), obtained from New England Nuclear, was diluted with water to an activity of 2 μCi/ml, frozen in small samples, and thawed for each day's experiment. The components indicated in the legends were placed on either side of a dialysis membrane in TechniLab dialysis cells and incubated at 37°C for 30 min on a rotary shaker, as described above. A 0.5-ml sample from the side containing the target organism was transferred to an 18 × 75-mm test tube containing 0.05 ml of [U-^{14}C]L-valine (0.1 μCi), and the tubes were incubated for 30 min at 37°C in a water bath oscillating 80 times/min. The reaction mixture was then diluted to 5.0 ml with sterile, distilled water and filtered through a 0.45-μm membrane filter in an E-8B Precipitation Apparatus (Tracerlab Div., LFE Electronics, Richmond, Calif.). The filters were washed with 10 ml of water, placed in liquid scintillation vials, and digested with 0.5 ml of NCS solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.). 10 ml of the toluene-ethanol-PPO-POPOP fluor were added, and the samples were counted in a liquid scintillation counter. The results were corrected for background and for counts retained by the filter in the absence of bacteria.

RESULTS

H_2O_2 Production by S. mitis.—The conversion of [^{14}C]formate to $^{14}CO_2$ by catalase and H_2O_2 can be employed as a measure of H_2O_2 formation. Table I demonstrates the conversion of [^{14}C]formate to $^{14}CO_2$ by catalase and *S. mitis*. Little or no formate oxidation was observed when the catalase or *S. mitis* was either deleted or heated at 100°C for 15 min. The addition of glucose to the incubation medium was required for optimum formate oxidation; this metabolite is presumably required for H_2O_2 formation by the streptococci. The optimal pH was 6.0–6.5 (Fig. 1). Formate oxidation increased linearly with the *S. mitis* concentration over the range 0.5–4 × 10^7 organisms/ml. Conditions optimal for H_2O_2 production by *S. mitis* were employed in the remainder of the study.

Antimicrobial Activity of the LPO-Iodide-S. mitis System.—Table II demonstrates the bactericidal effect of LPO, iodide, and *S. mitis* on *E. coli* and the requirement for each component of the antimicrobial system. Heating either the *S. mitis* or the LPO at 100°C for 15 min abolished the antimicrobial effect. Catalase (12 μg/ml) also was inhibitory whereas heated catalase at this concentration was not, which suggests that H_2O_2 is involved. Increasing the

TABLE I

H_2O_2 Production by S. mitis

Supplements	Formate oxidation
	nmol/10^7 organisms per h
S. mitis + catalase	76.0
S. mitis	0.5
Catalase	0.0
S. mitis (heated) + catalase	0.0
S. mitis + catalase (heated)	0.2

The reaction mixture contained [^{14}C]sodium formate, 1,000 nmol (0.25 μCi); glucose, 2 μmol; KRP, pH 6.5, to a final volume of 0.5 ml; and the supplements indicated below as follows: *S. mitis*, 2 × 10^7 organisms; catalase, 162 μg. Incubation period, 60 min.

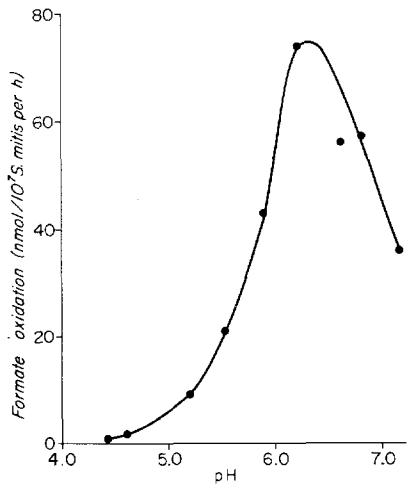

FIG. 1. Effect of pH on formate oxidation by *S. mitis*. The reaction mixture was as described in Table I (*S. mitis* + catalase) except that the pH of the KRP was varied. The pH of the final reaction mixture was as indicated. Incubation period, 60 min.

