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Efficacies of Novel *N*-Halamine Disinfectants against *Salmonella* and *Pseudomonas* Species

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Six novel *N*-halamine compounds of potential importance as disinfectants to the food-processing industry were tested against *Salmonella enteritidis*, *Salmonella gallinarum*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* in aqueous solution. Inactivation times for 10^6-fold reductions were determined as a function of water quality at pH 6.5 and 25°C. Phenol coefficients for the efficacies of the compounds against *S. enteritidis* have been reported also. When both stability and efficacy data are considered, as well as cost of production, two compounds, 1,3-dichloro-2,2,5,5-tetramethylimidazolidin-4-one and 1-chloro-2,2,5,5-tetramethylimidazolidin-4-one, offer the greatest potential as biocides for the food-processing industry.

The incidence of gastroenteritis in humans due to salmonellosis has increased markedly during the past decade (4, 13, 15, 16, 18). In the poultry industry, much of the *Salmonella* problem arises from consumption of contaminated eggs. There is evidence that *Salmonella* species and other organisms such as *Pseudomonas* species can penetrate egg shells or be transmitted to the eggs inside infected birds during the egg formation process (4, 8). Although egg shells can be fairly effectively decontaminated by washing with free chlorine, this disinfectant is corrosive toward processing equipment and does not remain stable for extended periods in wash water held at elevated temperatures (23). Furthermore, it has been found that *Salmonella* serotypes survive for long periods of time in artificially inoculated feeds (14), in drinking water (1), and on surfaces in poultry houses (12).

There is a need for a new disinfectant for the control of *Salmonella* and *Pseudomonas* species which can be utilized by the food-processing industry as a stable, noncorrosive, effective biocide. Considerable effort has been expended in our laboratories toward the development of such a biocide. Most of the work has focused upon the general class of compounds known as organic *N*-halamines. The structures of those *N*-halamine compounds which have proved to be effective as stable sources of "combined" halogen (as opposed to "free" halogen in hypochlorites) in a variety of disinfection applications (23) and which are the subjects of this study have been published (19, 23). Compound I (3-chloro-4,4-dimethyl-2-oxazolidinone), which was first prepared and shown to be bactericidal by Kaminski et al. (10), was tested extensively in our laboratories as a biocide against a variety of organisms as a function of concentration, pH, temperature, and water quality and was a very stable, long-term disinfectant in aqueous solution (11, 23). Compounds A (1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone) and AB (the 1,3-dibromo analog of compound A), which were prepared for the first time in our laboratories, were more stable in water than their oxazolidinone analogs (3-chloro and 3-bromo analogs, respectively) (24), and compound AB in particular killed all organisms against which it was tested in short contact times (21, 23). However, these imidazolidinone compounds are more expensive to prepare than are the oxazolidinone analogs (3). Compound DC (1,3-dichloro-2,2,5,5-tetramethylimidazolidin-4-one) and its monochloro (MC) and bromochloro (DBC) derivatives are the most recent compounds to be synthesized here, and they may be the best general-purpose *N*-halamine biocides yet tested in that they are inexpensive to prepare and adequately stable in aqueous solution (19). Compounds DC and DBC were shown to be effective against *Staphylococcus aureus* in solution and on hard surfaces (19). Compound MC is the most stable *N*-halamine biocide which has been reported. This communication will compare the disinfection efficacies of the above *N*-halamine biocides against three *Salmonella* serotypes and *Pseudomonas fluorescens*. The *Salmonella* species (*S. enteritidis*, *S. typhimurium*, and *S. gallinarum*) were chosen because of their importance to the poultry industry (4, 7–9).

MATERIALS AND METHODS

Chemistry. Compound I was prepared and purified by the methods outlined by Kaminski et al. (10). Compounds A, AB, and ABC were synthesized and purified as described by Barnela and coworkers (3). The preparation and purification of compounds DC, MC, and DBC were outlined in a recent article (19) and patent (22).

