The Alpha-Hemolysin of *Streptococcus gordonii* Is Hydrogen Peroxide

JOHN P. BARNARD,¹ AND MURRAY W. STINSON^{1,2*}

Department of Microbiology, School of Medicine and Biomedical Sciences,¹
and Department of Oral Biology, School of Dental Medicine,²
State University of New York at Buffalo,
Buffalo, New York 14214-3000

Received 18 April 1996/Returned for modification 4 June 1996/Accepted 1 July 1996

The alpha-hemolysin of viridans group streptococci, which causes greening of intact erythrocytes, is a potential virulence factor as well as an important criterion for the laboratory identification of these bacteria; however, it has never been purified and characterized. The alpha-hemolysin of *Streptococcus gordonii* CH1 caused characteristic shifts in the A_{403} , A_{430} , A_{578} , and A_{630} of sheep hemoglobin. A spectrophotometric assay was developed and used to monitor purification of alpha-hemolysin during extraction in organic solvents and separation by reverse-phase high-performance liquid chromatography (HPLC). The alpha-hemolysin was identical to hydrogen peroxide with respect to its effects on erythrocyte hemoglobin, oxygen-dependent synthesis by streptococci, insensitivity to proteases, inactivation by catalase, differential solubility, failure to adsorb to ion-exchange chromatography resins, and retention time on a reverse-phase HPLC column. The amount of hydrogen peroxide present in HPLC-fractionated spent culture medium was sufficient to account for all alpha-hemolytic activity observed.

The viridans group of oral streptococci contains several diverse species including the following: *Streptococcus pneumoniae*, the principal causative agent of pneumonia and otitis media (23, 24); *S. mutans* and *S. sobrinus*, the primary etiological agents of dental caries (2, 20, 37); and *S. gordonii*, *S. sanguis*, *S. oralis*, and *S. mitis*, which frequently cause bacterial endocarditis (27, 34, 41). The name viridans refers to the greenish discoloration (alpha-hemolysis) that these streptococci cause while growing aerobically on blood agar (29), a characteristic which has been recognized for over 90 years (28). Although alpha-hemolysin may be a significant virulence factor in the diseases caused by viridans streptococci, it is still poorly understood.

This study describes the development of a fluid-phase assay for the alpha-hemolysin of *S. gordonii* and its purification from spent culture medium. The data indicate that the alpha-hemolysin is hydrogen peroxide on the basis of their common chemical and biological properties. The amount of hydrogen peroxide that *S. gordonii* produced is similar to that produced by other viridans group streptococci (10, 11, 13, 38), indicating that it is also responsible for the alpha-hemolytic activity of these bacteria.

MATERIALS AND METHODS

Materials. Sheep erythrocytes, stabilized in Alsever's solution, were obtained from Crane Laboratories Inc., Syracuse, N.Y. Dehydrated brain heart infusion (BHI) and Todd-Hewitt broth (THB) media were from Difco Laboratories, Detroit, Mich., and tryptic soy broth (TSB) was from Becton Dickinson Microbiology Systems, Cockeysville, Md. Bovine liver catalase, proteinase K, pronase E, horseradish peroxidase, and 2,2'-azino-bis-(3-ethylbenz-thiazoline)-6-sulfonic acid (ABTS) were from Sigma Chemical Co., St. Louis, Mo. High-performance liquid chromatography (HPLC)-grade acetonitrile, 2-propanol, and water were purchased from J. T. Baker Inc., Phillipsburg, N.J. Reagent-grade HCl and 30% H_2O_2 were from Fisher Scientific, Fairlawn, N.J.

Bacterial strains and cultivation. *S. gordonii* Challis-1 was obtained from D. Clewell (University of Michigan, Ann Arbor), and strain G9B was obtained from B. Rosan (University of Pennsylvania, Philadelphia). *S. pneumoniae* UB1 was derived from a clinical isolate at Buffalo Children's Hospital. Unless specified

otherwise, bacterial cultures were inoculated with a 1% volume of a stationary-phase culture and grown at 37°C in THB on a gyratory shaker at 200 to 225 rpm. At the indicated intervals, bacteria were removed by 20 min of centrifugation at $7,650 \times g$, and the spent culture medium was collected, adjusted to pH 6.7, assayed as described below, and stored at -70°C . In some experiments, the spent culture medium was passed through a Centricon-10 ultrafilter (Amicon Inc., Beverly, Mass.), and the resulting retentate and ultrafiltrate were assayed for hemolytic activity.

