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Inactivation of *Salmonella enteritidis* on shell eggs by novel *N*-halamine biocidal compounds

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SUMMARY

Several new *N*-halamine compounds have been evaluated as potential replacements for free chlorine as disinfectants for the egg-processing industry. The compounds were tested against *Salmonella enteritidis* on the surfaces of egg shells. Test procedure included spraying inoculated egg shells with solutions of several of the *N*-halamine compounds and free chlorine for comparison, suspending the most stable *N*-halamine compound in a thin coating of mineral oil on the egg shell and subsequent inoculation, and measuring the rates of diffusion of the compounds and free chlorine through the egg shells. Compounds DBC (1-bromo-3-chloro-2,2,5,5-tetramethylimidazolidin-4-one) and DC (1,3-dichloro-2,2,5,5-tetramethylimidazolidin-4-one) were significantly more efficacious than free chlorine in inactivating *Salmonella* in the spray experiments, while compound MC (1-chloro-2,2,5,5-tetramethylimidazolidin-4-one), in a mineral oil suspension, provided disinfection of the egg shells within 72 h of contact. None of the disinfectant compounds penetrated egg shells at a rate greater than 1 mg/l over a period of 6 h. Compound MC is recommended as a possible replacement for unstable, corrosive-free chlorine as a bactericide for the egg-processing industry.

INTRODUCTION

Despite the implementation of the Egg Products Inspection Act in 1970, which requires pasteurization of bulk eggs and improved procedures for cleaning, sanitizing and grading eggs, there has recently been an upturn in the incidence of infections caused by *Salmonella enteritidis* in the United States [2,4,7,11,13,17]; the problem also exists elsewhere in the world [12,14–16]. Although it is generally believed that *S. enteritidis* organisms are usually present in the interior of infected eggs, transmitted there by infected laying hens, rather than on the shell surface [11,13], there remains concern that the organisms could be present on the shell [2,5], or even be transmitted through the shell [5]. Egg-washing and egg-sanitizing procedures are areas which should not be ignored in *S. enteritidis* control [4]. Indeed, the research to be reported herein proves that *S. enteritidis* was viable for extended periods on egg shells and that of those disinfectants studied, the widely employed disinfectant sodium hypochlorite was the least effective at inactivating it.

All of the compounds investigated in these studies fall under the general classification of organic *N-halamines*, which are biocidal, and which are more persistent in aqueous solution, less reactive with organic impurities, and less corrosive than free chlorine [19]. The structures of the compounds which were studied in this work have been shown in previous papers [18,21]. The properties and general biocidal characteristics of compounds I (3-chloro-4,4-dimethyl-2-oxazolidinone), A (1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone), and AB (1,3,-dibromo-4,4,5,5,-tetramethyl-2-imidazolidinone) have been summarized [21]. Compounds DC (1,3-dichloro-2,2,5,5-tetramethylimidazolidin-4-one), DBC (1-bromo-3-chloro-2,2,5,5-tetramethylimidazolidin-4-one), and MC (1-chloro-2,2,5,5,-tetramethylimidazolidin-4-one) have been reported only recently [18]. The D series compounds are more biocidal than their A series analogs [10,18,21], and they are considerably less expensive to prepare. These compounds are effective as bactericides against *S. enteritidis*, *S. gallinarum*, *S. typhimurium*, and *Pseudomonas fluorescens* in aqueous solution [10].

In this work the efficacies of several of the compounds

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were compared with that of free chlorine (sodium hypochlorite) against *S. enteritidis* on egg shell surfaces. Data were also obtained demonstrating that the *N*-halamine compounds did not diffuse through egg shells at an appreciable rate.

MATERIALS AND METHODS

Chemistry

The *N*-halamine compounds tested in this study were synthesized and purified in our laboratory. Compound I was prepare using the procedure outlined by Kaminski and co-workers [8]. The A and D series compounds were synthesized by methods developed in our laboratory [18,21]. Sodium hypochlorite was purchased from Clorox, Inc. (Oakland, CA), and used without purification. Aqueous solutions of the compounds were prepared using demand-free water (no chlorine demand) which was in turn prepared by chlorination of distilled, deionized water with sodium hypochlorite overnight, followed by exposure to direct sunlight until no total chlorine was detectable. Concentrations for the *N*-halamines and free chlorine to be tested as disinfectants were determined by iodometric total halogen titrations [1].