The water employed for disinfectant solutions was of three types. Chlorine-demand-free water (CDFW) was prepared by chlorination of distilled, deionized water with sodium hypochlorite overnight, followed by exposure to direct sunlight until no total chlorine was detectable by standard iodometric titration (2). Tap water (TW) was used without further purification except that dechlorination was performed in the same manner as for CDFW. Water from a domestic well (WW) was also employed in some experiments; the WW was not purified in any manner and was not subjected to analysis. All of the water samples were buffered to pH 6.5 using 0.05 M sodium phosphate. The TW and WW experiments were performed to simulate "real" conditions in the food-processing industry, while the CDFW experiments were carried out to provide a baseline comparison of the several *N*-halamine disinfectants. Disinfectant concentrations for the *N*-halamines were determined by iodometric total halogen titrations (2).

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Microorganisms and media. Cultures of *S. enteritidis* (ATCC 13076), *S. typhimurium* (ATCC 6994), *S. gallinarum* (ATCC 9184), and *P. fluorescens* (ATCC 17565) were purchased from American Type Culture Collection and maintained on nutrient agar. Cultures that had been grown for 24 h were used as the inocula in each experiment. A calibrated Klett-Summerson colorimeter equipped with a green filter was used to determine the cell densities in the various experiments. Initial cell densities of 10^8 CFU/ml were diluted to 10^6 CFU/ml in the solutions containing inoculum, buffer, and disinfectant.

Predicted disinfection times. The procedures utilized in these laboratories for measuring disinfection times for the various disinfectants acting upon the several bacteria have been discussed in depth (21) and thus will not be repeated here. Briefly, the times required to produce a 10^6-fold inactivation (initial cell density of 10^6 CFU/ml) as a function of disinfectant concentration and water quality were determined following quenching of the disinfectant by sterile 0.02 N sodium thiosulfate before plating. Predicted times necessary for the 10^6-fold decline in viable CFU/milliliter were calculated using a $\log_{10}$ (CFU/milliliter + 1) transformation of the CFU/milliliter data (6, 17). At least five contact times were employed for each compound, and duplicate assays were performed, unless the data were not in accord, in which case triplicate assays were made.

Phenol coefficients. Phenol coefficients were determined for the disinfectant compounds utilizing *S. enteritidis* (ATCC 13076) in CDFW at pH 6.0 and the standard method outlined by Engler (5). The phenol coefficient essentially represents the ratio of disinfection efficacy of a given disinfectant to the disinfectant phenol at a contact time of 10 min. Five milliliters of a 1:90 dilution of a 5.0% stock solution of phenol was found to inactivate 0.5 ml of 10^6 CFU of *S. enteritidis* per ml in 10 min, whereas a 1:100 dilution would not. Phenol coefficients were calculated by dividing by 90 (the value found for the phenol solution) the greatest dilution of 5 ml of a given disinfectant which would inactivate the 0.5 ml of 10^6 CFU of *S. enteritidis* per ml in 10 min. For example, if a 1:500 dilution of a given *N*-halamine were required, the calculated phenol coefficient would be 500/90 = 5.6.

RESULTS

The efficacies of the various *N*-halamine disinfectants against 10^6 CFU of *S. enteritidis* per ml as a function of quality of water are presented in Table 1. Compound MC was not studied in this portion of the work. A concentration of 5 mg of total chlorine per liter was employed for compounds DC, I, and A and the molar equivalents in total bromine (11.25 mg/liter) or total oxidant (8.125 mg/liter) for compounds AB and DBC, respectively. Thus, all of the compounds could be compared directly in terms of equivalent molar concentration of potential total halogen, although none of the compounds dissociate in water to produce appreciable amounts of "free" halogen. In general, inactivation times were least for CDFW, followed by TW, and WW.

Table 2 presents the predicted 10^6-fold inactivation times for all of the disinfectant compounds against three *Salmonella* species and *P. fluorescens*. In all cases, CDFW buffered to pH 6.5 and held at 25°C was employed. The concentration of disinfectant was 5 mg of total chlorine per liter for compounds DC, I, and A and its molar equivalent in total bromine (11.25 mg/liter) or total oxidant (8.125 mg/liter) for AB and DBC, respectively. Several general observations are apparent from the data in Table 2. Free chlorine inactivated all of the bacterial species within 10 s and was the most rapid biocide, although DBC and AB, the *N*-halamine compounds containing bromine moieties, were competitive with it. DC was the most rapid disinfectant of those compounds containing only combined chlorine, while MC was by far the slowest-acting biocide. Among the bacterial species, *S. gallinarum* seemed to be the most sensitive toward inactivation by the compounds, followed by *S. enteritidis*, *S. typhimurium*, and *P. fluorescens*, although there were several deviations from this ordering.