Absorbance spectra. Sheep erythrocytes were washed twice with phosphate-buffered saline (PBS) (150 mM NaCl, 7.7 mM Na_2HPO_4 , 2.3 mM KH_2PO_4) (pH 6.7) and resuspended to an A_{600} of 9.0. Forty microliters of erythrocyte suspension was mixed with 16 μl of 9.4 mM H_2O_2 or 80 μl of spent medium from a log-phase streptococcal culture (optical density at 600 nm of 0.45 to 0.65) and adjusted to a total volume of 0.2 ml with PBS. The mixtures were incubated for 15 min at room temperature before erythrocytes were sedimented by 5 s of centrifugation at $14,000 \times g$. The supernatant fluid was removed, and the erythrocytes were lysed by the addition of 0.1 ml of distilled water and vigorous pipetting. The lysate was added to 0.9 ml of PBS, and the absorbance spectrum was read versus a blank containing 0.9 ml of PBS and 0.1 ml of water.

Assay for alpha-hemolysis. Alpha-hemolytic activity was assayed as described above, except that variable amounts of culture supernatant or H_2O_2 were added and the A_{578} , A_{430} , and A_{403} of hemoglobin were recorded for both treated samples and a PBS-treated control. The mean of the changes in absorbance at these wavelengths was calculated by using the following formula: $\Delta\text{absorbance} = [(1 - (A_{578} \text{ test}/A_{578} \text{ control})) + [1 - (A_{430} \text{ test}/A_{430} \text{ control})] + [(A_{403} \text{ test}/A_{403} \text{ control}) - 1]]/3$. One unit of alpha-hemolytic activity is defined as that amount of hemolysin causing a $\Delta\text{absorbance}$ of 0.25. For assays at pH 6.0 and 7.4, all components in the reaction mixture were adjusted to the designated pH except for the erythrocyte lysates which were still diluted with PBS at pH 6.7 to avoid pH effects on the absorbance spectra. A standard alpha-hemolysis curve was generated each day to control for variations in the erythrocyte response.

Purification of alpha-hemolysin. Eighty milliliters of spent medium from an early-stationary-phase culture was mixed with 720 ml of acetonitrile and centrifuged for 5 min at $10,400 \times g$, and the supernatant was evaporated down to 10 ml under vacuum with constant swirling at 55 to 60°C. The concentrate was mixed with 90 ml of 2-propanol and concentrated to 0.5 ml under vacuum at 40 to 50°C; substantial loss of activity occurred if the sample was evaporated to dryness. The precipitate, formed in 2-propanol, adhered to the glass vessels, making centrifugation unnecessary for its removal. The 2-propanol concentrate was collected and mixed with 5 volumes of acetonitrile, concentrated under vacuum to 0.5 ml, and centrifuged for 1 min at $14,000 \times g$ to remove the precipitate. The resulting supernatant fluid was then vacuum evaporated to 0.2 ml, mixed with an equal volume of HPLC-grade water, and evaporated under vacuum to 0.1 ml to remove all traces of organic solvent. The aqueous sample was adjusted to 4 mM HCl. After the precipitate was removed by spin filtration (30 s at $14,000 \times g$) through 0.45- μm -pore-size HV syringe tip filters (Millipore Corp., Bedford, Mass.), 25- μl aliquots were subjected to reverse-phase HPLC on

* Corresponding author.

FIG. 1. Spectral changes induced in erythrocyte hemoglobin by alpha-hemolysin and hydrogen peroxide. Sheep erythrocytes were treated with 80 μ l of spent culture medium from a late-log-phase culture of *S. gordonii* (panel A, circles) or 150 nmol of H_2O_2 (panel B, circles) and then lysed with water. Squares represent PBS-treated controls, and closed symbols signify absorbance after adjustment of lysates to 0.5% KCN. The increase in A_{403} and decreases at A_{430} and A_{578} (arrows) were monitored in subsequent alpha-hemolysis assays.

a C_{18} column (4.6 by 250 mm) (Altex Scientific Inc., Berkeley, Calif.). The mobile phase was aqueous 1 mM HCl at a flow rate of 0.5 ml/min and was monitored for A_{225} . The eluate was collected in 87- μ l (3-drop) fractions, and 3 μ l of each fraction was assayed for alpha-hemolysin in 2 μ l of 0.5 M KH_2PO_4 - K_2HPO_4 (pH 6.7). The overall yield of alpha-hemolysin was typically 5 to 10% of the approximately 4,000 U present in the spent culture medium.

