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pH dependence of the in situ formation of an organic *N*-chloramine water disinfectant

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SUMMARY

Staphylococcus aureus was used to assess the bactericidal efficacy of aqueous solutions of the organic *N*-chloramine compound 3-chloro-4,4-dimethyl-2-oxazolidinone (agent I) formed in situ. The rate of in situ formation, accomplished by reacting free chlorine with the amine precursor, was a function of pH. When the reagents were combined under acidic conditions (pH $\leq$ 5.5) and allowed to react for 22 h, sufficient residual free chlorine was present to inactivate the bacteria in less than 5 min. When combined under less acidic conditions (pH $\geq$ 6.0), comparable bacterial inactivation required 30–60 min due to the extensive reaction of the free chlorine to form agent I. The kill rates present under less acidic and neutral conditions are equivalent to those for pre-formed agent I. In water disinfection applications for pH $\geqslant$ 6.0, in situ formation of agent I would provide a combination of rapid initial and slower long-term disinfection.

INTRODUCTION

The potential for using organic *N*-chloramine 3-chloro-4,4-dimethyl-2-oxazolidinone (referred to as agent I) as a water disinfectant has been discussed in several recent publications [2,3,6,10–16]. This compound was first prepared and found to be bactericidal by research groups working with Kaminski [5] and Kosugi [7]. Agent I has several properties that are desirable in a disinfectant, including effectiveness against species of *Enterobacter*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella* and *Sphaerotilus* [10,13,14,16], against species of *Giardia* and *Entamoeba* [16], and against polio virus type 1 [16]. Agent I is stable in neutral and acidic aqueous solutions and in its crystalline form [3,11,14,15]. This stability is a result of the dimethyl configuration at the 4 position in the oxazolidinone ring. The negative charge which develops as the Cl^+ ions are released is destabilized by the methyl groups. Also, the presence of the two methyl groups precludes the possibility of a dehydrohalogenation reaction or facile access to the N-Cl group by solvent molecules. The result is a stabilized N-Cl bond. The compound is thus 'slow-releasing' and stable for extended periods of time. When compared with disinfectants such as calcium

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hypochlorite, agent I is much less reactive with organic impurities, resulting in decreased trihalomethane formation [2,9,12]. Agent I is not toxic to chickens when used in drinking water [8]. Summaries of the properties [13] and possible mechanisms of the disinfection action [6] of agent I have been presented. These studies have all utilized agent I synthesized and purified in the laboratory.

A more recent investigation utilizing agent I formed in situ has indicated the potential for omitting the final step in the synthesis of the compound [4]. In situ chlorination of the 4,4-dimethyl-2-oxazolidinone precursor with calcium hypochlorite was found to be influenced by pH, with the acidic condition tested (pH 4.5) having the most effect. The stability of pre-formed agent I is enhanced under acidic and neutral conditions [11], but the reaction of precursor with free chloride proceeds best under alkaline conditions.

Based on this information, the current research compared pre-formed agent I with agent I formed in situ over a pH range of 4.5–7.0. The basis of comparison was challenges with *Staphylococcus aureus*, chosen for its resistance to disinfectants [10]. The objective was to determine what pH is necessary to produce agent I in situ efficiently.

MATERIALS AND METHODS

Agent I was synthesized and purified using the methods of Kosugi et al. [7] as modified by Williams et al. [10]. The in situ preparation was accomplished by combining calcium hypochlorite (HTH; Olin Chemicals Corp., New Haven, CT; 65% active chlorine, 35% inert ingredients) with the nonchlorinated precursor. Using reagent grade salts, acetate and phosphate buffer solutions were prepared with distilled, deionized water. To remove chlorine demand, sodium hypochlorite (Clorox; the Clorox Co., Oakland, CA) was added to form 2–3 mg/l Cl^+ solutions. The solutions were allowed to stand for 24 h. They were then dechlorinated by exposure to sunlight for 18–24 h. Chlorine concentrations were monitored using an *N,N*-dimethyl-*p*-phenylenediamine determination (DPD; Hellige, Garden City, NY) [1]. Appropriate amounts of the dechlorinated acetate and phosphate solutions were combined to form 0.05 M buffers of pH 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0. The buffers were sterilized by autoclaving for 15 min at 15 psi and 121°C. The pH of the buffers was monitored before and after sterilization using an Orion Model 601A pH meter with a glass combination electrode (Orion Research Inc., Cambridge, MA).

