FOOD BIOLOGICAL CONTAMINANTS

Effects of Salt and Serum on the Sporicidal Activity of Liquid Disinfectants

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This study compares the effects of various concentrations of salt or serum in the killing of *Bacillus subtilis* spores by either glutaraldehyde, sodium hypochlorite, cupric ascorbate, hydrogen peroxide, peracetic acid, formaldehyde, or phenol. Salt affected only glutaraldehyde, its sporicidal activity increasing with an increase in concentration of sodium bicarbonate or sodium chloride. The sporicidal activity of glutaraldehyde was minimal when the concentrations of aldehyde groups and lysine residues from protein were similar. We present an equation describing the effect of serum on spore survival as a function of glutaraldehyde concentration that fits the data with a regression coefficient of 0.9. Cupric ascorbate and peracetic acid were inhibited by serum, but this effect was linked to a rise in pH. Sodium hypochlorite was the agent most sensitive to protein, with its sporicidal activity nearly disappearing in the presence of 2% serum or an equivalent amount of purified protein.

Variables that may influence the antimicrobial activity of disinfectants include concentration (in-use dilution), treatment time, pH, working temperature, storage, nature of surface to be decontaminated, presence of solvents or surfactants, organic challenge, and Mg(II) and Ca(II) ions in the water (1). The effects of several of these variables on microbicidal agents commonly used to decontaminate medical devices have been examined previously (2). The effects of organic matter or inorganic salt have been studied for some substances but the relative effect of these variables on different disinfecting agents remains to be established.

Although reuse protocols include cleaning before disinfection, reusing medical devices may involve treatment with liquid disinfecting or sterilizing agents in the presence of residual organic matter such as blood or serum (3). This organic material may rapidly react and inactivate certain germicides such as chlorine-based disinfectants, which form chloramines with proteins (4), or peracetic acid, which is partially inhibited by organic matter (likely by reacting with sulfhydryl groups and disulfide bonds in proteins; 5). By contrast, and in view of its reactivity with proteins (6–8), the observed high resistance of glutaraldehyde to neutralization by organic matter remains puzzling (9, 10).

Increase in salinity enhances the antimicrobial activity of some organic acids and phenol (1, 11), but the effect of sodium salts on other disinfecting agents remains unclear.

The present study compares the effects of serum, purified protein, and sodium salt on the sporicidal activity of agents commonly used in commercial disinfectants and sterilants.

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Experimental

Bacteria

Spores of *Bacillus subtilis* var. *globigii* (ATCC 9372; AMSCO American Sterilizer Co., Erie, PA) were tested and used as previously described (2). We stained spores with trypan blue and examined them microscopically to confirm spore morphology. No vegetative cells (rods) were observed. Survival of *B. subtilis* in hydrochloric acid (2.5N) and killing by autoclave treatment was as expected for spores (2).

Chemical Agents

The presence of contaminating heavy metal ions, particularly copper and iron, was avoided by using deionized water and heavy-metal-free reagents. Glutaraldehyde (50%, w/v; Sigma Chemical Company, St. Louis, MO, or Aldrich Chemical Company, Milwaukee, WI) was alkalinized with sodium bicarbonate, pH 9.3. Formaldehyde (37.2%, w/v), sodium hypochlorite (5%, w/v), peracetic acid (32%, w/v), ascorbic acid, and hydrogen peroxide (30%, w/v) were purchased from Aldrich. Phenol ("Ultra Pure" molecular biology grade, Life Technologies, Gaithersburg, MD) was received frozen and maintained at −20°C until use. Cupric chlo-

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ride ($CuCl \cdot 2H_2O$) was purchased from Mallinkrodt Chemicals (Paris, KY) and dissolved before each experiment, as previously described (12). Sodium chloride and bovine serum albumin (BSA, fraction V) were purchased from Sigma. Fetal bovine serum (FBS) was purchased from Gibco Laboratories (AJ, Life Technologies, Inc., Grand Island, NY).

Fixed-Time Assays

We added 50 μL spore suspension, containing ca 10^8 colony-forming units (cfu)/mL, without touching tube walls, to the bottom of a 1.5 Eppendorf conical plastic tube (PGC Scientific, Gaithersburg, MD) maintained at 20°C in a water bath. We then added 25 μL chemical agent and 25 μL serum at 4 times the final test concentration to the spore suspension and mixed the suspension with a Vortex mixer. The sample was incubated for 30 min at 20°C, before 0.9 mL ice-cold Luria-Bertani (LB) broth was added. A 1/10 dilution in ice-cold LB broth stopped the reaction of all but 2 disinfecting agents. After incubation with spores, sodium hypochlorite and peracetic acid were immediately washed away by centrifuging the reaction mixture 2 min at 15 000 rpm (Microfuge Model 5414, Brinkman Instruments, Inc., Westbury, NY), carefully aspirating the supernatant with the disinfectants, and replacing it with new LB broth (1 mL). After the spore pellet was resuspended by shaking with a Vortex mixer, the spores were serially diluted 1/10, and then 0.1 mL of each dilution was spread over 100 mm diamter LB broth Miller-Difco plates. Typically, between 90 and 120 bacterial colonies were present in 0.1 mL untreated control (exposed to water) when diluted 1/100 000. The assay allowed measurement of bacterial survival of as low as 0.00001% of untreated controls (7 log reduction).

Kinetic Assays

The effect of time on spore inactivation by different disinfectants in the presence of FBS was determined in 1.5 mL plastic tubes by adding 310 μL spores (10^8 cfu/mL). Disinfectant (155 μL) and serum (155 μL) were added at 4 times the concentration under study. Immediately thereafter, a 100 μL portion was withdrawn from the mixture. The actual contact time of spores with disinfectant in a minimal-contact-time sample was less than 2 min before dilution in ice-cold LB broth. At various preestablished times, 100 μL portions were withdrawn from each reaction tube. To stop any reaction, each portion was immediately diluted to 1 mL with ice-cold media and kept in ice until the last time point was obtained.

