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Bactericidal Effect of Cysteine Exposed to Atmospheric Oxygen

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Peptostreptococcus anaerobius VPI 4330-1 was exposed to atmospheric oxygen in a dilution blank (0.2% gelatin, salts, resazurin) solution. The organisms were rapidly killed when the solution contained cysteine. The organisms were effectively protected by catalase and horseradish peroxidase as well as by the metal ion-chelating agents 8-hydroxyquinoline and 2,2'-bipyridine. Superoxide dismutase increased the rate of killing of the organisms, whereas singlet oxygen quenchers and scavengers of hydroxyl free radicals did not protect the organisms from the toxic effect of cysteine. Hydrogen peroxide was formed when cysteine was exposed to oxygen in the dilution blank solution, and the reaction was inhibited by metal ion-chelating agents. The organisms were rapidly killed by 20 μM hydrogen peroxide in anaerobic dilution blank solution. The toxic effect of hydrogen peroxide was completely abolished by catalase and metal ion-chelating agents. These results indicated that hydrogen peroxide was formed in the dilution blank solution in a metal ion-catalyzed autoxidation of cysteine and that hydrogen peroxide was toxic to *P. anaerobius* VPI 4330-1 in a reaction also catalyzed by metal ions.

Cysteine may be toxic to or may inhibit growth of *Escherichia coli* (11, 25, 46), *Bacillus subtilis* (52), yeasts (7, 33, 36), and fungi (4, 49). Cysteine appears to inhibit growth by two different mechanisms in *E. coli*. It may interfere with biosynthesis of leucine, isoleucine, threonine, and valine, or it may interact with the function of membrane-bound enzymes (25). The growth-inhibiting effect of cysteine in yeasts has been ascribed to its ability to chelate metal ions necessary for the activities of various enzymes (7, 36).

Cysteine is routinely used in many media for the cultivation of anaerobic bacteria (22). In a study on the bactericidal effects of various culture media exposed to atmospheric oxygen (13), it was observed that cysteine was toxic to *Peptostreptococcus anaerobius* VPI 4330-1. We now report that hydrogen peroxide is formed from cysteine in the presence of oxygen in a metal ion-catalyzed reaction and that hydrogen peroxide is toxic to *P. anaerobius* VPI 4330-1 in a reaction also catalyzed by metal ions.

MATERIALS AND METHODS

Microorganisms. *P. anaerobius* strain VPI 4330-1 (ATCC 27337) was used as the test strain (23). It was kept on blood agar plates at 4°C under strictly anaerobic conditions in an anaerobic box with an atmosphere of 10% H_2 and 5% CO_2 in nitrogen (54).

Chemicals. Peroxidase (from horseradish, grade I, 250 U/mg) and xanthine oxidase (from cow milk) were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany. Catalase (purified powder from bovine liver: C40), superoxide dismutase (SOD; from bovine blood), hypoxanthine, and bilirubin were from Sigma Chemical Co., St. Louis, Mo. Sephacryl S-200 superfine was from Pharmacia, Uppsala, Sweden. Hydrogen peroxide (30%, wt/wt; Perhydrol) and L-cysteine hydrochloride were from E. Merck AG, Darmstadt, West Germany, and 1,4-diazabicyclo-[2.2.2]octane was from Aldrich-Europe, Beerse, Belgium. 3-amino-1,2,4-triazole was from KEBO-GRAVE, Stockholm, Sweden.

Catalase was purified from SOD activity by dissolving 250 mg of the powder in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl. The solution was centrifuged at 48,000 × *g* for 20 min at 4°C, and the supernatant fluid was filtered through a Sephacryl S-200 superfine column (90 by 2.5 cm) in the same buffer. The purified catalase decomposed 40 mmol of hydrogen peroxide per min per mg of protein at pH 7.0 and 25°C.

Titanium stock reagent was prepared as described by Marklund (31). The 3-amino-1,2,4-triazole preparation contained substances which reacted with oxygen and formed hydrogen peroxide. These substances could be eliminated by dissolving the preparation to saturation in boiling ethanol, followed by filtration and recrystallization. The crystals were then dried at 80°C overnight. All chemicals and medium components were dissolved in double-distilled water from quartz vessels.