TABLE 1. Predicted inactivation times of *S. enteritidis* by *N*-halamine disinfectant compounds at molar equivalent concentrations as a function of water quality[a]

Compound	Inactivation time (SE)[b]		
	CDFW	TW	WW
DC	4.23 (0.49)	9.14 (0.30)	10.12 (1.48)
DBC	0.23 (<0.01)[c]	0.46 (0.12)	0.62 (0.17)
I	7.76 (0.36)	9.20 (0.30)	11.19 (0.95)
A	13.03 (2.02)	ND[d]	ND
AB	0.23 (<0.01)[c]	1.78 (0.07)	1.70 (0.11)

[a] Compounds were used at the following concentrations: 5 mg of total Cl per liter for compounds DC, I, and A; 8.125 mg of total Cl plus Br for compound DBC; and 11.25 mg of total Br per liter for compound AB. All compounds were buffered to pH 6.5 at 25°C.

[b] Contact time (in minutes) predicted for a 10^6-fold reduction in CFU/milliliter using the regression equation $\log_{10}$ (CFU/milliliter + 1) = time.

[c] Actual value may be lower; complete inactivation was found at 15 s, the lowest contact time tested.

[d] Not determined at 5 mg of total Cl per liter.

Table 3 presents phenol coefficients for the several biocides and free chlorine. The higher the phenol coefficient, the more effective was a given disinfectant relative to phenol against *S. enteritidis*. The data in Table 3 are entirely consistent with the predicted inactivation times for *S. enteritidis* presented in Table 2. All of the compounds were considerably more active as disinfectants than was phenol.

DISCUSSION

The results obtained for the efficacies of several disinfectants against *S. enteritidis* as a function of water quality were not surprising. As expected, inactivation times were extended as the quality of water diminished. More chlorine demand will be present in WW and municipal TW than in CDFW. Prior work in these laboratories (23) demonstrated that the inactivation times of the *N*-halamine compounds against a variety of microorganisms were considerably less affected by the presence of chlorine demand than are those for the disinfectant free chlorine which becomes completely ineffective at low concentration (e.g., 1 mg/liter) in the presence of organic load.

For the experiments comparing the efficacies of the disinfectants under uniform conditions against the three *Salmonella* serotypes and *P. fluorescens*, we have no explanation for the observed general trend of resistance to the disinfectants (*P. fluorescens* > *S. typhimurium* > *S. enteritidis* > *S. gallinarum*). All of the *N*-halamine compounds appear to be superior to the industrial biocide phenol, which is a fact worth emphasizing, given that there are questions being raised at this time concerning the toxicity of some of the phenolic compounds.

Upon initial consideration of the data in Tables 2 and 3, free chlorine would appear to be superior to all of the

TABLE 2. Predicted inactivation times of *Salmonella* species and *P. fluorescens* by *N*-halamine disinfectant compounds and free chlorine at molar equivalent concentrations in CDFW buffered to pH 6.5 at 25°C[a]

Disinfectant	Inactivation time (SE)[b]			
	S. enteritidis	*S. gallinarum*	*S. typhimurium*	*P. fluorescens*
DC	4.23 (0.49)	2.60 (0.66)	5.82 (1.08)	6.20 (1.54)
DBC	0.23 (<0.01)[c]	0.23 (<0.01)[c]	0.80 (0.11)	0.51 (0.03)
I	7.76 (0.36)	2.34 (0.32)	10.04 (0.09)	15.02 (0.17)
A	13.03 (2.02)	3.27 (0.37)	15.67 (1.50)	15.88 (2.86)
AB	0.23 (<0.01)[c]	0.53 (0.09)	0.49 (0.29)	0.78 (0.05)
MC	249.6 (21.6)	282.0 (16.8)	308.4 (16.8)	238.2 (4.2)
NaOCl[d]	0.17 (<0.01)[e]	0.17 (<0.01)[e]	0.17 (<0.01)[e]	0.17 (<0.01)[e]