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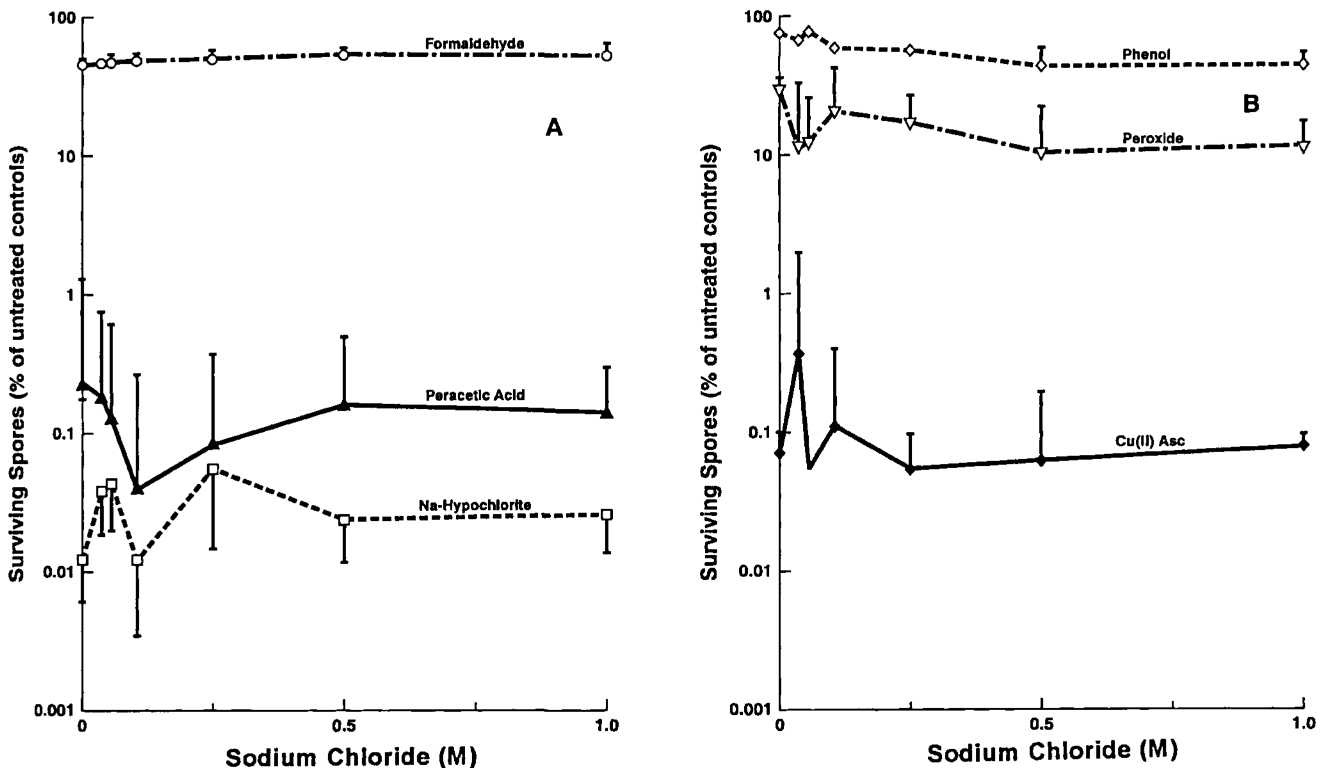

Figure 1. Effect of sodium chloride on sporicidal activity of liquid disinfectants. Spores were incubated with either (A) 8% formaldehyde, 0.03% peracetic acid, or 0.05% sodium hypochlorite or (B) 5% phenol, 10% hydrogen peroxide, 0.5% cupric ascorbate, 0.1% ascorbic acid or 0.003% hydrogen peroxide for 30 min at 20°C with 0–1.0M sodium chloride. Surviving spores were plated and counted. Mean survivals + standard error (SE) in 2–4 independent experiments are displayed.

Surviving spores were titrated as previously described. Colony-forming ability remained unaffected when spores were similarly treated with water.

Statistical Analysis

The relationship between serum inhibition and glutaraldehyde concentration was determined by a comprehensive curve-fitting software (CurveExpert 1.2 by D. Hyams, 1996). The slopes of linear curves were calculated with commercially available software (Excel, Microsoft Corporation, Bothell, WA), and differences among slopes were assessed by linear regression analysis (15). D-values, or the time required to reduce spore survival by one order of magnitude, were obtained graphically from initial slopes of log-survival versus time curves. Spore half life ($t_{1/2}$) was calculated from the relation $t_{1/2} = \ln 2/k$, where k is the reaction rate calculated by multivariate linear regression with best fit to N-order models (2).

Results

Effect of Salt

Spores were exposed to disinfectants in the presence of increasing concentrations of sodium chloride. Sporicidal activities of peracetic acid, sodium hypochlorite, hydrogen peroxide, and cupric ascorbate, as well as the

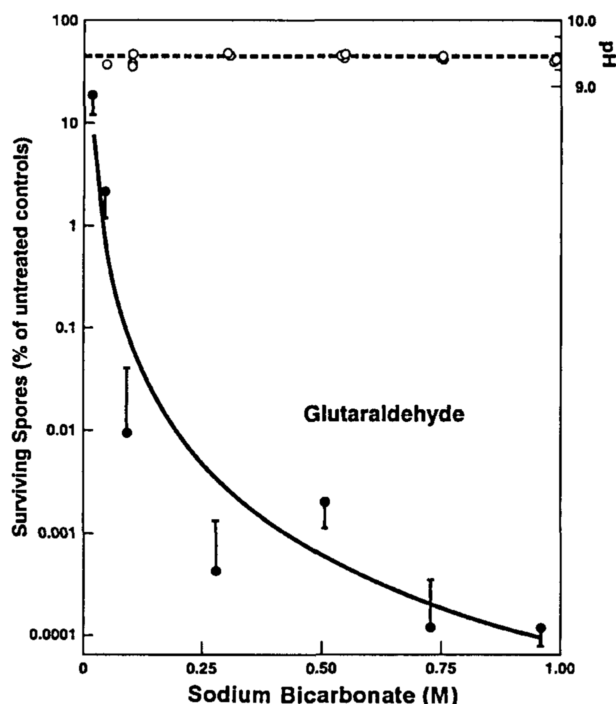

Figure 2. Effect of sodium bicarbonate on sporicidal activity of glutaraldehyde. Spore survival (solid line) is shown after incubation for 30 min at 20°C with 2% glutaraldehyde in the presence of 0–1M sodium bicarbonate. Solid circles represent mean survival ±SE in 5 independent experiments. Open circles represent pH at each treatment.