Bacteria and media

S. enteritidis ATCC 13076 was purchased from the American Type Culture Collection and maintained on nutrient agar. Twenty-four-hour cultures were used as the inoculum. A calibrated Klett-Summerson colorimeter equipped with a green filter was used to determine the cell densities. The detailed methodology used for preparing bacterial suspensions, calibration of the colorimeter, enumeration of CFU/ml, etc., has been described in detail [19,20].

Egg shell sanitization

Eggs were obtained from the Auburn University Poultry Science Research Center and maintained at 4 °C before use. Each egg was treated in the following manner. The diameter was estimated, to the nearest 0.1 mm, from measurements of its length and width by assuming that $d = 0.5\ (l + w)$. The surface area of the egg was then estimated as $A = \pi d^2$, i.e., by assuming a spherical egg. Each egg was washed thoroughly with detergent, boiled in water for 1 min, and dried in a sterile dish at ambient temperature. The eggs were coated with a thin film of noble agar (DIFCO) at 50 °C in order to enhance *S. enteritidis* survival at high density on the shell surface. Each egg was then inoculated with *S. enteritidis* by immersion for 5 min in a saline solution of the organism at a concentration of 10^6 CFU/ml at 10 °C. Each inoculated egg was dried on a sterile rack at ambient temperature for 10 min, and it was placed on a rotor which had four flexible metal prongs welded to he rotor shaft which could easily be molded to fit any egg. Each inoculated egg was rotated at 74 rpm, while either disinfectant or sterile demand-free water (for controls) was sprayed uniformly on it at constant pressure (supplied by nitrogen gas) at a volume of 0.8 ml/s for 15 s from a fixed chromatographic spray bottle (Fisher Scientific) positioned 14 cm from the rotating egg. The egg was then removed from the rotator and allowed to dry on a sterile rack for exactly 5 min. Each dried egg was placed in a sterile swirl bag (Whirl-Pak, Fisher Scientific) containing 10 ml of 0.02 N sterile sodium thiosulfate to quench disinfectant action, and it was gently massaged for 1 min in the bag, allowed to stand for 5 min, and then massaged for an additional 1-min period. One milliliter of solution was removed from the swirl bag, and log dilutions were plated in triplicate on nutrient agar. The plates were incubated at 37 °C and counted after 24 and 48 h. CFU/cm^2 values were computed for each egg. The disinfectant concentration was approx. 200 mg/l total chlorine or its molar equivalent in total bromine (for AB) or total oxidant (for DBC). The number of eggs investigated varied from six for MC to 14 for DBC, and a total of 24 controls. A standard *Q*-test [6] was employed to reject a few errant data points (see Results and Discussion).

Sanitation in an oil layer

Eggs were treated in the same manner as discussed in the previous section up to the inoculation step. For these experiments the eggs were immersed in a suspension containing 0.4% by weight compound MC in a mineral oil suitable for internal consumption (E.R. Squibb and Sons, Inc.) or just the mineral oil alone (for controls) at ambient temperature. After draining the excess oil, the eggs were rolled in a saline inoculum containing 10^6 CFU/ml of *S. enteritidis* and placed in sterile beakers. Four control eggs were then monitored for viable organisms after standing for 5 min, five additional eggs after standing for 24 h at 4 °C, two after standing for 48 h at 4 °C, five more after standing for 72 h at 4 °C, and one after standing for 168 h at 4 °C. Seven eggs coated with the suspension of MC were assayed after standing for 24 h at 4 °C, four after 48 h, seven after 72 h, and two after 168 h contact at 4 °C. The assay procedure incorporating the swirl bag containing sterile 0.02 N sodium thiosulfate was employed as described above.