Preparation of media. Aerobic and anaerobic cysteine-free dilution blank solutions (22) were used. The anaerobic dilution blank solution contained 125 ml of water, 125 ml of salt solution (22), 0.5 g of gelatin, and 0.5 ml of resazurin solution (22). The solution was boiled until the resazurin turned from pink to colorless. The solution was cooled to room temperature in an ice bath, all the while bubbling oxygen-free CO_2 through the solution in a gentle stream to exclude air. Then 1.5 ml of 7.5 M K_2CO_3 was added, and bubbling CO_2 through the solution was continued until the pH was 7.0. The solution was then dispensed under the protection of CO_2 gas into test tubes (9 ml in each). The tubes were tightly stoppered with rubber stoppers and autoclaved at 120°C for 20 min. The aerobic dilution blank solution contained 125 ml of water, 125 ml of salt solution, 0.5 g of gelatin, 1.5 ml of 7.5 M K_2CO_3, and 0.5 ml of resazurin solution. The solution was further buffered by 1.0 g of KH_2PO_4. The solution was dispensed under aerobic conditions into test tubes and autoclaved at 120°C for 20 min. The pH of the solution was 9.3 after autoclaving.

Peptone-yeast extract-glucose broth was prepared as described by Holdeman et al. (22). Horse blood (Gibco, Bio-Cult Ltd., Paisley, Scotland) was hemolyzed by freeze-thawing. This blood was used in the blood agar medium (22).

Bactericidal effect of cysteine. *P. anaerobius* VPI 4330-1 was grown at 37°C in peptone-yeast extract-glucose broth in the anaerobic box. When the culture was in logarithmic growth phase and had a density of 0.5 (E_{600}), it was diluted in the anaerobic dilution blank solution to a density of about 2×10^4 organisms per ml. A 0.2-ml sample of this suspension was added to 1.8 ml of a reaction mixture containing 1.6 ml of anaerobic dilution blank solution and 0.2 ml of various additions. Usually, eight different reaction mixtures were prepared in each experiment, and they were taken from the anaerobic box in tightly stoppered tubes. Fifteen minutes after the start of the dilution procedure, one of the inoculated reaction mixtures was aerated by pouring it into a 50-ml, round-bottomed flask submerged under continuous shaking in a thermostated (25°C) water bath. At 1-min intervals the other reaction mixtures were treated in the same way. Samples (0.1 ml) were taken at regular time intervals from the flasks and were spread over the surface of duplicate blood agar plates. The plates were taken from the anaerobic box just before use. The plates were returned to the box immediately after inoculation. The plates were incubated for 1 day at 37°C in the box, and the numbers of surviving organisms after various times of exposure to air in the reaction mixtures were determined.

The bactericidal effects of cysteine at various pH values were tested in a similar way, but the amount of reaction mixture was increased to 20 ml and the solution was kept together with a pH electrode in a 50-ml beaker. The pH of the solution was controlled by a titrator and autoburette (TTT2, ABU13; Radiometer, Copenhagen, Denmark) using 0.5 N HCl.

Exposure of *P. anaerobius* VPI 4330-1 to hydrogen peroxide. When *P. anaerobius* VPI 4330-1 was in logarithmic growth phase in peptone-yeast extract-glucose broth and the culture had a density of 0.4 (E_{600}), it was diluted in 10-fold steps in anaerobic dilution blank solution. A 20-μl sample of dilution 10^{-3} was inoculated into each of three reaction mixtures containing 1.5 ml of anaerobic dilution blank solution and 0.5 ml of various additions. From the culture in logarithmic growth this procedure was repeated five times at 7-min intervals. The culture usually had a density of 0.65 (E_{600}) at the start of the fifth dilution procedure. Two and three minutes, respectively, after the start of each dilution procedure, the organisms in two of the three inoculated reaction mixtures were exposed to 20 μM hydrogen peroxide. Samples (0.1 ml) were taken 2 min after the addition of 20 μl of a 2 mM hydrogen peroxide solution, and they were cultured on the surfaces of duplicate blood agar plates for determination of the numbers of surviving organisms in the reaction mixtures. Samples (0.1 ml) were also taken from the third reaction mixture and cultured on duplicate blood agar plates for determination of the original number of organisms in the three reaction mixtures of each series.

The anaerobic dilution blank solution used in the reaction mixtures was exposed to the atmosphere of the anaerobic box for at least 2 h before the start of the experiment. The pH of the solution was then 7.5, and the temperature of the solution was 28 ± 0.5°C.

