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Rapid bactericidal effect of low pH against *Pseudomonas aeruginosa*

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SUMMARY

Solutions with a pH of 3 or lower had a bactericidal effect within 60 s against *Pseudomonas aeruginosa* and other Gram-negative eubacteria in an assay based on the A.O.A.C. germicidal and detergent sanitizer assay. This effect was not observed on the Gram-positive eubacteria and yeasts examined. Acidification of disinfectants to improve their activity against Gram-negative eubacteria is a common practice. It is shown here that low pH itself can have a rapid bactericidal activity.

INTRODUCTION

An effect of low pH against Gram-negative eubacteria, in general, and *Pseudomonas* species, in particular, has been long recognized [5,7]. Most of the research on this topic has been related to preservation and/or inhibition of microbial activity and growth [1,2,5,6]. While acidification of hard-surface disinfectants to improve their efficacy against Gram-negative eubacteria is an accepted practice, few studies on the bactericidal effect of acidic solutions on microorganisms were found in the literature. The bactericidal effect of acids against *Pseudomonas aeruginosa* was reviewed by Morton [5]. The most rapid bactericidal activity observed was with acetic acid (15000 mg/l) after 15 min of exposure [7].

During a study of the activity of hard-surface disinfectants [8], an apparently synergistic bactericidal activity against *P. aeruginosa* was noted when hydrogen peroxide was combined with sodium meta-bisulfite (used to inactivate the hydrogen peroxide). This activity was not observed on *Staphylococcus aureus* or *Saccharomyces cerevisiae*. This phenomenon, and its underlying cause, are examined in this study.

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MATERIALS AND METHODS

Microorganisms and growth conditions

Enterobacter aerogenes ATCC 13048, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 15313, *P. aeruginosa* ATCC 15422, *Pseudomonas fluorescens* ATCC 13525, *S. aureus* ATCC 6538, *Candida albicans* ATCC 19231, and *S. cerevisiae* ATCC 18824 were obtained from the American Type Culture Collection. *Acinetobacter calcoaceticus* var. Iwoffi was obtained from the stock culture collection of the Department of Botany and Microbiology at the University of Oklahoma. Microorganisms were cultured as previously described [8]; cultures were incubated at 37 °C, except for *P. fluorescens*, *C. albicans*, and *S. cerevisiae*, which were grown at 30 °C.

Test procedures

The basic test procedure was adapted from the A.O.A.C. germicidal and detergent sanitizer test [4] as previously described [8]. The effect of a single compound on a microorganism was tested by adding the compound to synthetic hard water (100 ppm total hardness), adjusting the pH of the solution as required, and filter-sterilizing the test solution into a sterile disposable 150-ml flask. Most assays were initiated by adding the test microorganisms: 0.5 ml of culture for *P. aeruginosa*, 1.0 ml of culture for the other prokaryotes, and 5.0 ml of culture for the yeasts. The final assay volume was 50 ml. The effect of hydrogen peroxide and bisulfite in combination was determined by first adding the bisulfite to the synthetic hard water, filter-sterilizing this solution into a sterile flask, and

adding the test microorganism to the assay (bisulfite alone did not decrease viable cell counts in these assays). The assay was initiated by adding hydrogen peroxide. One milliliter of the assay mix was diluted into 4.0 ml of synthetic hard water containing 1 mM TES (pH 7.6) after 60 s under the assay condition had elapsed; the pH of a neutralized sample was between 7.0–7.6. The neutralized aliquot from each assay was serially diluted in culture medium containing 10 g/l purified agar (BBL, Cockeysville, MD) and plated. Viable cell counts were made after these plates were incubated at 37 °C for 2 days or 30 °C for 3 days, depending on the test microorganism. Each assay condition was examined in duplicate and each individual assay flask was plated in duplicate for determination of viable cell counts.