Assays for hydrogen peroxide. Hydrogen peroxide was assayed spectrophotometrically as previously described (7, 30). Briefly, samples containing 0.5 to 2 nmol of H_2O_2 were mixed with 2 U of horseradish peroxidase and 0.1 μ mol of ABTS in a total volume of 1 ml of 0.1 M NaH_2PO_4 - Na_2HPO_4 (pH 6.0). The colorimetric reaction was allowed to develop for 2 min, and then the A_{414} was read immediately versus a no-peroxide blank. Under these conditions the assay was linearly proportional to 72 absorbance units (AU)/(millimolar $\times$ centimeter) for concentrations up to 8 nmol of H_2O_2 per assay. The concentration of H_2O_2 in 30% stock solutions was determined spectrophotometrically in 50 mM NaH_2PO_4 - Na_2HPO_4 (pH 7.0) ($\epsilon_{240} = 0.0435$ AU/[millimolar $\times$ centimeter]) (31).

FIG. 2. Concentration dependence of alpha-hemolysis by *S. gordonii* spent culture medium and hydrogen peroxide at pH 6.0, 6.7, and 7.4. Alpha-hemolytic activity at pH 6.0 (circles), pH 6.7 (squares), and pH 7.4 (triangles) was measured as described in Materials and Methods. Results shown are the means $\pm$ SDs of three independent determinations at each concentration point.

RESULTS

Effect of alpha-hemolysin on erythrocytes. Sheep erythrocytes, treated with spent bacterial culture medium or low concentrations of hydrogen peroxide, showed a rapid greening that resembled the zone of alpha-hemolysis seen around colonies of *S. gordonii* on sheep blood agar. The spectral changes corresponding to this greening are shown in Fig. 1 and were due to oxidation of the heme iron of hemoglobin by both alpha-hemolysin (Fig. 1A) and H_2O_2 (Fig. 1B). This was most clearly demonstrated by the appearance of an absorbance peak at 630 nm which was abolished in the presence of 0.5% cyanide, two distinguishing characteristics of oxidized hemoglobin or methemoglobin (3). The absorbance of treated erythrocytes divided by the absorbance of mock-treated controls at 578, 430, and 403 nm (Fig. 1) proved to be a sensitive assay for alpha-hemolytic activity. It should be noted that this assay, described in Materials and Methods, incorporates absorbance decreases at the higher wavelengths and an absorbance increase at the lower wavelength which served as internal corrections for possible variations in erythrocyte numbers.

Hydrogen peroxide and alpha-hemolysin in streptococcal culture supernatants showed very similar titration profiles in the alpha-hemolysis assay at several pH values (Fig. 2). Maximum alpha-hemolysis was observed at pH 6.0 and was 2.7- and 7.8-fold greater than the values at pH 6.7 and 7.4, respectively; activities were compared at a Δ absorbance of 0.25, which defines 1 U of activity. It should be emphasized that erythrocytes were not lysed by alpha-hemolysin under any of these experimental conditions because hemoglobin readily and completely sedimented in alpha-hemolyzed cells during low-speed centrifugation, and the cells appeared normal in phase-contrast microscopy. The integrity of the erythrocyte membrane was also verified by the spectrophotometric alpha-hemolysin assay. For example, if treated erythrocytes released hemoglobin during the assay, the absorbance values for the cell-associated material would decrease proportionately at 578, 430, and 403 nm. We found that variations in Δ absorbance (e.g., dose-response curves in Fig. 2) resulted from a combination of increases and decreases at the selected wavelengths, indicating that the hemoglobin content was not diminished.

Maximum oxidation of the hemoglobin in erythrocytes occurred at 17.8 nmol of H_2O_2 per assay (Fig. 2, pH 6.7). On the basis of the hemoglobin content of erythrocytes (39), this corresponds to a stoichiometry of 6.3 mol of H_2O_2 per mol of hemoglobin.

Factors influencing alpha-hemolysin production. *S. gordonii* produced maximum amounts of alpha-hemolysin during the

FIG. 3. Production of alpha-hemolysin during growth of *S. gordonii* under high and low oxygen tension. Alpha-hemolytic activity and culture density (measured by optical density at 600 nm [OD₆₀₀]) during growth of *S. gordonii* in THB are shown. The bacteria were grown in air and shaken at 200 to 225 rpm (circles) or grown in air and not shaken (triangles). Results shown are typical of three independent determinations.

logarithmic phase of growth under well-aerated conditions (Fig. 3). When *S. gordonii* was grown in a variety of media with vigorous shaking to maintain oxygen tension, increased alpha-hemolysin production correlated with increased cell numbers (Table 1). Production of alpha-hemolysin by the Challis-1 strain was higher than that of the G9B strain of *S. gordonii* but was not improved by tripling the glucose concentration of the medium (Table 1, THBG medium). In contrast, the low oxygen tension in static culture and shaken anaerobic culture did not support alpha-hemolysin production despite increased yields of cells (Fig. 3; Table 1).