Oxidation of cysteine in the presence of oxygen, hydrogen peroxide, or horseradish peroxidase. The autoxidation of a 50 mM cysteine solution was studied by following the oxygen consumption in a biological oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio). The temperature of the system was kept at 25°C by a circulating-water pump. Aerated anaerobic dilution blank solution (4.0 ml), 0.5 ml of anaerobic 0.5 M cysteine solution, and 0.5 ml of various additions were added to the chamber of the oxygen monitor, and the chamber was immediately closed by the oxygen electrode. Oxygen consumption was followed for 5 min, and the rate of oxygen consumption was expressed as nanomoles of oxygen consumed per minute per milliliter of solution.

The oxidation of cysteine in the presence of hydrogen peroxide was studied under anaerobic conditions. Various amounts of hydrogen peroxide in 0.5 ml of solution were added to 4.5 ml of anaerobic dilution blank solution containing 64 or 640 μM cysteine. The concentration of cysteine in the reaction mixture was determined after 1 h by the method of Gaitonde (20).

The oxidation of cysteine in the presence of horseradish peroxidase was studied by adding horseradish peroxidase (40 μg/ml) to 5 ml of anaerobic dilution blank solution containing cysteine (640 μM). The solution was exposed to atmospheric oxygen under continuous shaking at ambient temperature. Samples were taken out after 0, 10, 30, and 60 min, and the cysteine concentration in the reaction mixture was determined (20).

Hydrogen peroxide generated in the autoxidation of cysteine. Catalase is inhibited by 3-amino-1,2,4-triazole in the presence of hydrogen peroxide (30). The generation of hydrogen peroxide in a reaction could thus be determined by adding catalase and 3-amino-1,2,4-triazole to the reaction mixture and measuring the decrease in catalase activity in the mixture.

To 5 ml of aerobic dilution blank solution, catalase (40 μg/ml), 3-amino-1,2,4-triazole (50 μmol/ml), and various amounts of cysteine were added, and the solution was aerated in 50-ml, round-bottomed flasks by continuous shaking at ambient temperature. Samples (0.1 ml) for determination of catalase activity in the reaction mixture were taken at regular time intervals. Each sample was first mixed with 0.5 ml of 0.45 M ethanol in 50 mM potassium phosphate buffer (pH 7.0) to decompose complex I of catalase-H_2O_2 and to stop the reaction with 3-amino-1,2,4-triazole (14). The solution was then incubated for 10 min to decompose any complex II of catalase-H_2O_2 (14, 30). It was then diluted to 10 ml with ice-cold water, and 0.5 ml of this solution was added to 2.0 ml of a 15 mM hydrogen peroxide solution. The catalase activity of the sample was determined by measuring the decrease in the concentration of hydrogen peroxide in the solution. Samples (0.2 ml) of the solution were taken at regular time intervals and added to 2.8 ml of water containing 50 μl of titanium stock reagent, and the extinction of the solution was measured at 405 nm (31).

The catalase-3-amino-1,2,4-triazole system was calibrated by determining the inhibition of catalase when hydrogen peroxide was generated by xanthine oxidase in the oxidation of hypoxanthine to uric acid. The rate of hydrogen peroxide generation was determined by measuring the rate of uric acid formation. The change of uric acid concentration in the reaction mixture was estimated from the change in the extinction of the solution at 293 nm ($\epsilon_{mM} = 12$). The formation of 1 mol of uric acid is equivalent to the generation of 2 mol of hydrogen peroxide in the oxidation of hypoxanthine.

RESULTS

Bactericidal effect of cysteine. *P. anaerobius* VPI 4330-1 was rapidly killed when exposed to atmospheric oxygen in the anaerobic dilution blank solution containing 260 μM cysteine. If cysteine was omitted from this solution or if the organisms were kept in the solution under anaerobic conditions, the organisms survived for more than 2 h. Catalase and horseradish peroxidase significantly decreased the rate of killing of the organisms, whereas SOD increased the rate (Fig. 1). The singlet oxygen quenchers 1,4-diazabicyclo[2.2.2]octane (20 mM) and bilirubin (2 mM) and the scavengers of hydroxyl free radicals mannitol (100 mM) and formate (100 mM) did not protect the organisms from the toxic effect of cysteine. The organisms were killed when the concentration of cysteine in the dilution blank solution was higher than 40 μM. The rate of killing was increased by increasing concentrations of cysteine (Fig. 2).