TABLE II

Microbicidal Effect of LPO-Iodide-S. mitis on E. coli

Supplements	Viable cell count
	organisms/ml × 10^6
None	5.2
LPO + iodide + *S. mitis*	0.027
LPO + iodide	5.6
LPO + *S. mitis*	5.4
Iodide + *S. mitis*	4.9
LPO (heated) + iodide + *S. mitis*	6.9
LPO + iodide + *S. mitis* (heated)	5.0
LPO + iodide + *S. mitis* + catalase (6 μg)	3.9
LPO + iodide + *S. mitis* + catalase (6 μg) (heated)	0.017
LPO + iodide + *S. mitis* + albumin (6 μg)	0.023
LPO + iodide + *S. mitis* + catalase (60 μg)	4.9
LPO + iodide + *S. mitis* + catalase (60 μg) (heated)	5.0
LPO + iodide + *S. mitis* + albumin (60 μg)	4.9

The reaction mixture contained KRP, pH 6.5, to a final volume of 0.5 ml; *E. coli*, 2.5 × 10^6 organisms; glucose, 2 μmol; and the supplements indicated below as follows: LPO, 15 U; sodium iodide, 5 nmol; *S. mitis*, 5 × 10^6 organisms. Catalase and crystalline bovine albumin were added in the amounts shown. Incubation period, 60 min.

catalase concentration 10-fold resulted in an inhibition that was unaffected by heat treatment, which suggests a nonspecific inhibitory effect of protein at this concentration. This is supported by the inhibitory effect of high (120 μg/ml), but not of low (12 μg/ml), concentrations of albumin.

The lactoperoxidase concentration employed in Table II was 30 *o*-dianisidine U/ml, which is comparable with that found in saliva (13). However, the iodide concentration employed in Table II was 10^{-5} M or 127 μg/100 ml, which is outside the physiological range (3.5 to 24 μg/100 ml [12]). Fig. 2 demonstrates the effect of a decrease in iodide concentration on the bactericidal activity of the LPO-iodide-*S. mitis* system. The fall in *E. coli* viable cell count varied from 33 to 92% over the physiological range of iodide concentration in saliva (shown in the shaded area).

In the experiments reported in Table II and Fig. 2, the *E. coli* viable cell count was determined in the presence of *S. mitis* by the addition of penicillin to the Trypticase soy agar in concentrations that killed *S. mitis* but did not

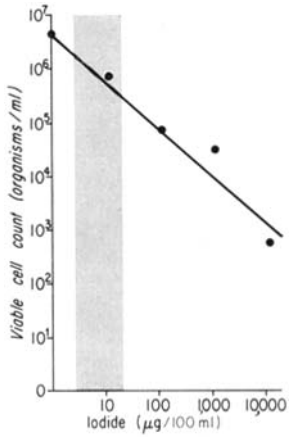

FIG. 2. Effect of iodide concentration on bactericidal activity of LPO-iodide-*S. mitis*. The reaction mixture was as described in Table II (LPO + iodide + *S. mitis*) except that the iodide was varied as indicated. Incubation period, 60 min.

affect *E. coli*. Testing the effect of the LPO-iodide-*S. mitis* system on the penicillin-sensitive organism *S. aureus* 502A required an alteration in technique. The *S. mitis* was separated from the target organism by a dialysis membrane, and the viable cell count of the target organism was determined by the use of agar that did not contain penicillin. The *S. mitis* was protected from the toxic affect of the microbicidal system since LPO was added only to the side containing the target organism.

Table III demonstrates that under these conditions *S. aureus*, *E. coli*, and *C. tropicalis* are killed only when the complete LPO-iodide-*S. mitis* system was employed. The separation of *S. mitis* and the target organism by a dialysis membrane indicates that the low-molecular-weight substance required for the microbicidal effect is generated by *S. mitis*. That this substance is H_2O_2 is suggested by the inhibitory effect of catalase (but not heated catalase) on the microbicidal system.

TABLE III

Microbicidal Effect of LPO-Iodide-S. mitis on S. aureus, E. coli, and C. tropicalis

Supplements	Viable cell count		
	S. aureus	*E. coli*	*C. tropicalis*
	organisms/ml × 10^5		
None	29	36	1.3
LPO + iodide + *S. mitis*	0.009	0.002	0.035
LPO + iodide	32	33	0.92
LPO + *S. mitis*	31	36	0.90
Iodide + *S. mitis*	30	41	1.1
LPO (heated) + iodide + *S. mitis*	29	40	1.1
LPO + iodide + *S. mitis* (heated)	27	38	0.86
LPO + iodide + *S. mitis* + catalase	28	40	1.3
LPO + iodide + *S. mitis* + catalase (heated)	0.005	0.004	0.019

To side A of the dialysis cell were added KRP, pH 6.5, to a final volume of 1.0 ml; glucose, 2 μmol; and, where indicated, *S. mitis*, 1×10^8 organisms and sodium iodide, 10 nmol. To side B were added KRP, pH 6.5, to a final volume of 1.0 ml; glucose, 2 μmol; either *S. aureus*, 3×10^6 organisms, *E. coli*, 4×10^6 organisms, or *C. tropicalis*, 3×10^5 organisms; and where indicated, sodium iodide, 10 nmol; LPO, 30 U; or catalase, 12 μg. Incubation period, 60 min.