Physical characteristics of alpha-hemolysin. To determine if alpha-hemolysis also involves a large protein like streptolysin-O (5) or pneumolysin (23), culture supernatants were fractionated by ultrafiltration in Centricon-10 units and assayed. The ratio of alpha-hemolytic activity which passed through the ultrafiltration membrane to that of the retentate was 0.95 ± 0.04 (mean $\pm$ standard deviation [SD] of three determinations), indicating that the alpha-hemolysin has a molecular mass below 10 kDa. Alpha-hemolysis did not seem to require a polypeptide, since treatment of 1 U of alpha-hemolysin with 5 mg of proteinase K or pronase E per ml failed to inhibit alpha-hemolytic activity. Instead, proteinase K and pronase E enhanced alpha-hemolytic activity $56\% \pm 4\%$ and $66\% \pm 2\%$, respectively (means $\pm$ SDs of five determinations), most likely as a result of proteolytic denuding of the erythrocyte surfaces which facilitated alpha-hemolysin uptake.

Several pieces of evidence indicated that alpha-hemolysin is not a charged molecule. It was freely soluble in 90% methanol, 2-propanol, acetone, and acetonitrile, but relatively low yields of activity were obtained upon removal of the two latter solvents under vacuum because of partial volatilization of the

TABLE 1. Peak production of alpha-hemolysin by *S. gordonii* under various growth conditions^a

Strain ^b	Medium ^c	Culture conditions	Alpha-hemolysin activity (U/ml) ^d	Turbidity ^d
CH1	CDM	Shaken, in air	93 $\pm$ 12	0.37 $\pm$ 0.01
	THB	Shaken, in air	104.5 $\pm$ 8.2	0.68 $\pm$ 0.01
	THBG	Shaken, in air	99.8 $\pm$ 15.9	0.77 $\pm$ 0.05
	BHI	Shaken, in air	142 $\pm$ 6	0.84 $\pm$ 0.04
	TSBY	Shaken, in air	238 $\pm$ 44	1.24 $\pm$ 0.06
G9B	TSBY	Shaken, in air	63.1 $\pm$ 2.8	0.91 $\pm$ 0.03
CH1	TSBY	Not shaken, in air	8.25 $\pm$ 0.56	2.10 $\pm$ 0.06
	TSBY	Shaken, no O ₂ ^e	3.23 $\pm$ 4.56	1.64 $\pm$ 0.53

^a Ten-milliliter cultures in 15-ml culture tubes were incubated at 37°C with samples removed at regular intervals for measurements of optical density at 600 nm and alpha-hemolysis assays as described in Materials and Methods.

^b Cultures were inoculated with a 1% volume of a stationary-phase culture of the Challis-1 (CH1) or G9B strain of *S. gordonii*.

^c CDM is chemically defined medium (36), THB is Todd-Hewitt broth, THBG is THB with 30 mM glucose, BHI is brain heart infusion, and TSBY is tryptic soy broth with 0.5% yeast extract.

^d Results shown are the alpha-hemolytic activity and cell density (measured by turbidity) at the peak accumulation of alpha-hemolytic activity (Fig. 3) and are the means $\pm$ SDs of determinations with triplicate cultures for each condition.

^e Culture medium was shaken overnight at 37°C in a GasPak anaerobic canister (BBL Microbiology Systems) and then inoculated under a stream of nitrogen gas and sealed to exclude oxygen. Only one time point was used with each culture tube to prevent oxygen contamination.

alpha-hemolysin (data not presented). In addition, alpha-hemolysin failed to bind to either cationic (AG 50W-X4) or anionic (AG 1-X8) exchange resins (Bio-Rad Laboratories, Richmond, Calif.) (data not presented).

Purification of alpha-hemolysin. The solubility of alpha-hemolysin in acetonitrile and 2-propanol was exploited for its purification. After THB constituents and streptococcal extracellular products were precipitated from spent culture medium with these solvents, alpha-hemolysin was subjected to reverse-phase HPLC in 1 mM HCl. As seen in Fig. 4, alpha-hemolysin and H₂O₂ had the same retention time of 6 min 8 s. No additional alpha-hemolytic activity was eluted by acetonitrile concentrations up to 100% (data not presented).