Diffusion of disinfectants

Large Grade A eggs purchased from a local supermarket were washed with detergent and rinsed with distilled, deionized water. The top one-third of the shell of each egg was delicately removed using surgical scissors. The yolk and albumen were discarded, and the membrane removed

from the inside of the shell using forceps and a spatula. Then 30 ml of DPD (diethyl-*p*-phenylene diamine) solution buffered at pH 6.5 were added to the interior of the shell, and the shell was suspended by means of a wire holder in an aqueous solution of one of the disinfectants at a measured concentration of approx. 200 ml/l total chlorine, or its molar equivalent in total oxidant for DBC, except in the case of MC where the disinfectant solutions contained only about 91 mg/l total chlorine. The levels of solution within and without the egg shell were held the same such that no disinfectant could reach the DPD solution except by diffusion through the porous shell. The egg shell and disinfectant solution were placed in a plastic box with a lid connected to a supply of flowing nitrogen to minimize air oxidation of the DPD solution and allowed to stand for 5–6 h. The contents of the egg shell were removed at the end of this timed interval and analyzed for free and total chlorine using the DPD-FAS method [1]. In some experiments a temperature differential was maintained across the egg shell by immersing a stainless steel heat-exchange coil inside the egg and placing the disinfectant container in a temperature-controlled water bath. In this case, nitrogen was swept over the surface of the egg shell, while using aluminum foil to exclude as much room air as possible. A temperature gradient of 15 °C (warmer solution at 25 °C, cooler solution at 10 °C) was maintained throughout the diffusion experiments. For some egg shells the warm solution was inside the shell; for others it was outside the shell.

RESULTS AND DISCUSSION

Egg shell sanitization

The greatest problem encountered in performing these experiments was variations in bacterial cell densities from egg to egg, probably due to variations in pore size from one egg shell to another even though an attempt was made to minimize this problem by using agar coating. For example, the CFU/cm^2 for the 24 control eggs employed in this portion of the study varied from 1.0×10^2 to 1.2×10^5; the latter data point was rejected using a statistical *Q*-test, but all other 23 data points were retained. The mean value for the retained 23 controls was 3200 CFU/cm^2 with a standard error of the mean of 1200 CFU/cm^2. Berrang and co-workers [3] recently reported a method of efficiently recovering *S. typhimurium* from egg shells involving inoculation with a suspension of the bacteria in 1% peptone. However, this report was published after the work described herein was completed, and it was applicable to low cell densities (1–100 cells). We tested our disinfectants against egg shells having high cell densities to provide a worst-case scenario. The standard errors of the mean of surviving CFU/cm^2 for the sets of eggs receiving each disinfectant were lower than for the control samples. The mean values and standard errors are presented in Table 1.

Although the standard errors of the mean values of surviving CFU/cm^2 caused overlapping of the data, inspection of the standard error data for the combined *N*-halamine compounds indicated no overlap with the controls, and the data for all of the *N*-halamine compounds tested, except compound AB, did not overlap with that for sodium hypochlorite. The data for sodium hypochlorite and the controls did overlap, however. When an analysis of variance procedure (ANOVA) was used to compare the efficacies of the several disinfectants, a *t*-test indicates that compounds DBC and DC were more efficacious (at the 0.05 significance level) than was sodium hypochlorite. All of the disinfectant compounds caused fractional inactivation of *S. enteritidis* on the egg shells, and sodium hypochlorite was the least efficacious biocide.

TABLE 1

Efficacies of disinfectant compounds against *S. enteritidis* on the surfaces of egg shells[a]

Compound	Conc.[b]	Trial eggs	Trials rejected[c]	Mean CFU/cm^2	SE[d]	%Reduction from control mean[e]
DBC	189 ± 5	14	2	240	69	92
DC	204 ± 5	9	1	550	260	83
MC	195 ± 0	6	1	610	340	81
AB	204 ± 3	9	0	1100	400	66
NaOCl	201 ± 2	8	0	2500	1400	22
Controls	0[e]	24	1	3200	1200	—

[a] Spraying time was 15 s; drying time before quenching was 5 min.
[b] Average concentration expressed as mg/l total Cl (molar equivalent for DBC and AB).
[c] A statistical *Q*-test was employed at a 99% confidence limit.
[d] SE = standard error of the mean CFU/cm^2.
[e] Sprayed with demand-free water only.