The toxic effect of cysteine exposed to atmospheric oxygen was prevented by the metal ion-chelating agents 8-hydroxyquinoline (25 μM), 2,2'-bipyridine (260 μM), and ethylenediaminetetraacetic acid disodium salt (10 mM) (Fig. 3). The anaerobic dilution blank solution contains a carbon dioxide-carbonate buffer (pH 7.0).

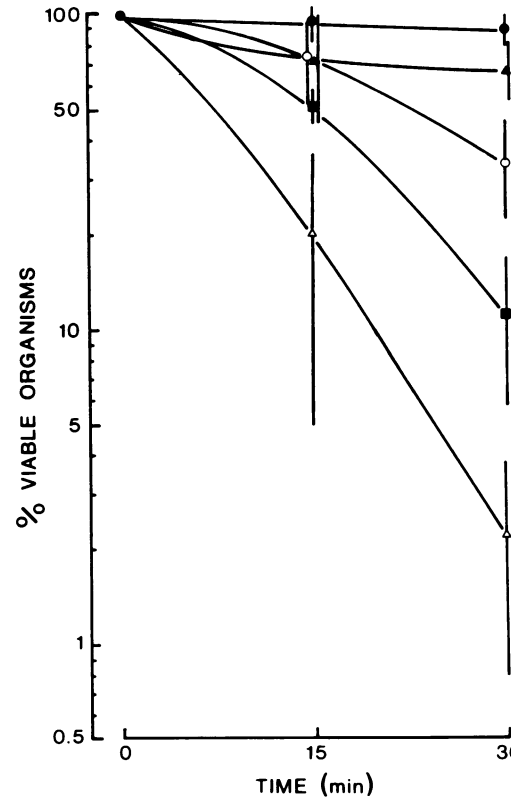

FIG. 1. *Effect of catalase, horseradish peroxidase and SOD on the survival of P. anaerobius VPI 4330-1 when exposed for 15 and 30 min to aerated anaerobic dilution blank solution containing 260 μM cysteine (Dil) at 25°C. Symbols: ●, Dil without cysteine; ■, Dil; ▲, Dil with horseradish peroxidase (100 μg/ml); ○, Dil with catalase (40 μg/ml); △, Dil with SOD (10 μg/ml). Means ± standard deviations of three experiments are given.*

When this solution was exposed to air, the pH increased to 8.65 in 10 min, 8.85 in 30 min, and 9.1 in 1 h. If the solution was kept at a lower pH, the rate of killing was significantly decreased (Fig. 4).

Oxidation of cysteine. Cysteine (50 mM) autoxidized when the anaerobic dilution blank solution was exposed to air (Table 1). The rate of autoxidation was decreased by metal ion-chelating agents (Table 1). No free hydrogen peroxide could be detected in the aerated anaerobic dilution blank solution containing cysteine, but formation of hydrogen peroxide in the autoxidation of cysteine was demonstrated by the hydrogen peroxide-dependent inactivation of catalase in the presence of 3-amino-1,2,4-triazole. The total amount of hydrogen peroxide formed for 30 min in the autoxidation of cysteine was determined (Fig. 5).

When hydrogen peroxide was added to a cysteine solution under anaerobic conditions, cysteine was oxidized. Two cysteine molecules were oxidized by one molecule of hydrogen peroxide. When horseradish peroxidase was added to a cysteine solution under aerobic conditions, peroxidase strongly catalyzed the oxidation of cysteine.

Bactericidal effect of hydrogen peroxide. When *P. anaerobius* VPI 4330-1 was exposed to 20 μM hydrogen peroxide in the anaerobic dilution blank solution under anaerobic conditions, more than 90% of the organisms were killed within 2 min. The toxic effect of hydrogen peroxide was completely abolished by catalase (26 μg/ml), 0.5 mM 8-hydroxyquinoline, and 0.1 mM 2,2'-bipyridine. 1,4-Diazabicyclo[2.2.2]octane (20 mM), bilirubin (2 mM), mannitol (100 mM), and SOD (10 μg/ml) had no effect on the toxicity of hydrogen peroxide. Cysteine in concentrations exceeding 100 μM protected the organisms to some extent from the toxic effect of hydrogen peroxide (Fig. 6).