Glassware used in the experiments was rendered chlorine demand-free by a 24-h soak in a 3–5 mg/l Clorox solution, followed by rinses with distilled, deionized, and demand-free waters, and drying in direct sunlight. All glassware was sterilized by autoclaving at 15 psi and 121°C for 15 min.

S. aureus (ATCC No. 25923) was maintained, as recommended by the American Type Culture Collection, on tryptic soy agar plates (Difco Laboratories, Detroit, MI). Bacteria for inocula were obtained from tryptic soy agar plates which had been incubated for 24 h at 37°C and were suspended in sterile 0.85% (w/v) saline. Calibration of the suspensions to approximately 1.0×10^8 cfu/ml was accomplished using a Klett-Summerson colorimeter (Klett Manufacturing Co., New York, NY) with a green filter.

Every experiment included duplicate in situ flasks, a control flask, and a flask containing the pre-formed compound. In each experiment 50 ml of buffer at the selected pH was transferred to a sterile 125-ml Erlenmeyer flask, covered with a gauze/cotton plug, and placed in a shaking water bath at 22°C operating at 160 orbits/min. An appropriate volume was removed and replaced with either calcium hypochlorite/nonchlorinated precursor or with pre-formed agent I to obtain 5 mg/l total chlorine concentration. After a 15-min equilibration period, 0.5 ml of the bacterial suspension was added to each experimental flask and to the control flask. Sample collection was initiated concurrently with the addition of the bacteria. All transfers were made using Gilson digital pipettes (Ranin Instrument Co., Woburn, MA). Each 1.0-ml sample was transferred to a tube containing 1.0 ml of 0.02 N thiosulfate (buffered to pH 7.0) to quench the disinfectant. Samples were collected

after 15 s and 5 min and the flasks were then continuously agitated in the water bath for 60 min.

A second challenge was initiated by the addition of 0.5 ml of the bacterial suspension to each of the experimental flasks. Control flasks were not inoculated during the second and third challenges because no bactericidal activity was observed during the first challenge. Samples were collected after 15 s, and 5 and 10 min. Upon completion of the second challenge the flasks were left undisturbed in the water bath overnight. For a third challenge, 0.4 ml of the bacterial suspension was added to the experimental flasks. Samples were collected after 15 s, and 5, 10, 30 and 60 min. Residual chlorine concentrations were determined using the DPD method after the completion of the third challenge [1].

Using freshly prepared saline dilutions, spot plates were made using three 25-μl samples per dilution. The tryptic soy agar plates, incubated at 37°C, were counted after 24 and 48 h to allow for colony formation by weakened cells. Reported counts were based on the average of the three 25-μl samples. Each colony counted represents 80 cfu/ml in the original reaction flask.

Test flasks were also established using the equivalent concentration of the amine precursor in the different buffers. These flasks were treated with the same protocol as listed above.

RESULTS AND DISCUSSION

During the first challenge, calcium hypochlorite was allowed to react with the precursor for 15 min prior to the addition of bacteria. For the second challenge, a period of 1 h had elapsed between the addition of the calcium hypchlorite to the precursor and the addition of the bacteria. In the in situ flasks 6-log decreases in viable cells were achieved in less than 15 s. The longest time period required for a 6-log decrease was less than 5 min. Based on the rate of formation of agent I [3], the rapid in situ kill rates are dependent on the presence of free chlorine. The slower kill rates (15 s–5 min) were for solutions at pH 7. As indicated in an earlier study of in situ formation [4], the rate of formation of agent I is more rapid at pH 7 than at pH 4.5 or pH 9.5, so less free chlorine is available to interact with the cells. Since in situ kill rates in the first and second challenges are the result of residual free chlorine, which has been discussed in numerous other papers, this phenomenon will not be addressed. In the flasks of pre-formed agent I, times required for 6-log decreases in viable cells were consistently longer than the 5- and 10-min sampling times used in these challenges.