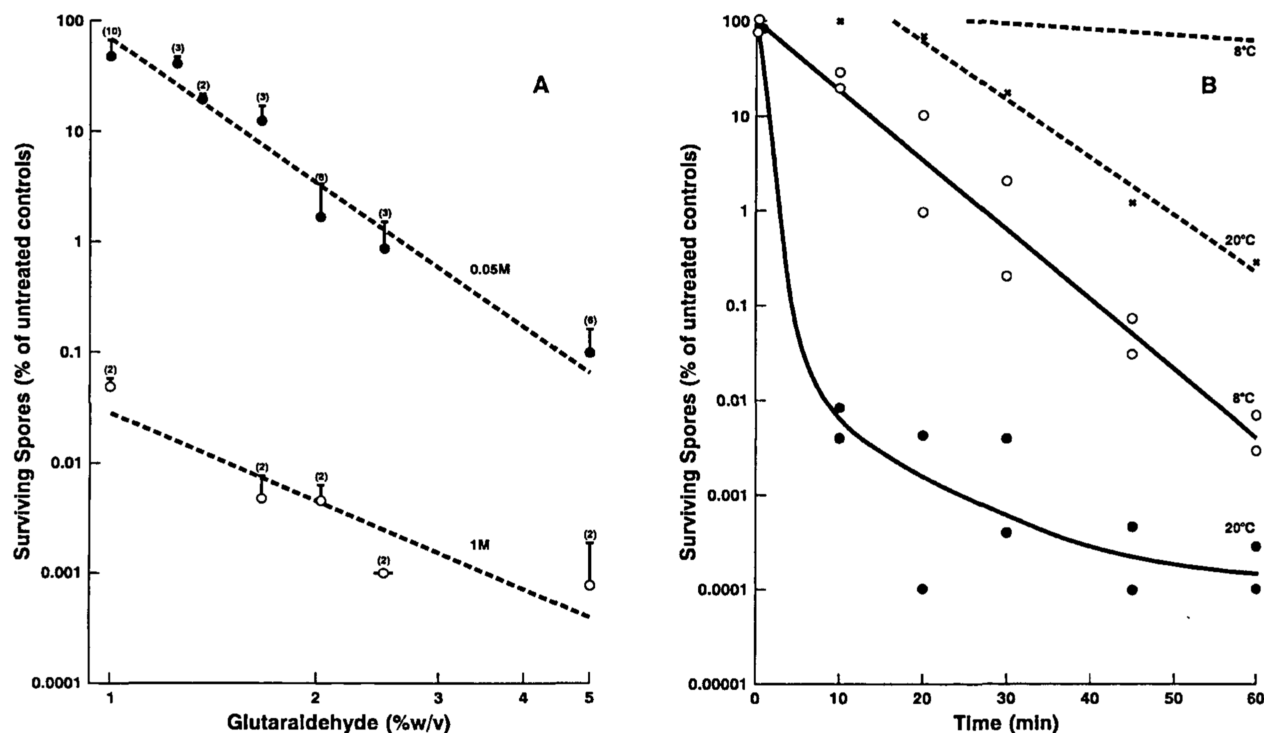

Figure 3. (A) Survival of spores incubated with 1–5% (w/v) glutaraldehyde at high (1.0M sodium bicarbonate; open circles) or low (0.05M sodium bicarbonate, solid circles) salt concentrations. (B) Effect of low (dashed line) and high (solid line) salt concentrations on the sporicidal kinetics at 8° or 20°C. Average relative survivals ±SE in the number of independent experiments indicated in brackets are displayed in A; independent results are displayed in B.

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low sporicidal activities of phenol and formaldehyde, were not affected by variations in salt ranging from 0 to 1M (Figure 1).

Addition of 0.7M sodium chloride to 2% glutaraldehyde in 0.02M sodium bicarbonate, pH 9.3, increased spore killing by 100-fold (data not shown). Addition of sodium bicarbonate (0.7M, pH 9.3) was 1000-fold more effective in increasing sporicidal killing than did addition of sodium chloride (in 3 independent experiments). Figure 2 shows that the logarithm of spore survival in glutaraldehyde (maintained at a constant pH) decreased with the third power of sodium bicarbonate concentration (regression coefficient, $r = 0.94$).

A decrease in sodium bicarbonate concentration between 1.0M and 0.05M resulted in more than 100-fold increase in the number of spores surviving treatment at 5% glutaraldehyde and over 1000-fold at 1% glutaraldehyde (Figure 3A). Similar changes in salt concentration altered sporicidal kinetics as shown in Figure 3B. Approximate D-values for glutaraldehyde were 1.7 and 14 min in high salt at 20° and 8°C, respectively, and 33 min in low salt at 20°C. The half-life of spores ($t_{1/2}$), calculated as previously described (2), decreased from 22 min in low salt (0.05M) to 7.3 min in high salt (1.0M) at 20°C, and from 63 min in low salt to 9.5 min in high salt at 8°C.

Effect of Serum

Adding serum (pH 7.1) at a final concentration of 25% reduced the pH of glutaraldehyde in low concentration buffer (0.05M sodium bicarbonate) from pH 9.2 to 7.8. The limited buffer capacity of 0.05M sodium bicarbonate and the relatively low sporicidal activity of glutaraldehyde at low ionic concentration precluded precise measurement of serum effect in these conditions.