DISCUSSION

P. anaerobius VPI 4330-1 was rapidly killed in a cysteine-containing solution exposed to at-

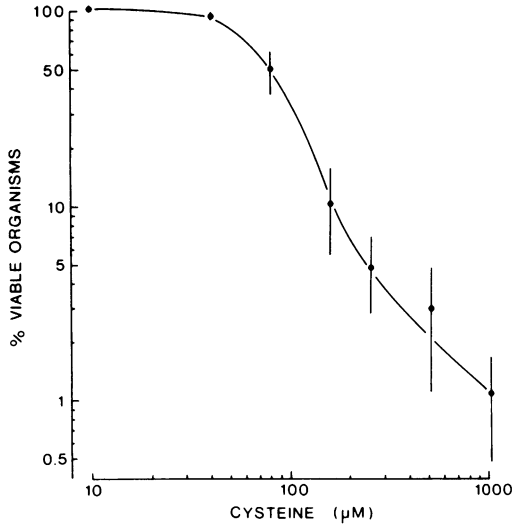

FIG. 2. *Survival of P. anaerobius VPI 4330-1 at 25°C when exposed for 30 min to aerated anaerobic dilution blank solution containing various concentrations of cysteine. Means ± standard deviations of three experiments are given.*

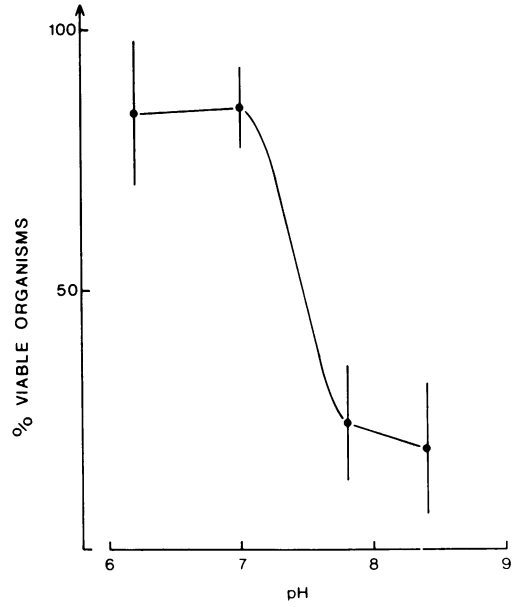

FIG. 4. *Survival of P. anaerobius VPI 4330-1 at various pH values when exposed for 30 min to aerated anaerobic dilution blank solution containing 260 μM cysteine at 25°C. Means ± standard deviations of three experiments are given.*

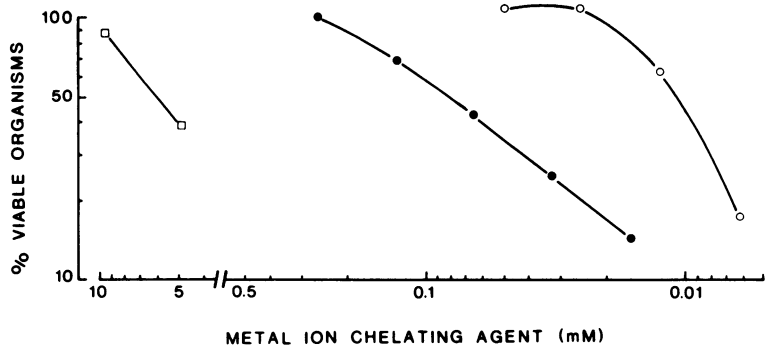

FIG. 3. *Protecting effect of various concentrations of the chelating agents 8-hydroxyquinoline, 2,2'-bipyridine, and ethylenediaminetetraacetic acid disodium salt on the survival of P. anaerobius VPI 4330-1 when exposed for 30 min to aerated anaerobic dilution blank solution containing 260 μM cysteine at 25°C. Symbols: ○, 8-hydroxyquinoline; ●, 2,2'-bipyridine; □, ethylenediaminetetraacetic acid disodium salt.*

TABLE 1. *Autoxidation of 50 mM cysteine in aerated anaerobic dilution blank solution, pH 9.3, in the presence of metal ion-chelating agents*

Metal ion-chelating agent	Autoxidation rate (nmol of O_2 consumed/ml per min)[a]
None	11.8 ± 0.9
2.5 mM 2,2'-bipyridine	6.6 ± 0.5
0.25 mM 8-hydroxyquinoline	5.4 ± 0.7

[a] Mean ± standard deviation of four experiments.