Materials and chemical assays

Concentrations of hydrogen peroxide and bisulfite (as sulfur dioxide after acidification) were determined with test kit assays (CHEMetrics (Calverton, VA) K5510C, 0–10000 ppm hydrogen peroxide, and CHEMetrics K9650, 0–500 ppm sulfite). All of the common chemicals were of reagent grade or better. Hydrogen peroxide (H325) and sodium meta-bisulfite (S244) were obtained from Fisher Scientific (Pittsburgh, PA). TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) was obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

An apparent synergistic effect between hydrogen peroxide and bisulfite against *P. aeruginosa* was observed during a comparative study of the activity of hard-surface disinfectants in a 60-s assay [8]. Low concentrations of hydrogen peroxide (1000 mg/l) or sodium meta-bisulfite (5000 mg/l) had little or no effect on the viability of *P. aeruginosa* in a 60-s assay, but in combination at lower concentrations (200 mg/l hydrogen peroxide and 1000 mg/l bisulfite) effected almost a total kill of a suspension of *P. aeruginosa* (Table 1). In this assay, hydrogen peroxide and bisulfite reacted rapidly to produce bisulfate:

$$H_2O_2 + HSO_3^- \text{-----> } H_2O + HSO_4^- \ .$$

Quantitative analysis, using test kits confirmed the disappearance of hydrogen peroxide and bisulfite in the stoichiometry given above after combination (data not shown). During this reaction, the pH decreased as bisulfite (second pK_a of 6.91) was converted to bisulfate (second pK_a of 1.92) [10].

An enhanced bactericidal effect of low concentrations of hydrogen peroxide and bisulfite in combination against *P. aeruginosa* was unexpected and was not indicated in a review of the literature [2,9]. Nor was this effect observed on two other microorganisms, *S. aureus* and *S. cerevisiae* [8]. This effect on *P. aeruginosa* could have been caused by a conversion of bisulfite into sulfur dioxide after acidification of the assay or caused directly by the lowered assay pH.

TABLE 1

Effect of hydrogen peroxide and sodium bisulfite against *Pseudomonas aeruginosa*

H_2O_2 (mg/l)	$NaHSO_3$[a] (mg/l)	pH[b]	Viable cells/ml[c]	Control
1000		7.6	2×10^5	4×10^5
	5000	5.2	4×10^5	4×10^5
50	1000	4.5	3×10^6	5×10^6
100	1000	4.0	2×10^6	5×10^6
200	1000	3.2	2×10^0	5×10^6

[a] Added as sodium meta-bisulfite ($Na_2S_2O_5$).

[b] pH of the assay after all additions.

[c] The effect of hydrogen peroxide and sodium bisulfite was determined in a 60-s bactericidal assay [8]. Cells were added to a bisulfite solution, followed by the addition of hydrogen peroxide to initiate the assay. After 60 s, an aliquot of the assay was diluted in synthetic hard water containing TES buffer (pH 7.6) and then plated for enumeration of viable cells. Control counts were obtained by incubating cells in the absence of hydrogen peroxide and bisulfite.

The possibility that sulfur dioxide was causing this rapid bactericidal effect on *P. aeruginosa* was examined by varying the amount of sodium meta-bisulfite in an assay (0–2000 mg/l), adding cells, and initiating the assay with the addition of 500 mg/l hydrogen peroxide. Complete kill of *P. aeruginosa* was observed in assays with no detectable sulfite or sulfur dioxide remaining after constituting the assay. These data suggested that the bactericidal effect of hydrogen peroxide and bisulfite in combination against *P. aeruginosa* was not due to the formation of free sulfur dioxide. While sulfur dioxide is used as a preservative, no indication of it having a rapid bactericidal effect was found [1,2]. However, in the assays (above) where a bactericidal effect was observed, the assay pH was 3.2 or lower after all additions were made.

The effect of acidic pH alone against *P. aeruginosa* was examined (Table 2). At pH 3.5, 10 mM solutions of citric acid, lactic acid, or phthalic acid had little effect on the viability of *P. aeruginosa* after 60 s. However, at pH 2.5, a pronounced bactericidal effect was observed. Solutions of succinic acid, phosphoric acid, and sulfurid acid at a pH of 3.0 also had rapid bactericidal effects on cells of *P. aeruginosa*. These results indicate that at a low enough pH, in the presence of either organic acids or inorganic

TABLE 2

Effect of acids against *Pseudomonas aeruginosa*

Acid[a]	pH	Viable cells/ml[b]	Control
Citric acid	3.5	5×10^6	8×10^6
Citric acid	3.0	3×10^6	8×10^6
Citric acid	2.5	1×10^3	8×10^6
Lactic acid	3.5	7×10^6	8×10^6
Lactic acid	3.0	5×10^6	8×10^6
Lactic acid	2.5	$< 1 \times 10^0$	8×10^6
Phthalic acid	3.5	2×10^5	8×10^6
Phthalic acid	3.0	9×10^3	8×10^6
Phthalic acid	2.5	$< 1 \times 10^0$	8×10^6
Succinic acid	3.0	9×10^0	1×10^7
Phosphoric acid	3.0	1×10^0	1×10^7
Sulfuric acid	3.0	3×10^3	1×10^7

[a] Acids were tested at a concentration of 10 mM. The pH of each assay was adjusted as indicated.