To isolate the contribution of serum, glutaraldehyde (2%, w/v) was tested at a constant pH and relatively high (1M) salt concentration. Serum (25%, v/v) had only a modest effect on glutaraldehyde (2%, w/v) in 1M sodium bicarbonate (Figure 4).

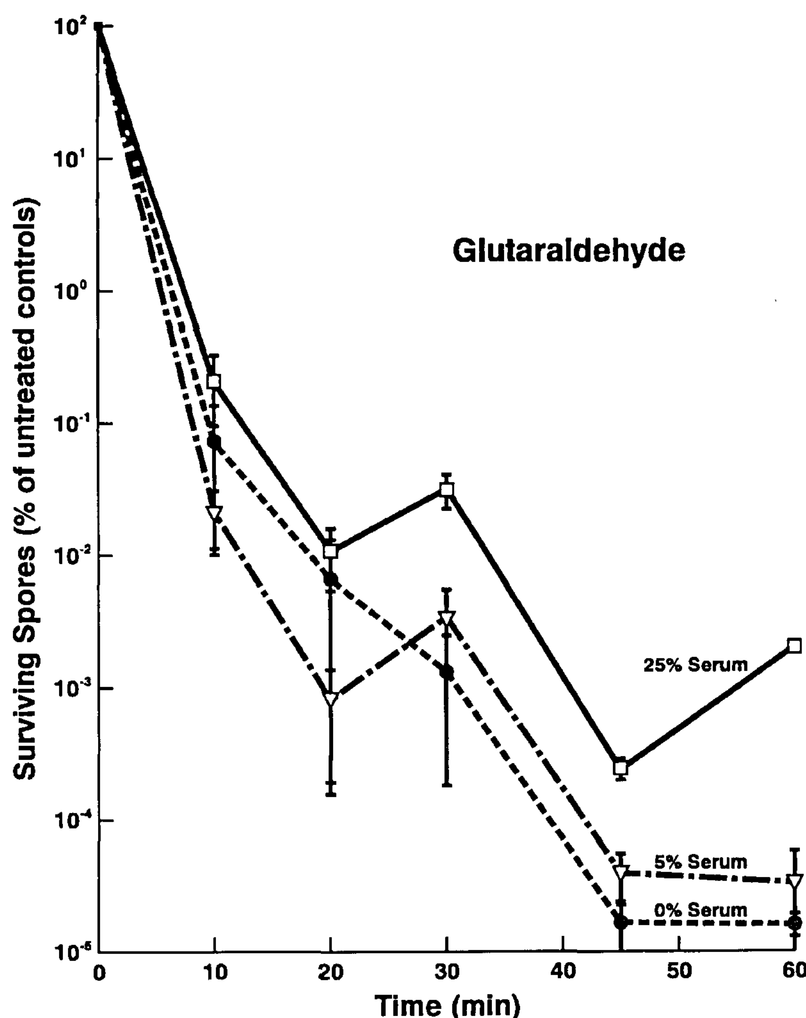

Figure 4. Effect of serum on sporicidal activity of glutaraldehyde. Spores were treated at pH 9.4 with 2% glutaraldehyde in 1M sodium bicarbonate in the presence of 0% (circles), 5% (triangles), or 25% (squares) serum. Average relative survivals ±SE in 3–5 independent experiments are displayed.

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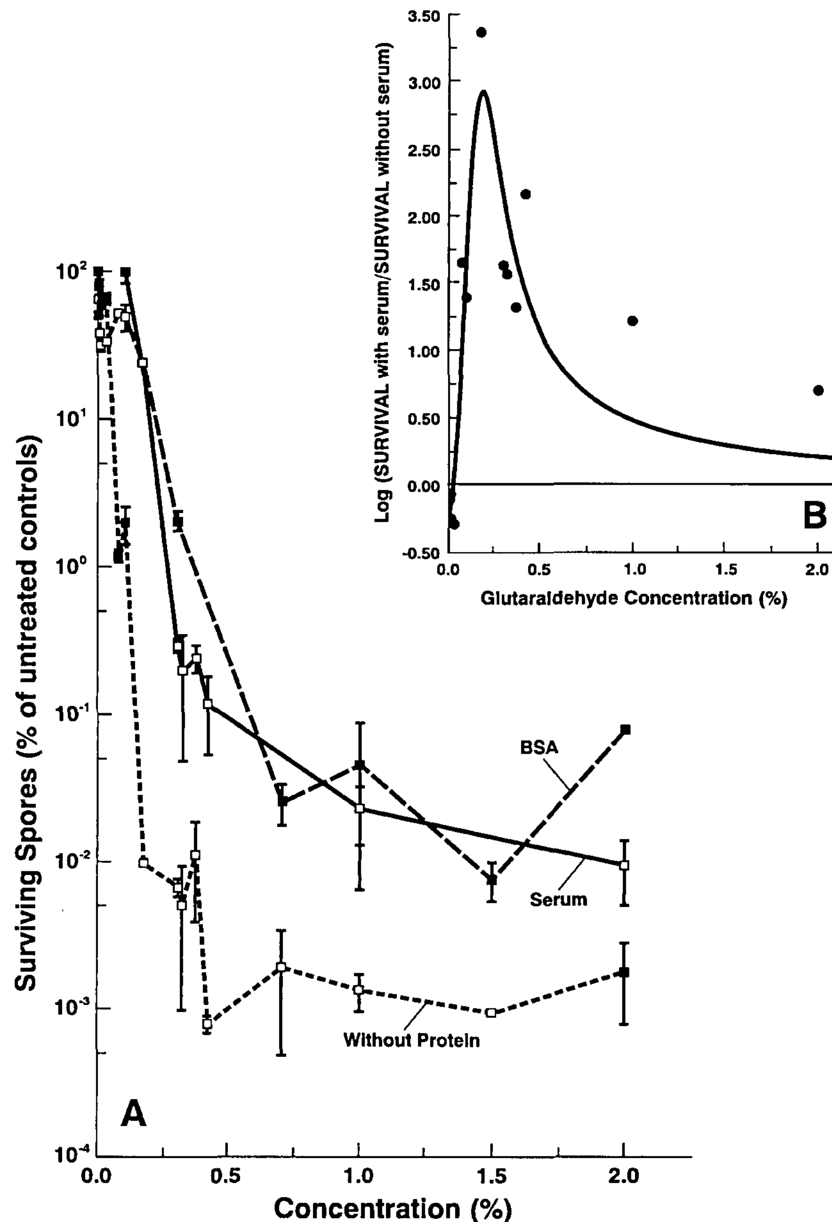