Antimicrobial Activity of the LPO-Thiocyanate-S. mitis System.—When iodide ions were replaced by thiocyanate ions under the conditions employed in Tables II and III, no decrease in the viable cell count of the target organism was observed. However, an antimicrobial effect was demonstrable with thiocyanate ions when an inhibition of growth in complete growth medium or an inhibition of the uptake of amino acid was employed as an indication of microbial damage.

Table IV demonstrates the inhibitory effect of the LPO-thiocyanate-*S. mitis* system on the growth of *E. coli* in complete growth medium. In this experiment, *S. mitis* was separated from *E. coli* (and LPO) by a dialysis membrane during a 60-min incubation with components of the antimicrobial system. The rate of growth of the *E. coli* in complete growth medium over a 3-h period was then determined. The complete LPO-thiocyanate-*S. mitis* system was required for the inhibition of the growth of *E. coli* under these conditions, and heat treatment of either LPO or *S. mitis* abolished the inhibitory effect. Catalase, but not heated catalase, protected the target organism, which suggests the involvement of H_2O_2. Similar results were obtained when *S. aureus* replaced *E. coli* as the target organism.

Fig. 3 shows that the LPO-thiocyanate-*S. mitis* system also inhibits the uptake of L-valine by *E. coli*. *E. coli* was separated from *S. mitis* by a dialysis membrane during a 30-min incubation with components of the antimicrobial system. The *E. coli* was then transferred to a tube containing [^{14}C]L-valine, and the uptake of amino acid by the bacteria was determined. As in the growth

TABLE IV

Effect of LPO-Thiocyanate-S. Mitis on Growth of E. coli

Supplements	*E. coli* growth
	Δ *OD* $\times 10^3$
None	195
LPO + thiocyanate + *S. mitis*	20
LPO + thiocyanate	190
LPO + *S. mitis*	170
Thiocyanate + *S. mitis*	190
S. mitis	180
LPO (heated) + thiocyanate + *S. mitis*	180
LPO + thiocyanate + *S. mitis* (heated)	185
LPO + thiocyanate + *S. mitis* + catalase	195
LPO + thiocyanate + *S. mitis* + catalase (heated)	20

To side A of the dialysis cell were added KRP, pH 6.5, to a final volume of 1.0 ml; glucose, 2 μmol; phosphate buffer pH 7.0, 40 μmol; and where indicated, *S. mitis*, 8×10^8 organisms; sodium thiocyanate, 4 μmol; and catalase, 24 μg. To side B were added KRP, pH 6.5, to a final volume of 1.0 ml; glucose, 2 μmol, phosphate buffer pH 7.0, 40 μmol; *E. coli*, 5×10^7 organisms; and where indicated, sodium thiocyanate, 4 μmol; catalase, 24 μg; and LPO, 30 U. Growth period, 3 hr.

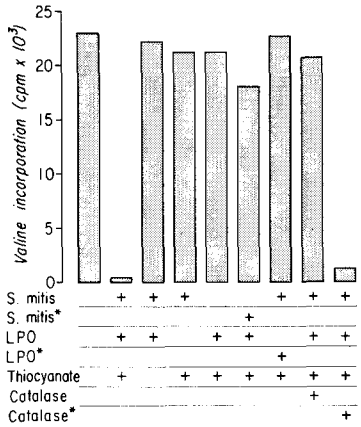

FIG. 3. Effect of LPO-thiocyanate-*S. mitis* on valine incorporation by *E. coli*. To side A of the dialysis cells were added KRP pH 6.5 to a final volume of 1.0 ml; glucose, 2 μmol; and where indicated, *S. mitis*, 8×10^8 organisms; sodium thiocyanate, 1 μmol; or catalase, 24 μg. To side B were added KRP pH 6.5 to a final volume of 1.0 ml; glucose, 2 μmol; *E. coli*, 5×10^7 organisms; and, where indicated, sodium thiocyanate, 1 μmol, or LPO, 30 U. Components were heated (*) where indicated.

experiments, all components of the antimicrobial system were required for inhibition, the effect of LPO or *S. mitis* was decreased by heat treatment, and catalase protected the target organism whereas heated catalase did not.