Correlation of alpha-hemolytic activity with hydrogen peroxide. When 1.5 U of alpha-hemolysin in culture supernatant was incubated for 5 min at 37°C with 100 U of catalase, 101% $\pm$ 2% of the alpha-hemolytic activity was inactivated (mean $\pm$

FIG. 4. Reverse-phase HPLC elution profiles of alpha-hemolysin and hydrogen peroxide. Contaminants in spent culture medium were precipitated with 2-propanol and acetonitrile before HPLC as described in Materials and Methods (upper trace). The lower trace shows H₂O₂ chromatographed under identical conditions. The peak of alpha-hemolytic activity eluted at the same position as pure H₂O₂, as indicated by the horizontal bar.

TABLE 2. Correlation of alpha-hemolytic activity with hydrogen peroxide concentration

Sample	Alpha-hemolysin activity (U/ml) ^a	H ₂ O ₂ concn (nmol/ml) ^b	H ₂ O ₂ /alpha-hemolysin ratio (nmol/U)
Spent culture medium	24.9 ± 0.9	157 ± 14	6.31 ± 0.80
Purified alpha-hemolysin ^c	318 ± 25	3,220 ± 69	10.12 ± 1.02
H ₂ O ₂ standard ^d	220 ± 2.3	2,100 ± 56	9.55 ± 0.35

^a Results are the means ± SDs of three independent determinations of alpha-hemolytic activity assayed with erythrocytes as described in Materials and Methods.

^b Results are the means ± SDs of four independent determinations of peroxide using horseradish peroxidase and ABTS.

^c Alpha-hemolysin was purified by differential solubility and reverse-phase HPLC.

^d Reagent-grade 30% H₂O₂ diluted 5,000-fold in PBS.

SD of five determinations). The same experiment, performed with 1.5 U of HPLC-purified alpha-hemolysin, yielded 103% ± 1% inhibition (mean ± SD of five determinations), demonstrating that H₂O₂ was responsible for all of the alpha-hemolytic activity present in culture supernatant and HPLC-purified samples. This observation was confirmed by the failure of catalase, which had been heat inactivated for 15 min at 100°C, to inhibit alpha-hemolytic activity. Replacement of catalase with heat-inactivated catalase led to -5% ± 3% and 2% ± 5% inhibition of the alpha-hemolytic activity in the culture supernatant and HPLC-purified samples, respectively (mean ± SD of five determinations). Quantitation of both H₂O₂ and alpha-hemolysin in selected samples revealed that 1 U of pure alpha-hemolysin is equivalent to 10.1 ± 1.02 nmol of H₂O₂ (Table 2).

Interestingly, spent culture medium showed 52% ± 17% more alpha-hemolytic activity than could be accounted for by the amount of peroxide present (Table 2). This enhancement seemed to be partly due to medium components, because commercial H₂O₂ diluted in fresh THB medium gave a 35% ± 8% stimulation of alpha-hemolytic activity relative to equal amounts of H₂O₂ in PBS (mean ± SD of three determinations). However, spent culture medium also contains several proteases (26, 33) that may enhance H₂O₂ uptake by erythrocytes, similar to our observations with proteinase K and pronase E. Thus, under optimal culture conditions, *S. gordonii* CH1 produced 238 ± 44 U of alpha-hemolysin per ml (Table 1), which, on the basis of the conversion factor of 6.31 nmol of H₂O₂ per U of alpha-hemolysin in culture supernatant (Table 2), corresponds to 1.50 ± 0.28 mM H₂O₂. Under identical conditions *S. pneumoniae* UB1 produced 1.64 ± 0.25 mM H₂O₂ (mean ± SD of three determinations).

DISCUSSION

This study is the first devoted to defining the chemical nature of the alpha-hemolysin of viridans streptococci, and the results presented here provide conclusive evidence that this hemolysin is hydrogen peroxide. Hydrogen peroxide and the alpha-hemolysin were identical with regard to their ability to oxidize the hemoglobin in erythrocytes, pH profile for alpha-hemolytic activity, resistance to proteases, inactivation by catalase, differential solubility, inability to adsorb to ion-exchange resins, and retention time on a C₁₈ reverse-phase HPLC column. Direct quantitation of the H₂O₂ present in culture supernatants and HPLC-purified samples further supports the contention that H₂O₂ accounts for all the alpha-hemolytic activity produced by *S. gordonii*. Viridans streptococci are known to produce H₂O₂ by using an NADH oxidase which reduces mo-

lecular oxygen to H₂O₂ (1, 9, 12, 15, 38, 45). Limiting the availability of oxygen during growth reduced the ability of streptococci to produce alpha-hemolysin as expected.

