Bactericidal Effect of Various Combinations of Gamma Radiation and Chloramine on Aqueous Suspensions of *Escherichia coli*

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Methods of combining gamma radiation with chloramine to disinfect aqueous suspensions of *Escherichia coli* were investigated. Logarithmically grown cells were exposed to the bactericidal agents sequentially (i.e., radiation followed by chloramine, and chloramine followed by radiation) and simultaneously. Regardless of which combination was used, the bactericidal effect was always less than additive. During the phase of work involving the simultaneous addition of both agents, it was observed that chloramine was destroyed more rapidly by radiation than were the organisms. Since an increase in the bactericidal effectiveness of either disinfectant by prior or simultaneous treatment of the cells with the other disinfectant was not achieved in buffered distilled water, it was concluded that disinfection of wastewater effluents by combining ionizing radiation with chlorine would not be economically feasible.

Elimination of pathogenic microorganisms from water and wastewater effluents depends upon improvement of existing disinfection techniques or development and application of new economical processes. In response to this need, several studies are underway to evaluate ionizing radiation as a potential wastewater disinfectant.

In early studies, Ridenour and Armbruster (12), using gamma radiation from a cobalt-60 source, determined that a dosage of 8.4 × 10⁴ rad was sufficient to reduce the total bacterial population in effluent from primary wastewater treatment plants by 99%. Secondary effluent required only half that amount for similar reductions. Lowe et al. (9) found that the 99% microbial inactivation dosage was 1.5 × 10⁴ rad, while that for sterilization was 5.0 × 10⁴ rad. More recently, Touhill et al. (14) reported a 99.9% reduction in both total bacterial plate count and coliform numbers in secondary wastewater treatment plant effluent treated with 10⁵ rad.

Ballantine et al. (5) estimated that the cost of providing a dosage of 10⁵ rad for a 10 million gallon per day sewage treatment plant using cobalt-60 as the gamma source would approximate $1.04/10⁴ gal (based on assumptions of $0.40/Ci of cobalt-60, 75% utilization of energy, and 10-year amortization). If the price of radiation decreased to $0.10/Ci, the cost would still be $0.26/10⁴ gal.

In order for radiation to be an economically feasible wastewater disinfectant, its ability to inactivate microorganisms must be substantially improved. In 1963 Sidorenko et al. (13) reported that when chlorine was combined with gamma radiation to disinfect water, the organisms were inactivated much more quickly than when either agent acted alone. Other means, both physical and chemical, for increasing the effectiveness of radiation in killing bacteria have been reported. Dharkar (7) reported that by treating *Micrococcus radiodurans* and *Streptococcus faecalis* with sublethal doses of ultrasound prior to radiation treatment, the radiation dose necessary for sterilization was reduced 50%. Cotton and Lockingen (6) described conditions under which the lethal effect of X rays on T2 r bacteriophage was increased 500,000-fold in the presence of small amounts of chloroform.

Chlorination is presently the most widely accepted method of disinfecting water and wastewater effluents. However, concern has been expressed recently regarding the possible formation of chlorinated compounds that are toxic to the receiving water biota as a result of
treating domestic and industrial waste discharges with chlorine. Evidence that chlorinated effluents adversely affect aquatic life has been reported (J. Arthur, personal communication). This project was undertaken to test the feasibility of combining gamma radiation and chloramines (those forms of chlorine dominant in most wastewaters) as a potential method of disinfecting wastewater effluents.

MATERIALS AND METHODS

Organisms. Two strains of Escherichia coli were used in this study, namely, E. coli ATCC 11299 and E. coli K-12, C-600. The latter was kindly provided by J. Trela of the University of Cincinnati.

In initial experiments, the organisms were grown under static conditions in nutrient broth (NB) (Difco; The mention of commercial products here and elsewhere does not imply endorsement by the U.S. Environmental Protection Agency) at 37 C for 18 to 24 h. At the end of this time they were centrifuged, washed twice with 3.1 \times 10^{-4} M phosphate buffer (pH 7.2), and resuspended in the same buffer. The turbidity was adjusted to a reading of 130 to 150 Klett units in a Klett-Summerson photoelectric colorimeter (green filter). This corresponded to a viable cell count of approximately 1.5 \times 10^{8} cells/ml. After appropriate dilutions, the cell numbers used in all test flasks were 3.5 to 5.0 \times 10^{8} cells/ml.

It was difficult to obtain a standardized uniform cell crop with this procedure. Therefore, logarithmically grown cells were prepared in the following manner. A 16-h culture of E. coli, grown in NB under static conditions, was centrifuged, washed once with fresh NB, and suspended in 25 ml of fresh NB. A 1-ml inoculum of these organisms was added to 25 ml of NB and incubated at 37 C on a rotary water bath shaker at 150 rpm (Fermentation Design, Inc., Allen-town, Pa.). After 4 h of exponential growth, the cells were harvested, washed twice with 3.1 \times 10^{-4} M phosphate buffer, and suspended in the same buffer. The turbidity was adjusted to approximately 300 Klett units (3.0 to 5.0 \times 10^{8} cells/ml). The suspension was diluted to provide 1.0 to 1.5 \times 10^{9} cells/ml in the experiments.