Antimicrobial Activity of the Saliva-S. mitis System.—Saliva and *S. mitis* did

not exert a bactericidal effect on *E. coli* or *S. aureus* under conditions (see Tables II and III) in which LPO, iodide, and *S. mitis* were strongly bactericidal; indeed, saliva at a concentration of 10% or greater inhibited the bactericidal activity of the LPO-iodide-*S. mitis* system. The nature of the inhibitors of this system in saliva is unknown; they may include low-molecular-weight reducing agents (8), alternate iodine acceptors (e.g., protein), or catalase.

Saliva, however, could replace LPO and thiocyanate ions in the *S. mitis*-dependent inhibition of bacterial growth and amino acid uptake. Table V demonstrates the inhibitory effect of saliva and *S. mitis* on the growth of *E.*

TABLE V

Effect of Saliva-S. mitis on Growth of E. coli

Supplements	*E. coli* growth	
	Mixed saliva	Parotid saliva
	$\Delta OD \times 10^8$	
Saliva	170	110
Saliva + *S. mitis*	35	35
Saliva (heated)	125	105
Saliva (heated) + *S. mitis*	140	120
Saliva (heated) + *S. mitis* + LPO	20	30
Saliva (dialyzed)	110	110
Saliva (dialyzed) + *S. mitis*	95	95
Saliva (dialyzed) + *S. mitis* + thiocyanate	15	25
Saliva + *S. mitis* + catalase	190	155
Saliva + *S. mitis* + catalase (heated)	50	50

The reaction mixture was as described in Table IV except that mixed or parotid saliva (intact, heated, or dialyzed), 80%, was added to side B and the amount of sodium thiocyanate employed was 0.5 μmol. Growth period, 2 h.

coli in complete growth medium. Growth inhibition was abolished by heat treatment of the saliva; full activity was restored by the addition of LPO in physiologic amounts. Growth inhibition was also decreased (but not abolished) when the saliva was dialyzed; activity was restored when thiocyanate was added in physiologic concentrations. Catalase, but not heated catalase, protected the target organism from the antimicrobial effect of the saliva-*S. mitis* system.

Fig. 4 shows that the uptake of valine by *E. coli* was stimulated by either intact, dialyzed, or heated saliva. The further addition of *S. mitis* to intact saliva resulted in a marked reduction in valine uptake. This inhibitory effect of saliva and *S. mitis* was considerably reduced when either heated or dialyzed saliva was employed. However, the addition ot physiological concentrations of LPO to heated saliva or of thiocyanate to dialyzed saliva decreased valine incorporation to the level observed with whole saliva. As with previous systems, catalase protected the target organisms whereas heated catalase did not.

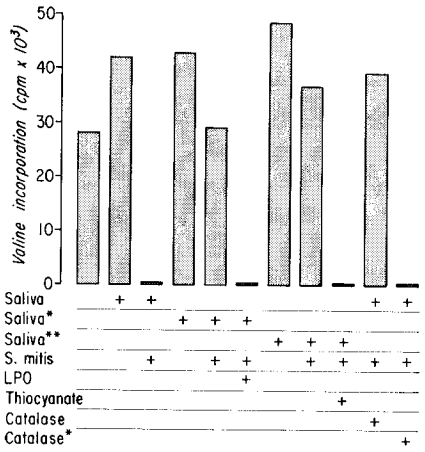

FIG. 4. Effect of saliva-*S. mitis* on valine incorporation by *E. coli*. The reaction mixture was as described in Fig. 3 except that mixed saliva (intact, heated, or dialyzed) was added to side B. Components were heated (*) or dialyzed (**) where indicated.

DISCUSSION

The oral microbial flora is normally maintained under careful control by a variety of mechanical and chemical factors. It is probable that among these is an antimicrobial system composed of the salivary peroxidase, thiocyanate ions, and the H_2O_2 produced by viridans streptococci and other salivary microorganisms.

Alpha-hemolytic streptococci were isolated from the saliva of 98% of 50 schoolchildren by Ross (23). The concentration of this organism ($1.6–1.7 \times 10^7$/ml) was higher than that of any other organism detected. Other H_2O_2-generating organisms in saliva include pneumococci, aerobic lactobacilli, *S. fecalis* and beta-hemolytic streptococci. Kraus et al. (24) have reported that most (58%) of the aerobic salivary bacteria form H_2O_2 in vitro. However, these investigators could not detect free H_2O_2 in saliva in concentrations in excess of 1 μg/ml (the lower limit of sensitivity of their assay procedures). They concluded that this was due to the high threshold of their assay procedures and to the presence in saliva of agents that destroy H_2O_2, namely, peroxidase and catalase. They detected catalase activity in mixed saliva, but not in saliva collected directly from the parotid or submaxillary glands, which suggests that the source of the salivary catalase is not glandular secretions (25). Oral microorganisms were suggested. It is of interest in this regard that patients with acatalasia often have ulcers and gangrenous lesions in the oral cavity, possibly caused by an accumulation of H_2O_2 to toxic levels.