Because viridans group streptococci are catalase negative (14), they eliminate H₂O₂ by release into the surrounding medium, where accumulated levels are reported to reach 0.51 to 7.3 mM from *S. oralis*, *S. mitis*, and *S. sanguis* (11, 13, 38). These levels are consistent with the values of 0.45 to 1.5 mM H₂O₂ from *S. gordonii* and 1.64 mM H₂O₂ from *S. pneumoniae* observed in this study and indicate that H₂O₂ is the alpha-hemolysin of all the viridans streptococci. McLeod and Gordon (21) were the first to suspect that H₂O₂ from *S. pneumoniae* was responsible for intergeneric bacterial antagonism and alpha-hemolysis seen on blood agar plates because similar effects could be caused by commercial H₂O₂. More recently, Holmberg and Hallander (16) correlated bacteriocin-like activity and alpha-hemolysis with H₂O₂ production by *S. sanguis* because incorporation of catalase into sheep blood agar or anaerobiosis diminished both phenomena. However, the role of H₂O₂ as the alpha-hemolysin has not been generally accepted, because unheated blood agar contains indigenous catalase that is expected to inactivate H₂O₂ as it is produced by the growing streptococci (4, 44). Although erythrocytes do contain a significant amount of catalase (4), the low affinity of catalase for H₂O₂ ($K_m = 1.1$ M) (22) enables millimolar levels of H₂O₂ to accumulate and cause the oxidative damage corresponding to alpha-hemolysis. Hydrogen peroxide production by oral streptococci correlates closely with alpha-hemolysis around colonies on blood agar plates. The alpha-hemolytic *Streptococcus* species (*S. gordonii*, *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. sanguis*, and *S. sobrinus*) produce extracellular H₂O₂ concentrations up to 7 mM, whereas the nonhemolytic *Streptococcus* species (*S. anginosus*, *S. constellatus*, *S. intermedius*, *S. mutans*, and *S. salivarius*) accumulate less than 0.05 mM H₂O₂ under aerobic conditions (11, 13, 17, 38, 42).

Although the H₂O₂ produced by viridans streptococci has long been known to inhibit the growth of competing bacteria in vitro (16, 18, 40, 43), the possibility that this peroxide plays a role in pathogenesis has only recently been recognized (10). It has been reported that concentrations of H₂O₂ well below those produced by viridans streptococci are sufficient to cause lethal damage to human cells (8, 10, 32, 35). In addition, the lethal effects of peroxide in vivo may be potentiated by lactic acid production by the viridans group streptococci (14). Acidic conditions dramatically enhance the effects of H₂O₂ on erythrocytes (Fig. 2), cultured epithelial cells (19), and renal proximal tubule cells (25). The nature of this enhancement has not been elucidated, although reduced pH may increase cell permeability, inhibit antioxidant defenses (19), or supply protons needed as reagents in the oxidation of heme iron (i.e., $\text{H}_2\text{O}_2 + 2\text{Fe}^{2+} + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O} + 2\text{Fe}^{3+}$). Curiously, acidic conditions have the opposite effect on hepatic endothelial cells, which are protected from oxidative injury at pH 6.4 (6). Hence, the influence of streptococcal lactic acid production on the pathology induced by hydrogen peroxide will have to be determined for each cell type exposed to viridans streptococci during infection.

The identification of H₂O₂ as the alpha-hemolysin of viridans streptococci renders its designation as a hemolysin somewhat of a misnomer. Erythrocyte membranes were not lysed by the concentrations of H₂O₂ produced by viridans streptococci in this study and had to be lysed osmotically by the addition of distilled water before accurate spectral data could be obtained. Nevertheless, the term alpha-hemolytic streptococci has been used for over 90 years (28), and the convention is not likely to

change even though the designation peroxidogenic streptococci would be more accurate.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant R01-DE05696 from the National Institute of Dental Research.