[b] The effect of acid was determined in a 60-s bactericidal assay [8]. Each assay was initiated by the addition of cells to the acidic solution. After 60 s, an aliquot of the assay was diluted in synthetic hard water containing TES buffer (pH 7.6) and then plated for enumeration of viable cells. Control counts were obtained by incubating cells in synthetic hard water.

acids, the viability of cells of *P. aeruginosa* can be reduced rapidly.

The effect of hydrogen peroxide and sodium meta-bisulfite on other Gram-negative eubacteria, Gram-positive eubacteria, and yeasts was determined (Table 3). A microbicidal effect of hydrogen peroxide and bisulfite in combination, with the concomitant reduction in pH, was observed against each of the Gram-negative eubacteria tested but not on the Gram-positive eubacteria or yeasts. The effect of lactic acid at pH 2.5 on *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. cerevisiae* was examined (Table 4). Again, a rapid microbicidal effect was observed against the Gram-negative eubacteria but not on the other microorganisms.

These results indicated that exposure to a pH of about 3.0 or lower had a rapid bactericidal effect on Gram-negative eubacteria but not Gram-positive eubacteria or yeasts. A pH having this effect could be attained by combining hydrogen peroxide and bisulfite. Inhibitory and/or bactericidal effects of acids on Gram-negative microorganisms are recognized [3,5,7] and the ability of Gram-positive eubacteria to survive exposure to low pH is also noted [6]. However, this is the first report of a bactericidal

TABLE 3

Effect of hydrogen peroxide (200 mg/l) and sodium bisulfite (1000 mg/l) on microorganisms

Microorganism	pH[a]	Viable cells/ml[b]	Control
Gram-negative eubacteria			
Acinetobacter calcoaceticus	3.5	8×10^3	3×10^5
Enterobacter aerogenes	3.2	3×10^2	1×10^7
Escherichia coli	3.2	3×10^5	2×10^7
Pseudomonas fluorescens	3.2	2×10^0	4×10^6
Gram-positive eubacteria			
Listeria monocytogenes	3.4	4×10^5	5×10^5
Staphylococcus aureus	3.2	1×10^4	1×10^4
Yeasts			
Candida albicans	3.2	2×10^5	6×10^5
Saccharomyces cerevisiae	3.2	6×10^5	2×10^5

[a] pH of the assay after all additions.

[b] The effect of hydrogen peroxide and sodium bisulfite (added as sodium meta-bisulfite) was determined in a 60-s bactericidal assay [8]. Cells were added to the bisulfite solution, followed by the addition of hydrogen peroxide to initiate the assay. After 60 s, an aliquot of the assay was diluted in synthetic hard water containing TES buffer (pH 7.6) and then plated for enumeration. Control counts were obtained by incubating cells in the absence of hydrogen peroxide and bisulfite.

TABLE 4

Effect of lactic acid on microorganisms

Microorganism	Viable cells/ml[a]	Control
Escherichia coli	5×10^2	4×10^6
Pseudomonas aeruginosa	$< 1 \times 10^0$	5×10^6
Staphylococcus aureus	2×10^5	3×10^5
Saccharomyces cerevisiae	1×10^5	2×10^5

[a] The effect of lactic acid, 10 mM at pH 2.5, on microorganisms was determined in a 60-s microbiocidal assay [8]. Each assay was initiated by the addition of cells to the lactic acid solution. After 60 s, an aliquot of the assay was diluted in synthetic hard water containing TES (pH 7.6) and then plated for enumeration of viable cells. Control counts were obtained by incubating cells in synthetic hard water.

effect of low pH against Gram-negative eubacteria in a 60-s assay. This effect may be useful for the control of Gram-negative eubacteria in circumstances where a brief exposure to an acidic pH is acceptable.

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