By the initiation of the third challenge the calcium hypochlorite had reacted with the precursor for 22 h. As shown in Table 1 and Fig. 1, more variation in kill rates was present for the third challenge. In flasks containing pre-formed agent I, 6-log decreases in viable cells were achieved in less than 60 min. These values are consistent with earlier studies using agent I [16] and with the predicted times (Table 1). For the in situ flasks pH becomes an important consideration. Under more acidic conditions (pH 4.5 and 5.0), the rapid kill rates were still evident. At pH 5.5 the kill rate was comparable to the rate for in situ pH 7.0 flasks in the second challenge. At pH 6.0, 6.5, or 7.0 the in situ kill rates were consistent with the rates for pre-formed agent I.

Free chlorine concentrations were determined at the end of the third challenge. For in situ flasks at pH 4.5 or 5.0 30% of the initial chlorine was present in the free form. For in situ flasks at pH 5.5, 15% of the initial chlorine was still in the free form. Where kill rates were consistent with pre-formed agent I (in situ pH 6.0, 6.5, and 7.0) and in all of the flasks of pre-formed agent I, any free chlorine present was below detectable concentrations. The consistency of the results with those found with pre-formed agent I and the decrease in free chlorine indicate agent I formation.

Samples were collected and processed for control flasks during each challenge. With bacteria added to the control flasks only at the initiation of the first challenge, counts made following the third challenge represent approximately 24-h exposure to the buffers. Comparisons of first challenge counts with third challenge counts indicated less than

Table 1

Action of agent I against the third challenge (22 h) with *S. aureus*

Samples were collected after 15 s, and 5, 10, 30 and 60 min.

pH	Flask[a]	Log 10[b] t_0	Time required for 6-log decrease in viable cells (min)	Predicted time for 6-log decrease in viable cells (min), $r^2 > 0.9$[c]
4.5	I	0.00	0.25	0.25 (–)
	P	6.19	30–60	56.23(±3.41)
5.0	I	0.00	0.25	0.25 (–)
	P	5.98	30–60	57.44(±3.39)
5.5	I	5.89	0.25–5	5.10(±0.02)
	P	5.93	30–60	57.20(±3.32)
6.0	I	6.13	30–60	57.48(±3.40)
	P	5.90	30–60	57.12(±3.41)
6.5	I	6.21	30–60	57.44(±4.53)
	P	6.09	30–60	58.30(±7.71)
7.0	I	6.25	30–60	60.63(±6.12)
	P	6.38	30–60	56.07(±7.23)

[a] I indicates flasks containing in situ agent I at 5 mg total chlorine per liter; P indicates flasks containing preformed agent I at 5 mg total chlorine per liter.

[b] Timing was initiated at the introduction of cells into the flasks. The cells were added, and a t_0 aliquot was removed within 15 s of the addition.

[c] Predicted time for a 6-log decrease in viable cells (± S.E.M.) was calculated by using the regression relationships generated by the SAS Proc GLM where the specified model was log 10 (cfu/ml) = contact time (min).

0.5-log decreases in viable cells. Samples were also collected from test flasks containing only the precursor compound. In the time course of the experiments the precursor did not show any antimicrobial activity, nor did it act as a substrate for growth. Since the buffers and precursor did not exhibit antimicrobial activity, these data are not presented.

It is clear from this study that rapid, appreciable agent I formation in situ requires a pH > 5.5. Thus, to use in situ-formed agent I to the greatest advantage, i.e., as a combination rapid (from unreacted free chlorine) and long-term (from formed agent I) disinfectant, the water should not have a pH value lower than approx. 6.0. This should be the case for many water disinfection applications such as swimming pools, hot tubs, cooling towers, air conditioning systems, and even for potable water supplies.

CONCLUSIONS

This study has compared the use of the organic *N*-chloramine 3-chloro-4,4-dimethyl-2-oxazolidinone, agent I, formed in situ with the preformed compound as a disinfectant against *S. aureus*. With in situ formation accomplished by combining the precursor with calcium hypochlorite, free chlorine is present in the intitial challenges and results in rapid disinfection. As the reaction progresses with the formation of agent I, free chlorine-dependent kill rates are succeeded by long-term disinfection