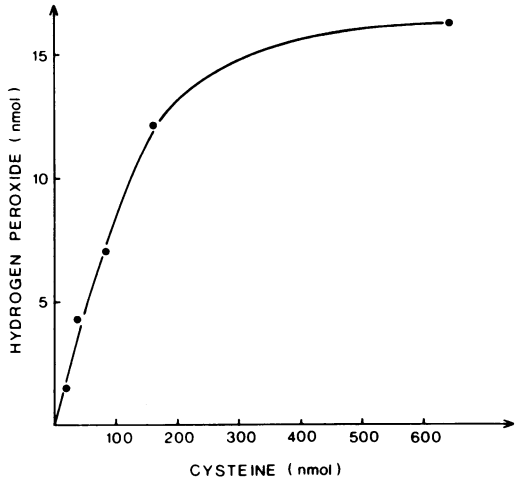

FIG. 5. *Hydrogen peroxide produced by autoxidizing cysteine in aerated anaerobic dilution blank solution at 25°C. The amount of hydrogen peroxide formed for 30 min from various amounts of cysteine was determined by the catalase-aminotriazole method.*

mospheric oxygen. The organisms were effectively protected by catalase and horseradish peroxidase as well as by metal ion-chelating agents. This indicated that hydrogen peroxide mediated the bactericidal effect of cysteine and that metal ions were also involved.

It is a well-established fact that cysteine autoxidizes to cystine in the presence of transitional metal ions (35, 37, 50). Although hydrogen peroxide has been predicted as a product of this reaction, hydrogen peroxide has not been demonstrated as a stable product of the reaction (37, 50). One reason could be that hydrogen peroxide reacts with cysteine (38, 43, 51). The finding in the present study that catalase was inhibited in the presence of 3-amino-1,2,4-triazole in an autoxidizing solution of cysteine (Fig. 5) strongly indicated that hydrogen peroxide was formed in the reaction. The protecting effects of catalase and peroxidase against the bactericidal effect of autoxidizing cysteine are further evidence for the involvement of hydrogen peroxide. The rate of autoxidation of cysteine increases when the pH of a cysteine solution is increased from 7 to 9 (27, 34, 43). The higher toxicity of cysteine at an alkaline pH than at a neutral pH (Fig. 4) may be explained by this increased rate of cysteine oxidation.

Horseradish peroxidase is an efficient scavenger of hydrogen peroxide, and, in addition, peroxidase is able to use cysteine as a substrate in the reaction (40). This makes horseradish peroxidase especially suited to protect *P. anaerobius* VPI 4330-1 from the toxic effect of cysteine. The use of catalase as a protector may have some drawbacks. Catalase has a high K_m for hydrogen peroxide (39), and it is inactivated by

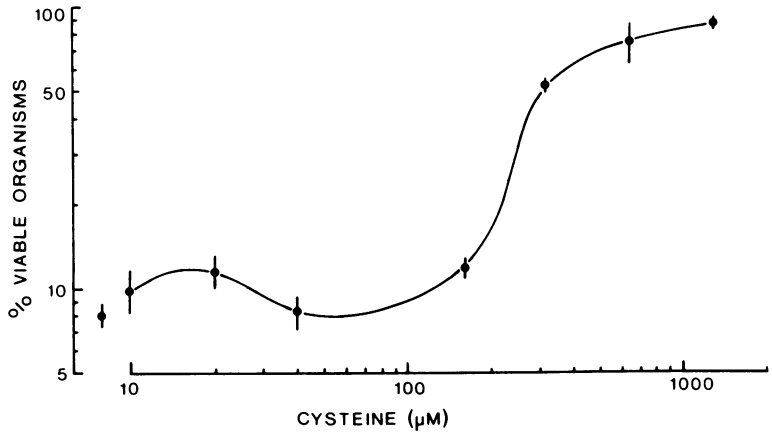

FIG. 6. *Survival of P. anaerobius VPI 4330-1 after exposure for 2 min to 20 μM hydrogen peroxide at 28°C in anaerobic dilution blank solution containing various concentrations of cysteine. Means ± standard error are given.*

cysteine (3, 8, 16). It has been reported that catalase has a catalytic effect on the oxidation of cysteine (8), but this characteristic of catalase has significance only in reaction mixtures where the catalase/cysteine ratio is higher than that in the present study (8). An unexpected finding was that SOD increased the death rate of *P. anaerobius* VPI 4330-1 in the presence of autoxidizing cysteine. Subsequent studies have shown that heat-inactivated SOD is even more potent than the native enzyme. The native as well as the inactivated SOD actually catalyze the oxidation of cysteine (G. K. Nyberg, G. P. D. Granberg, and J. Carlsson, manuscript in preparation).