Figure 5. Inhibition of sporicidal activity of glutaraldehyde by 25% serum or 1.75 g bovine serum albumin (BSA)/100 mL. (A) Average spore survivals ±SE in 2–5 independent experiments at different concentrations of glutaraldehyde. (B) Log of spore survival in presence of serum divided by survival in absence of serum as a function of glutaraldehyde concentration.

To determine whether some serum component was masking the expected reaction of glutaraldehyde with serum proteins, additional experiments were done with purified protein. A similar effect was observed on sporicidal activity of 2% glutaraldehyde when protein in 25% serum was replaced by an equivalent amount (1.75 g/100 mL) of purified serum albumin (Figure 5A).

Inhibition of sporicidal activity by serum became noticeable at glutaraldehyde concentrations below 1%. At a glutaraldehyde concentration near 0.2%, spore survival was 1000-fold higher in the presence than in the absence of serum (Figure 5B). The relationship between serum inhibition and glutaraldehyde concentration can be expressed by the best-fit function $y = (a + bX)/(1 + cX + dX^2)$ where y = log spore survival in serum divided spore survival in absence of serum; a = −0.364, b = 15.13, c = −7.70, and d = 36.85 are constants; and X is the concentration (%, w/v) of glutaraldehyde. The data fit with a regression coefficient $r = 0.8978$, and convergence was reached after 14 iterations.

Spores were incubated with sodium hypochlorite, cupric ascorbate, peracetic acid, or hydrogen peroxide

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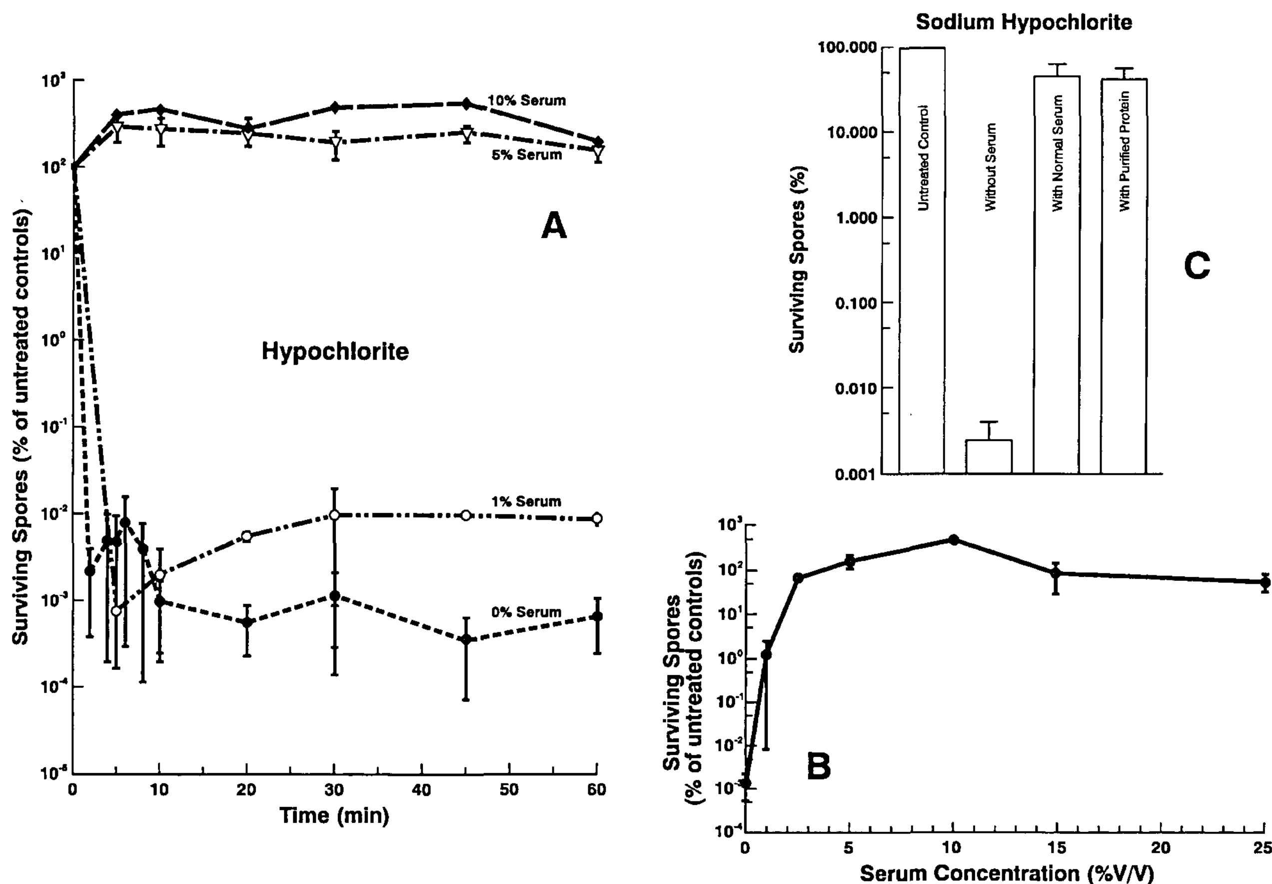

Figure 6. Elimination of the sporicidal activity of sodium hypochlorite by serum. Spores were exposed 0–60 min to 0.05% sodium hypochlorite, pH 7.0 in the presence of 0% (solid circles), 1% (open circles), 5% (triangles), or 10% (diamonds) serum. (A) Average survivals ±SE in 2–5 independent experiments. (B) Inhibition of sporicidal activity as a function of serum concentration (*n* = 5–7, values are means ±SE). (C) Similar inhibition of sporicidal activity by 25% serum and 1.75 g purified BSA/100 mL (height of bars correspond to means of 4 independent experiments; SE is indicated above bars).

in the presence of variable concentrations of serum. Results are shown in Figures 6–9.