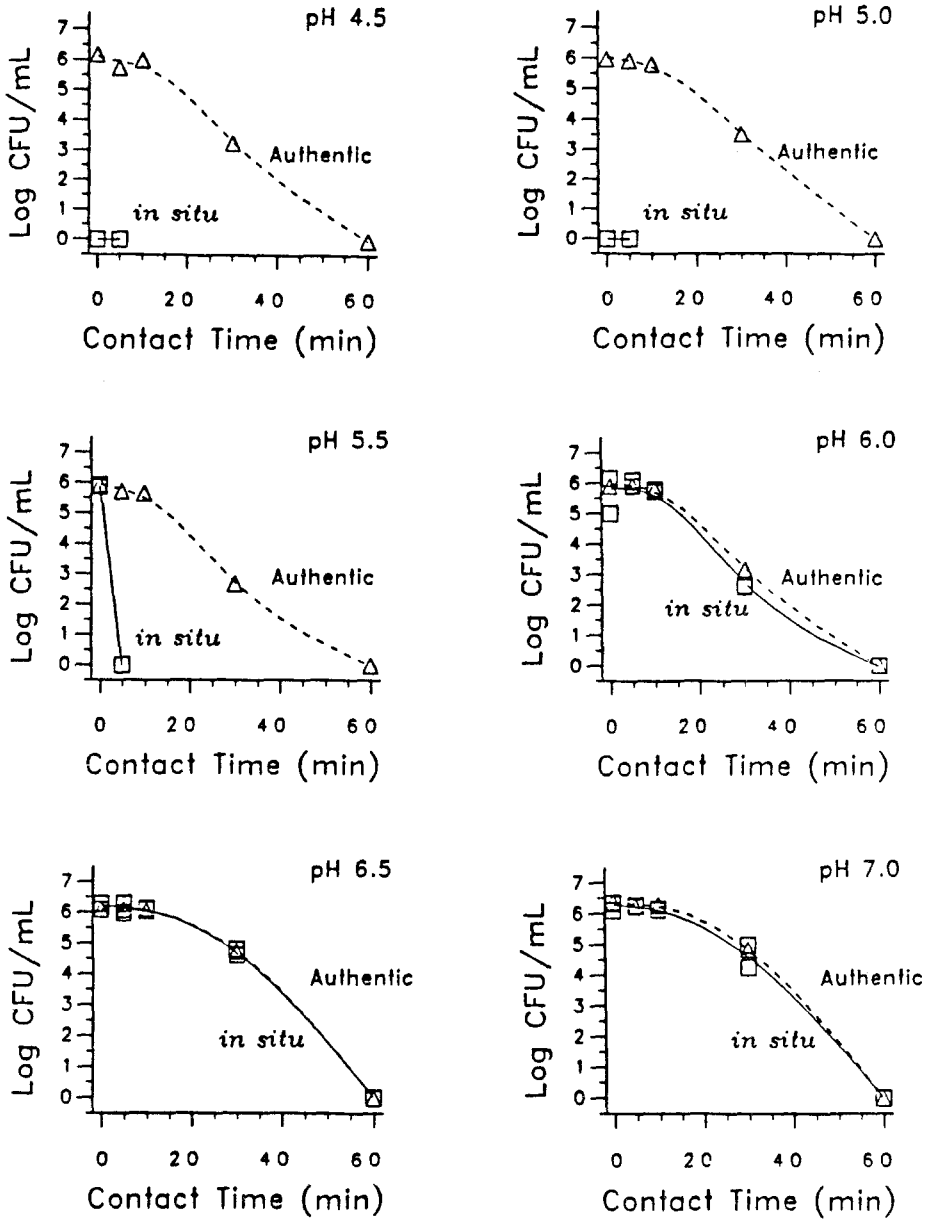

Fig. 1. Comparison of action of pre-formed agent I formed in situ in aqueous solution against *S. aureus* as a function of pH. Chlorine demand-free buffers with pH values of 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 were used at 22°C. Data were taken following the third challenge of *S. aureus* which represents 22 h contact between the amine precursor and the free chorine. □, agent I formed in situ; △, pre-formed agent I (authentic agent I).

consistent with the disinfection rates observed for laboratory-synthesized agent I.

With in situ formation of agent I, the pH of the reaction becomes very important. Under acidic to neutral conditions the rate of formation is slowest at pH 4.5 and 5.0, intermediate at pH 5.5, and most rapid at pH 6.0, 6.5, and 7.0. However, based on earlier work [11], once the agent is formed, its stability is enhanced under acidic and neutral conditions.

In situ formation of the organic *N*-chloramine affords both rapid and sustained disinfection. De-

pending upon the intended use, bypassing the final step in synthesis may produce a more economically useful disinfectant.

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