The organism was rapidly killed when it was exposed to 20 μM hydrogen peroxide in an anaerobic, cysteine-free solution. Cysteine did not potentiate the toxic effect of hydrogen peroxide. Instead, cysteine in concentrations higher than 100 μM protected the organisms to some extent from the toxic effect of 20 μM hydrogen peroxide. This indicated that the toxic effect of autoxidizing cysteine was not mediated by cysteine–peroxide adducts. The toxic effect of hydrogen peroxide could be abolished by metal ion-chelating agents, whereas scavengers of singlet oxygen, superoxide radicals, and hydroxyl free radicals afforded no protection. This implied that the organisms were killed via a metal ion-catalyzed reaction with hydrogen peroxide. The protecting effect of cysteine in the anaerobic environment could probably be ascribed to the tendency of cysteine to react with hydrogen peroxide (38, 43, 51) and to the ability of cysteine to chelate metal ions (2). It is not possible from the present data to decide what type of reaction the metal ions catalyzed. It could be reactions between hydrogen peroxide and essential sulfhydryl groups (15, 24, 28) or formation of free radicals from hydrogen peroxide (29).

The site of damage of bacteria injured by hydrogen peroxide has not been established. In *E. coli*, paralysis of the respiratory chain (18), deoxyribonucleic acid (DNA) strand breakage (44), and DNA degradation (26) have been reported. Such changes are also induced, however, in cells exposed to various forms of physical and chemical stresses (6, 42). An interesting recent finding is that cells sublethally stressed (e.g., by heating) have an increased susceptibility to hydrogen peroxide and that hydrogen peroxide appears to interfere with the ability of the cells to recover after the stress (9, 17, 32). Hydrogen peroxide present in ordinary culture media is thus highly toxic for sublethally stressed cells of species like *Staphylococcus aureus, Salmonella typhimurium, Pseudomonas fluorescens* and *E. coli* (32). In addition, this toxic effect of hydrogen peroxide is significantly potentiated by components of selective culture media, such as sodium chloride in media for staphylococci (9, 17, 45). These findings indicate that hydrogen peroxide toxicity is not a problem confined only to the area of anaerobic bacteriology. Recognition of the effects of hydrogen peroxide could be imperative for accurate interpretation of many types of microbiological data. Measures should therefore routinely be taken for minimizing the production and accumulation of hydrogen peroxide in culture media. Addition of hemolyzed blood, catalase, peroxidase, or sodium pyruvate to the media could be an effective way to handle this problem (9, 12, 19, 45).

The present study demonstrated that autoxidizing cysteine could be an important source of hydrogen peroxide formation in culture media. We have previously shown that heating glucose and phosphate together in culture media gives autoxidizing products which release hydrogen peroxide into the media (13). The facts that hydrogen peroxide is almost ubiquitous in autoclaved complex culture media exposed to air (13) and that heat-treated bacteria are highly susceptible to hydrogen peroxide (9, 17, 32, 45) may give a clue to one site where bacteria are injured by hydrogen peroxide. Heat induces breaks in bacterial DNA, and this is accompanied by a loss in viability if the repair of DNA is inhibited (10, 48). The DNA breaks are repaired with high efficiency if the bacteria are kept at physiological temperatures in buffer or in minimal medium (41, 53), but if the heat-treated bacteria are exposed to aerobic complex culture media, the DNA repair is inhibited and there is a significant loss in viability (21). These findings together with the demonstration of the efficient protection of heat-treated bacteria by catalase on aerobic complex culture media (9, 17, 32, 45) suggest that hydrogen peroxide in some way interferes with the repair of DNA. It is then of interest that bacterial strains with various deficiences in their DNA repair systems have different resistances to the toxic effect of aerobic complex culture media (1, 5, 47). These DNA-repair-deficient strains will be valuable experimental requisites in studies aimed at elucidating a possible inhibiting effect of hydrogen peroxide on DNA repair.

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