Sodium hypochlorite at neutral pH was very sensitive to serum (Figure 6), and this inhibition was mimicked by a similar amount of purified protein. The low sporicidal activity of hydrogen peroxide apparently was not affected by serum (Figure 7).

Twenty-five percent serum raised the pH of cupric ascorbate to nearly 4 and reduced sporicidal activity by about 1000-fold after 30 min (Figure 8). Serum or BSA were not inhibitory when acidified so that the final pH of the sporicidal mixture remained near 2 (Figure 8, inset). Similarly, 25% serum at neutral pH raised the pH of the reaction mixture to 5 and increased spore survival in peracetic acid by nearly 1000-fold after 30 min incubation (Figure 9). Serum acidified below pH 2 maintained the sporicidal activity of peracetic acid (Figure 9, inset).

Discussion

Among the disinfectants tested, sodium hypochlorite was most sensitive to serum (2% serum completely abolishing sporicidal activity). Because the optimal pH for sporicidal activity of sodium hypochlorite is similar to that of serum (near neutral), the inhibitory effect of normal serum is not related to pH but likely due to a reaction and neutralization of hypochlorite with serum proteins. Our results demonstrate that inhibition by serum is mediated by proteins, because a similar loss of sporicidal activity was observed in the presence of purified BSA, pH 7. Our sporicidal data agrees with previous observations indicating that organic material rapidly forms chloramines in chlorine solutions, consuming available chlorine, and reducing its bactericidal activity (4).

The curves of log-survival as a function of time were linear within 60 min for glutaraldehyde at 8°C in high or low salt and in low salt at 20°C (Figure 3) and within 30 min at 20°C for hydrogen peroxide, cupric ascorbate, and peracetic acid (Figures 7–9). The most notable departure from linearity was the sporicidal kinetics of hypochlorite (Figure 6). We investigated whether this response was due to a heterogeneous population of spores that included some that were highly resistant to treatment. Spores were exposed to hypochlorite for

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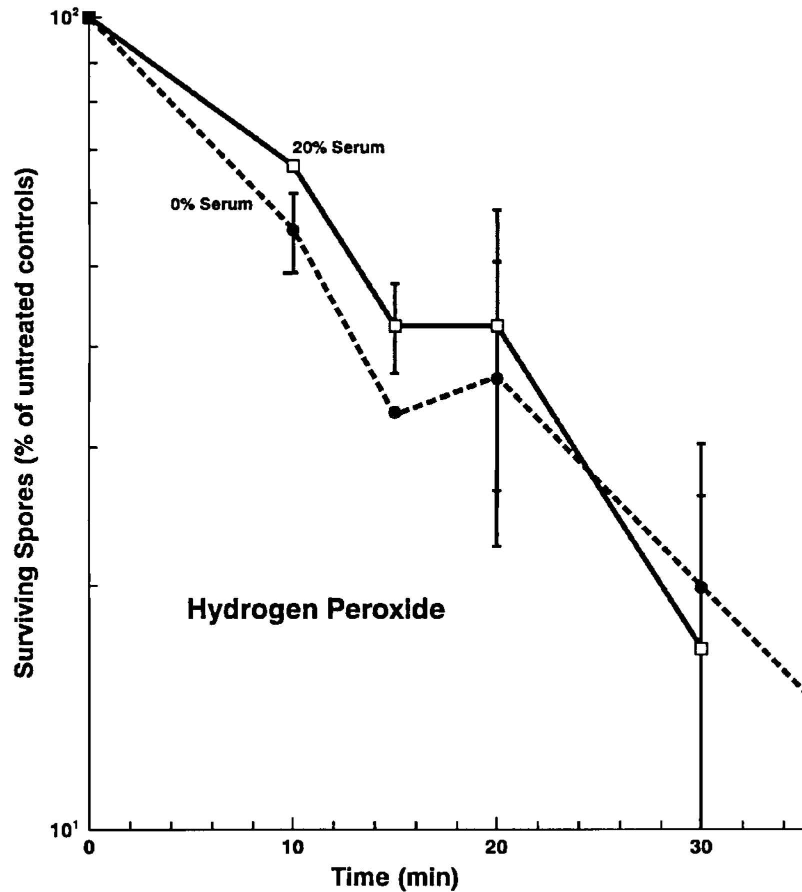

Figure 7. Undetectable effect of serum on low sporicidal activity of hydrogen peroxide. Spores were treated with 10% hydrogen peroxide for 30 min at 20°C in the absence (solid circles) or (open squares) presence of 25% serum (average ±SE, *n* = 3–5).

30 min at 20°C, diluted in broth, and centrifuged. The hypochlorite in the supernatant discarded. The surviving spores were resuspended in water and treated for a second time with new hypochlorite under identical conditions. The spore suspension was similarly sensitive to both hypochlorite treatments (data not shown), indicating that our spores did not include a highly resistant subpopulation and suggesting that the nonlinear curve may be related to consumption of disinfectant.

Peracetic acid was inhibited by serum. Our results appear to disagree with reports indicating that peracetic acid remains effective in the presence of organic matter (5). This discrepancy could be explained by our findings suggesting that the inhibitory effect of serum on the sporicidal activity of peracetic acid relates to a rise in pH. This view is supported further by data in Figure 9 indicating that acidified serum or BSA does not inhibit sporicidal activity.