TABLE 2

Efficacy of compound MC in mineral oil coatings against *S. enteritidis* on egg shells

No. of eggs	Coating[a]	Contact time (h)	Mean CFU/cm^2	SE[b]
4	oil	0.08	1.4×10^5	5.3×10^4
5	oil	24	6.0×10^4	2.0×10^4
2	oil	48	4.8×10^4	4.4×10^4
5	oil	72	4.1×10^4	2.6×10^4
1	oil	168	4.6×10^4	—
3	MC + oil	0.08	2.2×10^4	1.2×10^4
7	MC + oil	24	4.0×10^2	2.7×10^2
4	MC + oil	48	1.1×10^2	1.1×10^2
6[c]	MC + oil	72	0[d]	0
2	MC + oil	168	0[d]	0

[a] Controls consisted of mineral oil only; 0.4% by weight MC added to mineral oil for disinfection experiments.
[b] SE = standard error of the mean CFU/cm^2.
[c] A seventh egg contained 150 CFU/cm^2, this datum point can be rejected using a standard *Q*-test.
[d] 0 indicates no growth on plates after 48-h incubation at 37°C.

The highest efficacy was exhibited by compound DBC which contains one bromine and one chlorine moiety. This compound serves as a combination initial rapid biocide due to the more labile Br moiety and as a long-term biocide due to the very stable N–Cl bond. *N*-halamine compounds containing Br are, in general, more rapid biocides than are their Cl counterparts, but are less stable in solution because of their greater tendency to react with organic load [21]. Compounds DC and MC were next in bactericidal efficacy; containing no Br, they are less toxic toward most organisms, but are quite stable in the presence of organic load [18]. Compound MC is the most stable *N*-halamine compound developed in these laboratories or elsewhere and is the decomposition product of compound DC [18]. Compound AB was the least efficacious of the *N*-halamines studied. Even though AB contains two Br moieties, it is the least stable of these *N*-halamine compounds in the presence of organic load [21]. Undoubtedly the reason why NaOCl was a poor bactericidecide (only 22% inactivation) was that free chlorine is less stable than any *N*-halamine in the presence of organic load.

While even compound DBC only provided a 1-log reduction in *S. enteritidis* under the conditions of the experiment, it is probable that an increase in spraying time, contact time after spraying, or concentration of the disinfectant would be effective in increasing inactivation of the organism on egg shells, even at high cell density ($> 10^3$). Compound MC would probably be the best of the *N*-halamines for this application because of its long-term stability in aqueous solution in the presence of organic load and its lack of corrosiveness to the surface of materials, such as might be found in egg-processing plants. All of the *N*-halamine compounds are less corrosive and reactive than free chlorine [21]. A possible perceived limitation in this experiment might be the coating of the egg shells with Noble agar, in that this could conceivably cause entrapment of organisms or disinfectant in pores of the shell. However, this can be discounted as a factor affecting the trend observed in Table 1. A limited number of eggs was tested as described herein without the Noble agar step in the procedure. The same qualitative trend as noted in Table 1 was obtained in these experiments; the Noble agar merely served to enhance the cell density on the egg shells.

Sanitation in an oil layer

This portion of the study was undertaken to ascertain whether an *N*-halamine compound could be effective at inactivating *S. enteritidis* introduced following the oiling

TABLE 3

Diffusion of biocidal compounds through egg shells at ambient temperature with no temperature gradient imposed

Compound	Concentration[a]	Time (h)[b]	Trial eggs	mg/l[c]	SE[d]
MC	91 ± 1	6.0 ± 0	6	0.024	0.004
DBC	176 ± 4	6.0 ± 0	6	0.031	0.007
NaOCl	203 ± 3	6.0 ± 0	11	0.067	0.012
DC	199 ± 15	6.0 ± 0	11	0.114	0.019
A	227 ± 10	5.7 ± 0.3	6	0.121	0.026
I	228 ± 8	5.6 ± 0.5	7	0.133	0.034

[a] Initial concentration outside the egg shell expressed as mg/l total Cl (DBC corrected from total oxidant to total Cl).
[b] Time in h for diffusion observed.
[c] Amount of total Cl in mg/l which diffused through the egg shell during the time indicated.
[d] SE = standard error of the mean diffusion concentration.