[a] Compounds were used at the following concentrations: 5 mg of total Cl per liter for compounds DC, I, A, MC, and NaOCl; 8.125 mg of total Cl and Br per liter for compound DBC; and 11.25 mg of total Br per liter for compound AB.
[b] Contact time (in minutes) predicted for a 10^6-fold reduction in CFU/milliliter using the regression equation $\log_{10}$ (CFU/milliliter + 1) = time.
[c] Actual value may be lower; complete inactivation was found with 15-s contact, the first time point.
[d] NaOCl, sodium hypochlorite.
[e] Actual value may be lower; complete inactivation was found with 10-s contact, the first time point.

N-halamine disinfectants for inactivation of *Salmonella* species and *P. fluorescens* in CDFW. However, this is not necessarily true in industrial applications. It has been shown in these laboratories through use of Le Chatelier's principle (20) that the combined *N*-halamine compounds themselves are the active disinfectants rather than the minimal amounts of free halogen produced in hydrolysis equilibria. The enhanced stabilities of the *N*-halamine compounds in water may be attributed to the presence of the methyl groups on the ring carbons adjacent to the N-X moieties. There are at least three possible explanations for this: (i) electronic destabilization of the negative charge upon nitrogen after the loss of free X^+ groups caused by the electron-donor methyl groups, (ii) steric hinderance of approaching water molecules necessary for hydrolysis caused by the bulky methyl groups, and (iii) no chance of dehydrohalogenation in the ring because of no adjacent hydrogen atoms to the N-X functional groups.

In any case, the *N*-halamine compounds developed in these laboratories are considerably more stable than commercial analogs such as cyanurates or hydantoins, and as such, should persist for extended periods of time in food-processing applications, thus necessitating only infrequent replenishment. Furthermore, the compounds would not be expected to be corrosive to industrial equipment because little corrosive free halogen is produced, i.e., the compounds are weak oxidizing agents (23). The compounds containing bromine (AB and DBC) do liberate more free halogen (ca. 2% for AB, less for DBC) than do their chlorinated analogs because the N-Br bond is more labile than the N-Cl bond (23). This explains why AB and DBC inactivated the bacteria more rapidly than did the combined *N*-chloramines, i.e., the *Salmonella* and *Pseudomonas* species are more susceptible to free-halogen disinfection than to combined-halogen disinfection.

For the food-processing industry, we would recommend use of one of the combined *N*-chloramines discussed herein because of their great stabilities and adequate disinfection efficacies. Compound MC appeared the least efficacious against the bacteria in this study, but it was employed at a very low concentration (5 mg of total chlorine per liter). Its inactivation time for *S. aureus* at pH 7.0 and 25°C in CDFW was less than 1 min when a 0.17% solution was employed (22). Also, compound MC is soluble and stable in alcohol solvents which renders it applicable to disinfection of environmental surfaces. Compounds DC and MC are much less expensive to prepare than are compounds I and A (22, 23). Thus, compounds DC and MC should be the disinfectants of choice dependent upon application. If moderate stability and short contact times are needed, we would recommend DC; for long-term applications where contact time is not particularly important such as preservatives or preventatives, compound MC could be employed.

TABLE 3. Phenol coefficients for the inactivation of *S. enteritidis* in CDFW at pH 6.0 and 20°C by *N*-halamine compounds and free chlorine[a]

Disinfectant	Dilution	Phenol coefficient
DC	2.0×10^5	2,222
DBC	1.61×10^6	17,921
I	1.0×10^5	1,111
A	6.67×10^4	741
AB	1.0×10^6	11,111
MC	1.54×10^4	171
NaOCl[b]	3.33×10^6	37,037

[a] See Materials and Methods for explanation of calculation of phenol coefficient.
[b] NaOCl, sodium hypochlorite.

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