Cupric ascorbate was inhibited by serum in a manner similar to that observed for peracetic acid and only when pH was elevated. Cupric ascorbate retained sporicidal activity in the presence of serum or purified protein at acidic pH. This finding agrees with a report (13) indicating that the virucidal activity of cupric ascorbate is not inhibited by BSA.

Chemical studies have demonstrated that the high reactivity of glutaraldehyde with proteins is due to reaction with free amino groups, particularly in the side chain of lysine. Subsequent condensation of glutaraldehyde leads to rapid formation of 1,3,4,5-substituted pyridinium salt analogues (6–8). Hence, large amounts of proteins like those present in serum could neutralize reactive groups in glutaraldehyde and should reduce its microbicidal activity.

Surprisingly, our experiments and several previous reports indicate a high resistance of glutaraldehyde to neutralization by organic matter, such as 20% blood serum or 1% whole blood (9, 10). Our results also agree with previous observations indicating that the inhibitory effect of organic matter is more important at lower concentrations of glutaraldehyde (14).

About 10% of amino acids in serum proteins are lysine (15). The proportion of lysine is also near 10% in BSA (57 lysine amino acids of 583 amino acids in a molecule of molecular weight 68 000; 16). The concentration of lysine in our experiments (25% serum or 1.75 g/100 mL BSA, with 57 mol of lysine per mol of BSA) was approximately 15 mM.

A solution of 1% glutaraldehyde is 100 mM (at 2 groups per molecule, the concentration of individual

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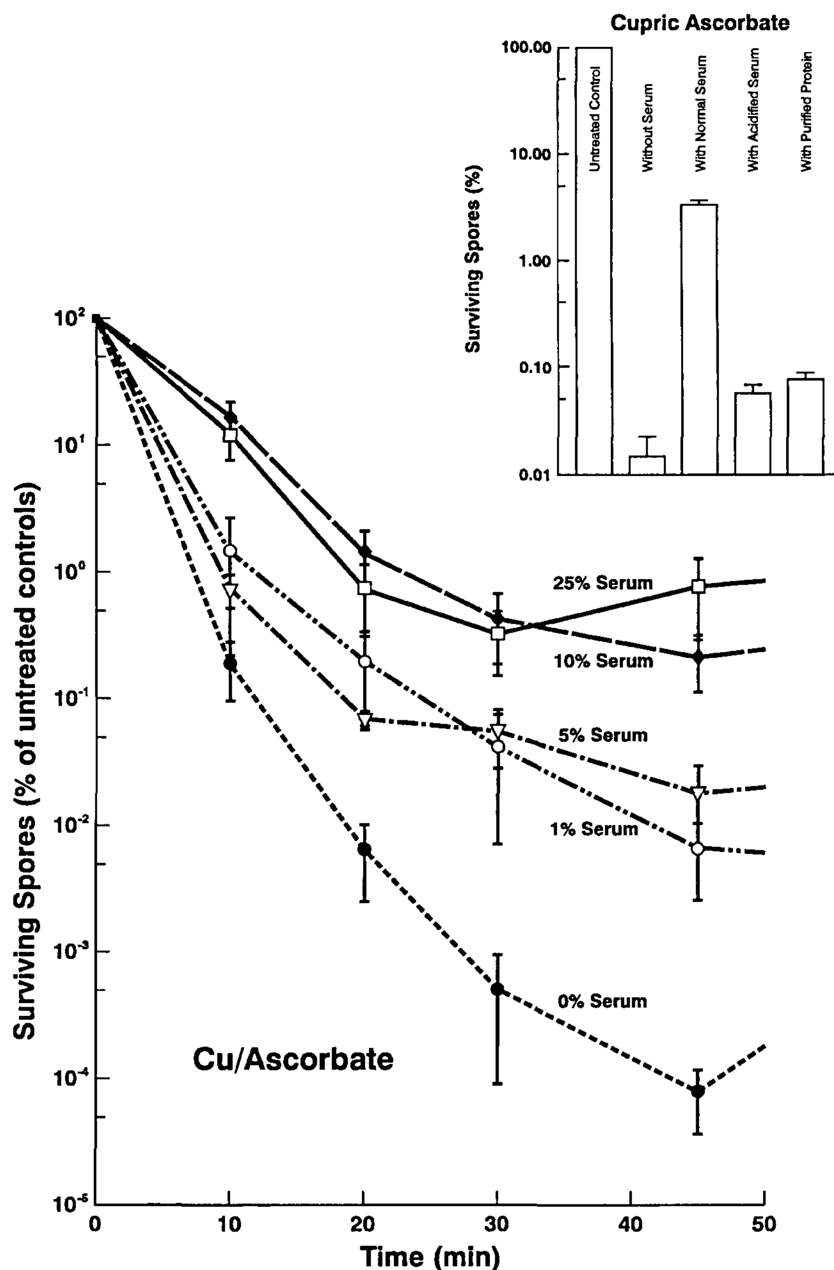

Figure 8. Kinetics of spore inactivation by copper ascorbate in the presence of serum. Spores were treated at 20°C with 0.5% cupric ions, 0.1% ascorbic acid, and 0.003% hydrogen peroxide for 0–50 min in the presence of 0, 1, 5, 10, or 25% final serum concentration (average ±SE, n = 3–5). (Inset) Spores were exposed to cupric ascorbate in acidified 25% serum or in 1.75 mg BSA/100 mL, both at final pH 1.8, or in normal 25% serum, which raised the final pH to 3.6–4.0 (averages of 6 experiments + SE are displayed).

aldehyde groups is then 200 mM). Accordingly, at concentrations of glutaraldehyde below 0.1%, there is an excess of lysine residues, whereas at concentrations above 1%, aldehyde groups are in excess. Thus, at higher glutaraldehyde concentrations (above 1%), excess aldehyde groups maintain high sporicidal activity, and the inhibitory effect of serum is negligible. At glutaraldehyde concentrations between 0.1 and 0.3%, lysine and aldehyde residues are present in similar amounts. Thus, glutaraldehyde activity is neutralized, leading to higher spore survival. The loss of sporicidal activity could be due to formation of a stable pyridinium salt or derivative between glutaraldehyde and proteins (7). The equation relating spore survival, presence of serum, and concentration of glutaraldehyde fit data with a correlation coefficient near 0.9.