A number of examples of the antagonistic effect of one bacterial species on another through the formation and secretion of H_2O_2 have been described (26–32). We have reported here that the antimicrobial activity of *S. mitis* is greatly

increased by LPO and either iodide or thiocyanate ions in the concentrations present in saliva.

The formation of H_2O_2 by *S. mitis* was demonstrated, and its utilization in the antimicrobial system was suggested by the inhibitory effect of catalase. The activity of the LPO-iodide-*S. mitis* system was measured by the decrease in the viable cell count of the target organism (*E. coli*, *S. aureus*, *C. tropicalis*), and that of the LPO-thiocyanate-*S. mitis* system, by the decrease in the growth of the target organisms (*E. coli*, *S. aureus*) in complete growth medium or by the inhibition of L-valine uptake by these organisms. Attempts to substitute whole saliva for LPO and iodide ions were unsuccessful; indeed, saliva inhibited the microbicidal activity of LPO, iodide, and *S. mitis*. Mixed or parotid saliva, however, could substitute for LPO and thiocyanate ions in an *S. mitis*-dependent antimicrobial system. The activity of saliva was reduced by dialysis or heat treatment; thiocyanate ions in physiological concentrations increased the antimicrobial activity of dialyzed saliva + *S. mitis*, and LPO in physiological concentrations increased the antimicrobial activity of heated saliva + *S. mitis*, which suggests the involvement of these two agents.

The overgrowth and suprainfection that result from the suppression of the oral viridans streptococci by penicillin therapy (17) is compatible with a physiological role for the *S. mitis*-dependent, peroxidase-mediated, antimicrobial system in saliva. The factors that control the activity of this system are complex. The activity may be influenced by the level of the components (LPO, thiocyanate ions, H_2O_2) in saliva. For example, depression of salivary thiocyanate levels by the oral administration of potassium perchlorate is associated with a decrease in salivary antilactobacillus activity, whereas the increase in thiocyanate levels produced by the oral administration of potassium thiocyanate is associated with an increase in activity (12). H_2O_2-generating organisms other than *S. mitis*, e.g., beta-hemolytic streptococci, *S. fecalis*, lactobacilli, and pneumococci, may contribute to the H_2O_2 of saliva and to the antimicrobial activity of the salivary system. H_2O_2-generating as well as non-H_2O_2-generating organisms are susceptible to the antimicrobial activity of the system, and thus the formation as well as the utilization of H_2O_2 is affected by its action. A number of antagonists, e.g., catalase or low-molecular-weight reducing agents, are present in saliva. These inhibitors may be supplied by either the microorganisms, the oral mucosa, or the salivary glands. For example, all of the staphylococcal and diphtheroid strains that antagonized the inhibition of diphtheria bacilli by viridans streptococci or saliva have been found to produce catalase (29). The control of the microbial species of the oropharynx by this complex, peroxidase-mediated, antimicrobial system may be an important host defense mechanism.

SUMMARY

H_2O_2 formation by *Streptococcus mitis* was measured by the catalase-dependent conversion of [^{14}C]formate to $^{14}CO_2$; it was optimal at pH 6.0–6.5

and required glucose. The H_2O_2 formed by *S. mitis* could be employed as a component of an antimicrobial system that also included lactoperoxidase (LPO) and either iodide or thiocyanate ions in the concentrations present in saliva. The antimicrobial effect of the LPO-iodide-*S. mitis* system was measured by the decrease in the viable cell count of the target organisms (*Escherichia coli*, *Staphylococcus aureus*, *Candida tropicalis*). The antimicrobial effect of the LPO-thiocyanate-*S. mitis* system was measured by the decrease in the rate of growth or the rate of uptake of [^{14}C]valine by the target organisms (*E. coli*, *S. aureus*). Mixed or parotid saliva could replace LPO and thiocyanate ions in the *S. mitis*-dependent inhibition of bacterial growth and valine uptake. The presence in saliva of a peroxidase-mediated, antimicrobial system dependent on microbial metabolism for H_2O_2 and its role as a natural host defense mechanism are considered.

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