Tryptone glucose extract agar was used for enumeration of colonies by the pour plate method with incubation at 37 C for 48 h.

Preparation of chloride demand-free water. The test water used throughout this study was double-distilled, the second distillation being achieved in a Bellco Loughborough glass still (Bellco Glass, Inc., Vineland, N.J.). This water was made chloride-demand-free by adding 3 to 5 \mu g of chlorine per ml, boiling, cooling to room temperature, and dechlorinating with either ultraviolet light or Na_2SO_4. No further treatment was necessary, as sterilization was achieved by this process. All glassware was acid-cleaned with chromic acid potassium dichromate cleaning solution, scrupulously rinsed with distilled water, and sterilized in a hot air oven (steam sterilization imparts a demand).

Preparation of chloramine. A stock ammonium chloride solution was prepared by dissolving 3.82 g of reagent grade NH_4Cl in 1,000 ml of double-distilled water, resulting in a solution containing 1,000 \mu g of ammonia-nitrogen per ml. The chlorine used was standard commercial sodium hypochlorite (Chlorox, 5.25% available chlorine).

The chloramine was always prepared on the day of the test. The ammonia-nitrogen (NH_4Cl) was added to the double-distilled demand-free water in a three- to fivefold excess by weight to that of chlorine. To assure that sufficient time was allowed for all of the chlorine to react to form chloramine, the NH_4Cl and NaOCl were allowed to react with each other for at least 1 h before the experiment was initiated. The chloramine was analyzed amperometrically according to standard methods (1). At pH 7.8 approximately 82% of the chloramine is in the form of monochloramine (NH_2Cl), the remaining 18% consisting of dichloramine (NHCl)_2 (3). In all experiments with chloramine, the 1.0-ml test samples withdrawn for plating were neutralized immediately with 9.0 ml of sodium thiosulfate (500 \mu g/ml) to insure that no excess residual chloramine would further affect the viability of the organisms in the sample.

Radiation source. A 3-kCi cobalt-60 source (located at the General Electric Co., Nuclear Systems Programs, Evendale, Ohio) was used in this study. The radiation chamber was situated at the bottom of a water-shielded well 13 feet (457.20 cm) deep. It consisted of a series of 36 rods containing cobalt-60 pellets arranged in three concentric tiers of 6, 12, and 18 rods (Fig. 1). The dose rate was varied by positioning the sample at specified distances from the cobalt-60 source. The stainless-steel cylindrical exposure chamber (Fig. 2) measured 12.5 cm outer diameter (OD) by 6.5 cm in height and 1.0 mm thick, and was fitted with an aluminum lid. A Kimax glass beaker (12 cm OD and 2.0 mm thick) was cut to provide a glass insert that fit snugly inside the stainless-steel receptacle. A glass lid was positioned atop the glass insert. Two small holes were drilled through the aluminum and glass lids so that samples could be withdrawn at various time intervals through sterile stainless-steel hypodermic needles attached to Tygon tubes (20 ft long) fastened at the other end to 50-ml sterile plastic syringes. Samples were irradiated by placing the exposure chamber inside a water-tight cylindrical aluminum container three feet (91.44 cm) in length connected to a 12-foot (365.76 cm) pipe handle through which the Tygon tubes were threaded (one tube per sample).

A special rig was designed for the phase of work requiring simultaneous exposure of the organisms to gamma radiation and chloramine (Fig. 3). A stainless-steel solenoid-activated valve (0.25 inch [0.64 cm] OD) connected the round-bottom flask above with the exposure chamber below through Teflon tubes (0.25 inch [0.64 cm] OD) and stainless-steel Swagelok union connectors. The electric cord from the solenoid was threaded through the pipe handle and plugged into an electric outlet above the well. In a typical experiment, a 3.0-ml suspension containing the desired number of organisms was placed in the exposure chamber below the solenoid, while a 300-ml sample of...
test water containing a predetermined concentration of chloramine was placed in the container above the solenoid. When the unit was lowered into the radiation field, the solenoid was activated and the test water was drained into the exposure chamber, thus exposing the organisms to chloramine and gamma radiation at the same time (the drainage time was 20 s). Mixing was achieved by manually rotating the pipe handle clockwise and counterclockwise.

All experiments were performed at ambient temperature which averaged 25 ± 3 °C. The test water was adjusted to pH 7.8 with 5 × 10^{-3} M phosphate buffer. Survival controls (test water with no disinfectant added) paralleled each test.

Dosimetry. Dosimetry of the cobalt-60 source was performed by the ferrous-ferric Frick dosimeter method (2). A 300-ml volume of dosimeter solution (same volume used in all disinfection experiments) was irradiated for 1 h in the same radiation field used in bacteriological tests. The change in optical density was recorded in a Beckman DU spectrophotometer (305-nm wavelength and 1.0-cm path length). The dose rate, calculated from the optical density reading, was 1.46 × 10^4 rad/h.