REFERENCES

- Anders, R. F., D. M. Hogg, and G. R. Jago. 1970. Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. *Appl. Microbiol.* **19**:608–612.
- Asikainen, S., and S. Alaluusua. 1993. Bacteriology of dental infections. *Eur. Heart J.* **14**(Suppl. K):43–50.
- Beutler, E. 1983. Carboxyhemoglobin, methemoglobin, and sulfhemoglobin determinations, p. 1632–1634. In W. J. Williams, E. Beutler, A. J. Erslev, and M. A. Lichtman (ed.), *Hematology*, 3rd ed. McGraw Hill Book Co., New York.
- Beutler, E. 1984. Catalase, p. 105–106. In E. Beutler (ed.), *Red cell metabolism: a manual of biochemical methods*, 3rd ed. Grune & Stratton, Inc., Orlando, Fla.
- Bhakdi, S., M. Roth, A. Sziegoleit, and J. Trantum-Jensen. 1984. Isolation and identification of two hemolytic forms of streptolysin-O. *Infect. Immun.* **46**:394–400.
- Bronk, S. F., and G. J. Gores. 1991. Acidosis protects against lethal oxidative injury of liver sinusoidal endothelial cells. *Hepatology* **14**:150–157.
- Childs, R. E., and W. G. Bardsley. 1975. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. *Biochem. J.* **145**:93–103.
- Cochrane, C. G., I. U. Schraufstatter, P. A. Hyslop, and J. H. Jackson. 1988. Cellular and biochemical events in oxygen injury, p. 125–136. In P. A. Cerutti, I. Fridovich, and J. M. McCord (ed.), *Oxy-radicals in molecular biology and pathology*. Alan R. Liss, Inc., New York.
- Diguiseppi, J., and I. Fridovich. 1982. Oxygen toxicity in *Streptococcus sanguis*: the relative importance of superoxide and hydroxyl radicals. *J. Biol. Chem.* **257**:4046–4051.
- Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff. 1993. Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. *Infect. Immun.* **61**:4392–4397.
- Dubreuil, D., J. G. Bissailon, and R. Beaudet. 1984. Inhibition of *Neisseria gonorrhoeae* growth due to hydrogen peroxide production by urogenital streptococci. *Microbios* **39**:159–167.
- Fukui, K., K. Kato, T. Kodama, H. Ohta, T. Shimamoto, and T. Shimono. 1988. Kinetic study of a change in intracellular ATP level associated with aerobic catabolism of ethanol by *Streptococcus mutans*. *J. Bacteriol.* **170**:4589–4593.
- Garcia-Mendoza, A., J. Liebana, A. M. Castillo, A. De La Higuera, and G. Piedrola. 1993. Evaluation of the capacity of oral streptococci to produce hydrogen peroxide. *J. Med. Microbiol.* **39**:434–439.
- Hardie, J. M. 1986. Genus *Streptococcus* Rosenbach, p. 1043–1047. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. II. Williams and Wilkins, Baltimore.
- Higuchi, M., M. Shimada, Y. Yamamoto, T. Hayashi, T. Koga, and Y. Kamio. 1993. Identification of two distinct NADH oxidases corresponding to H₂O₂-forming and H₂O-forming oxidase induced in *Streptococcus mutans*. *J. Gen. Microbiol.* **139**:2343–2351.
- Holmberg, K., and H. O. Hallander. 1973. Production of bactericidal concentrations of hydrogen peroxide by *Streptococcus sanguis*. *Arch. Oral Biol.* **18**:423–434.
- Kilian, M., L. Mikkelsen, and J. Henriksen. 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982) and *Streptococcus mitis* (Andrews and Horder 1906). *Int. J. Syst. Bacteriol.* **39**:471–484.
- LeBien, T. W., and M. C. Bromel. 1975. Antibacterial properties of a peroxidogenic strain of *Streptococcus mitior* (mitis). *Can. J. Microbiol.* **21**:101–103.
- Link, E. M. 1988. The mechanism of pH-dependent hydrogen peroxide cytotoxicity *in vitro*. *Arch. Biochem. Biophys.* **265**:362–372.
- Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**:353–380.
- McLeod, J. W., and J. Gordon. 1922. Production of hydrogen peroxide by bacteria. *Biochem. J.* **16**:499–506.
- Nicholls, P., and G. R. Schonbaum. 1963. Catalases, p. 147–225. In P. D. Boyer, H. Lardy, and K. Myrback (ed.), *The enzymes*, 2nd ed. Academic Press, New York.
- Paton, J. C., P. W. Andrew, G. J. Boulnois, and T. J. Mitchell. 1993. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. *Annu. Rev. Microbiol.* **47**:89–115.