TABLE 4

Diffusion of biocidal compounds through egg shells with a 15 °C temperature gradient imposed

Compound	Concentration[a]	High temp.	Time (h)[b]	Trial eggs	mg/l[c]
DC	190 ± 3	outside	6	3	0.634
DC	191 ± 5	inside	6	3	0.313
DBC	179 ± 0	outside	6	1	0.215
DBC	177 ± 2	inside	6	2	0.300
NaOCl	200 ± 2	outside	6	2	0.174
NaOCl	202 ± 1	inside	6	2	0.204
Control[d]	0	outside	6	1	0.087

[a] Initial concentration outside the egg shell expressed as mg/l total Cl (DBC corrected from total oxidant to total Cl).
[b] Time in h for diffusion observed.
[c] Amount of total Cl in mg/l which diffused through the egg shell during the time indicated.
[d] The control consisted of distilled, deionized water only.

process which is often performed to seal egg shell pores in processing plants [9]. Compound MC was chosen as the test biocide because it is the one most resistant to reaction with organic load such as might be present on the egg shell or in the oil. The eggs were stored at 4 °C during this portion of the study because it has been shown that *S. enteritidis* survives on eggs for up to 12 days at 7 °C, but for only 1 day at ambient temperature [2]. The results are shown in Table 2. The control eggs yielded approx. 1.4×10^5 CFU/cm^2 5 min following inoculation into the oil layer. After 24 h the controls indicated approximately a 1-log loss of viable cells, but no appreciable decline in viable cells was noted thereafter in the controls. For eggs which contained compound MC in the oil layer, after 24 h the viable cell concentration had declined to 0–1700 CFU/cm^2 for the seven eggs, after 48 h to 0–440 CFU/cm^2 for four eggs, after 72 h to 0 for six eggs and 150 for one egg, and after 168 h to 0 for two eggs. The 150 CFU/cm^2 datum point for 72 h contact can be rejected with 99% confidence using the standard Q-test [6]. Thus, compound MC used at 0.4% by weight in a thin oil layer was effective at inactivating *S. enteritidis* over a 3-day period. It is probable that MC in an oil layer could be used effectively to not only kill bacteria on egg shells during application, but also to prevent colonization by bacteria during shipment and storage.

Diffusion of disinfectants

This portion of the study was undertaken in order to assess the tendencies of several *N*-halamines and free chlorine to diffuse through egg shells. Results for egg shells for which a temperature gradient was not imposed are shown in Table 3. The lowest diffusion rate, which was 0.024 mg/l total chlorine for the 6 h period, was observed for compound MC; the highest rate of 0.133 mg/l total chlorine occurred for compound I.

When a temperature gradient of 15 °C was established across the egg shell for a limited number of trials, the diffusion rate increased somewhat for the compounds tested (Table 4). However, the diffusion rate was still low, being only 0.634 mg/l for 6 h in the worst case (compound DC, high temperature outside and low temperature inside). A control egg in which no biocide was employed gave measurable total chlorine concentration of 0.087 mg/l for 6 h demonstrating that the DPD solution inside the egg shell was minimally oxidized by ingredients in the shell or air which was not totally excluded in the temperature gradient experiments. These diffusion experiments suggest that there would not be a problem with the biocidal compounds diffusing through the shells into the yolk and albumen of the eggs when exposure times to spraying or soaking are only a few seconds in processing plants.

As a result of this study, we believe that the new *N*-halamine compounds, especially compound MC, have potential as biocides for the egg-processing industry. They could be employed in sprayers, soak tanks, and/or added to oil coatings at the conclusion of the processing.

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