Our results show that sodium chloride residues that could be left after washing and drying some medical devices should not affect decontamination by sodium hypochlorite, cupric ascorbate, hydrogen peroxide, or peracetic acid. None of these agents was inhibited at high or low sodium chloride concentration. By contrast,

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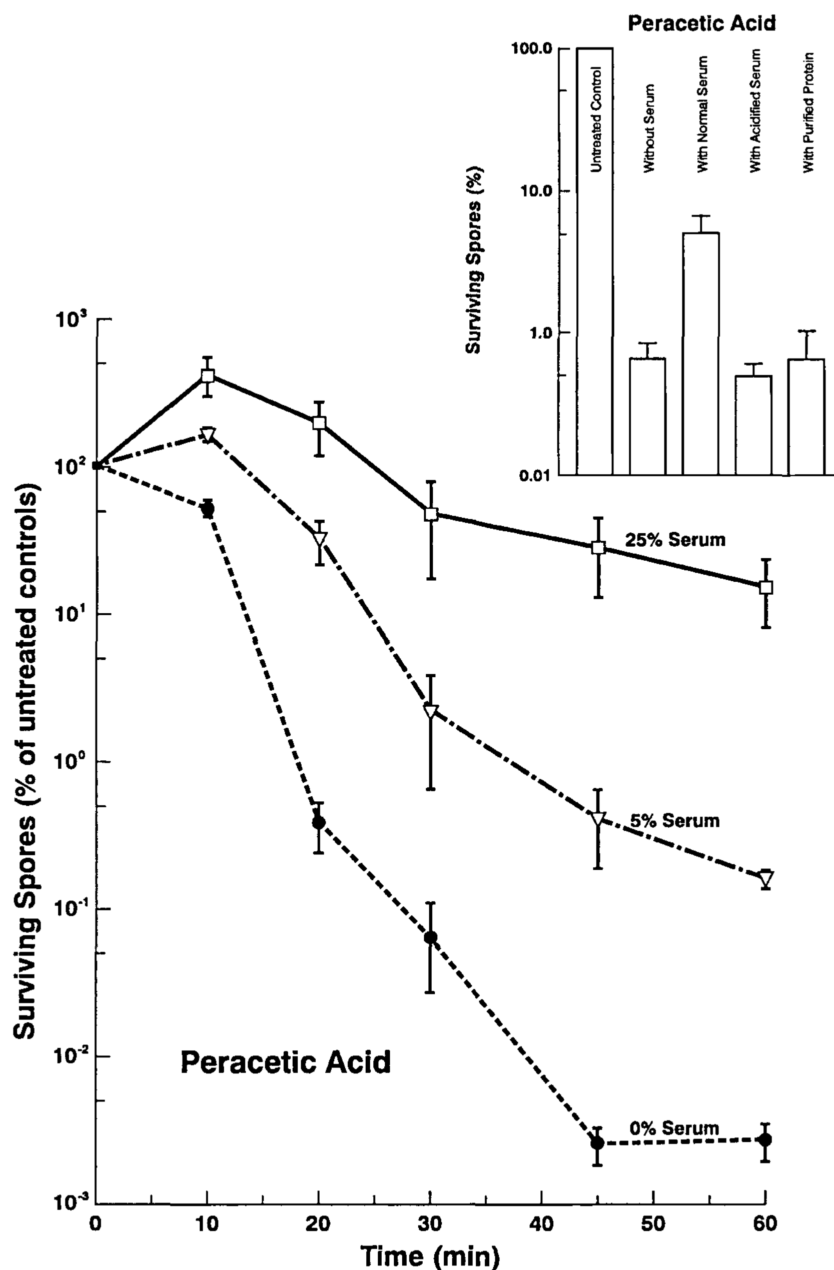

Figure 9. Kinetics of spore inactivation by peracetic acid in the presence of serum. Spores were exposed to 0.03% peracetic acid at 20°C in the presence of 0, 5, or 25% final concentration of serum for 0–60 min. Surviving spores (averages ±SE in 3–5 experiments) are shown in the ordinate. (Inset) Spores were treated with peracetic acid for 30 min in the absence of serum or in either 25% normal serum (which brought the incubation mixture to pH 5), 25% acidified serum, or 1.75 mg BSA/100 mL. The last 2 reactions were acidified to maintain the final pH of the reaction mixture near pH 2. Heights of bars represent the means (+ SE) in 4 independent experiments.

substantial amounts of sodium bicarbonate or sodium chloride must be present for glutaraldehyde to show considerable sporicidal activity.

In this study, we selected spores of *B. subtilis* var. *niger* because this organism is used in the AOAC sporicidal method used to test efficacy of liquid sterilants and because the high resistance of *B. subtilis* spores made our measurement of alterations in disinfectant activity more precise. Although *B. subtilis* spores were the target in our study, the chemical interaction between disinfectant and salt or serum should be independent of the organism studied.

Although some fragmented data on the effect of serum or salt have been previously available for some disinfectants, the present study provides a direct comparison of these 2 variables on a panel of chemical agents. Our results demonstrate that the absence of sodium salts or the presence of protein can substantially alter the sporicidal activities of some biocidal agents, perhaps leading to failure of the disinfection.

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Our findings further characterize variables affecting sporicidal activity and should help predict sporicidal kill under a broader range of conditions. Better understanding and control of these variables should improve the reproducibility of sporicidal tests. The results also could assist in selection of disinfectants and sterilants for particular applications.

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