- Roberts, R. B. 1985. *Streptococcus pneumoniae*, p. 1142–1152. In G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), *Principles and practice of infectious diseases*, 2nd ed. John Wiley and Sons, New York.
- Rodeheaver, D. P., and R. G. Schnellmann. 1993. Extracellular acidosis ameliorates metabolic-inhibitor-induced and potentiates oxidant-induced cell death in renal proximal tubules. *J. Pharmacol. Exp. Ther.* **265**:1355–1360.
- Rogers, A. H., P. S. Zilm, N. J. Gully, and A. L. Pfennig. 1990. Some aspects of protease production by a strain of *Streptococcus sanguis*. *Oral Microbiol. Immunol.* **5**:72–76.
- Scheld, W. M., and M. A. Sande. 1985. Endocarditis and intravascular infections, p. 504–530. In G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), *Principles and practice of infectious diseases*, 2nd ed. John Wiley and Sons, New York.
- Schottmuller, H. 1903. Die Artunterscheidung der für den Menschen Pathogenen Streptokokken durch Blutagar. *Muench. Med. Wochenschr.* **50**:849–853, 902–912.
- Sherman, J. M. 1937. The streptococci. *Bacteriol. Rev.* **1**:3–97.
- Shindler, J. S., R. E. Childs, and W. G. Bardsley. 1976. Peroxidase from human cervical mucus: the isolation and characterisation. *Eur. J. Biochem.* **65**:325–331.
- Sigma Chemical Co. 1995. Catalase, p. 221. In *Biochemicals, organic compounds for research and diagnostic reagents*. Sigma Chemical Co., St. Louis.
- Simon, R. H., C. H. Scoggin, and D. Patterson. 1981. Hydrogen peroxide causes the fatal injury to human fibroblasts exposed to oxygen radicals. *J. Biol. Chem.* **256**:7181–7186.
- Straus, D. 1982. Protease production by *Streptococcus sanguis* associated with subacute bacterial endocarditis. *Infect. Immun.* **38**:1037–1045.
- Sussman, J. I., E. J. Baron, M. J. Tenenbaum, M. H. Kaplan, J. Greenspan, R. R. Facklam, M. B. Tyburski, M. A. Goldman, B. F. Kanzer, and R. A. Pizzarello. 1986. Viridans streptococcal endocarditis: clinical, microbiological, and echocardiographic correlations. *J. Infect. Dis.* **154**:597–603.
- Tenovu, J., and H. Larjava. 1984. The protective effect of peroxide and thiocyanate against hydrogen peroxide toxicity assessed by the uptake of [³H]-thymidine by human gingival fibroblasts cultured *in vitro*. *Arch. Oral Biol.* **29**:445–452.
- Terlechy, B., N. P. Willett, and G. D. Shockman. 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. *Infect. Immun.* **11**:649–655.
- Thibodeau, E. A., and D. M. O'Sullivan. 1995. Salivary mutans streptococci and incidence of caries in preschool children. *Caries Res.* **29**:148–153.
- Thomas, E. L., and K. A. Pera. 1983. Oxygen metabolism of *Streptococcus mutans*: uptake of oxygen and release of superoxide and hydrogen peroxide. *J. Bacteriol.* **154**:1236–1244.
- Tietz, N. W. 1970. Table of normal values, p. 944. In *Fundamentals of clinical chemistry*. W. B. Saunders Co., Philadelphia.
- Vernazza, T. R., and T. H. Melville. 1979. Inhibitory activity of *Streptococcus mitis* against oral bacteria. *Microbios* **26**:95–101.
- Weinberger, I., Z. Rotenberg, D. Zacharovitch, J. Fuchs, E. Davidson, and J. Agmon. 1990. Native valve infective endocarditis in the 1970's versus the 1980's: underlying cardiac lesions and infecting organisms. *Clin. Cardiol.* **13**:94–98.
- Whiley, R. A., and D. Beighton. 1991. Emended descriptions and recognition of *Streptococcus constellatus*, *Streptococcus intermedius* and *Streptococcus anginosus* as distinct species. *Int. J. Syst. Bacteriol.* **41**:1–5.
- Willcox, M. D., and D. B. Drucker. 1988. Partial characterization of the inhibitory substances produced by *Streptococcus oralis* and related species. *Microbios* **55**:135–145.
- Wilson, G. S., and A. Miles. 1975. The streptococci, p. 712–763. In Topley and Wilson's principles of bacteriology, virology and immunity, 6th ed., vol. 1. The Williams and Wilkins Co., Baltimore.
- Zitzelberger, W., F. Gotz, and K. H. Schleifer. 1984. Distribution of superoxide dismutases, oxidases and NADH peroxidase in various streptococci. *FEMS Microbiol. Lett.* **21**:243–246.