Mathematical analysis. The first-order destruction rate constants (these rate constants are calculated from the linear portion of the kill curves where the organisms exist as individual cells; the initial shoulders of most of the curves shown are due to the unavoidable clumping of the cells, which causes a departure from classical first order destruction rate kinetics) in all figures are computed from the following equation:
the probability of killing. Therefore, the combined survival in the double-disinfectant system is the product of both survivals, and these points are computed on the theoretical combined survival curves shown.

**RESULTS**

Effects of gamma radiation and chloramine on *E. coli*. Figures 4 and 5 depict the base-line disinfection patterns of gamma radiation and chloramine, respectively, on the two strains of *E. coli*. The data indicate that *E. coli* K-12, C-600 is slightly more resistant to the action of ionizing radiation (Fig. 4) but more sensitive to the effect of chloramine (Fig. 5) than *E. coli* ATCC 11229.

Combined effect of chloramine and gamma radiation applied simultaneously. Data presented in Fig. 6 show the effect of exposing *E. coli* ATCC 11229 (log-phase cells) to 0.55 μg of chloramine per ml and ionizing radiation simultaneously. To facilitate comparison, the disinfection curves from Fig. 4 and 5 are repeated on the same graph. Also included is the calculated theoretical additive effect that should or would be anticipated if each disinfectant acted independently of the other.

A pronounced shoulder is observed with the chloramine curve, whereas only a brief lag is noted with the radiation curve. When both
radiation and chloramine are applied simultaneously, the resultant line, instead of being exponential, is a complex curve not characteristic of typical logarithmic death. More important, however, is the fact that the combination of ionizing radiation and chloramine in this test system results in a less-than-additive effect. This is readily apparent when the experimental curve is compared with the calculated theoretical curve (Fig. 6). Comparable results (not shown) were obtained when the cells were grown under static conditions.

The apparent antagonistic effect between the two disinfectants when applied simultaneously is explained in the following manner. As shown in Fig. 7, only 62% of the original chloramine remains following a 15-min exposure to gamma radiation (same dosage rate used in disinfection runs). During that time period, the chloramine in the control flask has been virtually ineffective (Fig. 6), whereas the radiation alone has inactivated 65% of the test organisms. The net result of simultaneously combining the two disinfectants is that for at least the first 30 min of exposure the inactivation curve is almost identical with that of radiation alone. After 30 min, the chloramine (now reduced to less than 50% of its original concentration) begins to affect the cell population, causing an increase in the death rate slightly greater than that for radiation alone, but significantly less than that which would occur if both agents were acting independently.

Effect of sequential exposure of strain ATCC 11229 to radiation followed by chloramine. The second phase of study involved irradiating log-phase cells of E. coli before subjecting them to the action of chloramine to see whether such treatment would enhance the effect of the chloramine. Two 300-ml samples of test water were transported to the cobalt-60 well. One sample, containing 10-fold more organisms per ml than the other, was irradiated for 40 min (corresponding to one log inactivation). At this time, the number of viable cells in both samples was equal. Each was divided into two 100-ml fractions (irradiated and non-irradiated). One fraction from each pair was treated with chloramine (0.35 µg/ml final concentration); no additions were made to the others. Samples were removed periodically for bacteriological plating.

The results of this second phase are presented in Fig. 8. The arrows mark the time that exposure to irradiation was terminated and chloramine was added. It is clear that irradiating the cells did not significantly sensitize them to the lethal effect of chloramine. It is also evident that a slight decrease in survival (33%) occurs in the control test after the organisms are removed from the radiation field.

Effect of sequential exposure of E. coli K-12 to chloramine followed by radiation. The procedure used in this series of experiments differed significantly from that described above. Whereas the above method necessitated addition of concentrated chloramine to irradiated cells, in this sequence the cells were added to test water already containing 0.31 µg of chloramine per ml; after 30 min exposure, they were irradiated for 40 to 60 min. The results from these experiments are summarized in Fig. 9. It is clear that an additive bactericidal effect occurs when E. coli is exposed to chloramine prior to gamma radiation.

DISCUSSION

The experimental protocol in this study was divided into three main phases: exposure of E. coli in pure water to chloramine and gamma radiation separately, simultaneously, and sequentially. The experiments involving sequential exposure to both disinfectants were further subdivided: (i) chloramine followed by radiation, and (ii) radiation followed by chloramine.
Combined chlorine was used instead of free chlorine because chloramines are the dominant forms existing in wastewater effluents. A study with free chlorine would have very limited practical significance because breakpoint chlorination is seldom practiced in wastewater disinfection.

The reason for using chlorine-demand-free water as the test menstruum was to insure that all chloramines formed were inorganic rather than the less germicidally effective organic chloramines which might have formed from the reaction of chlorine with traces of organic material present in water that was not demand free.

The test organisms were suspended in phosphate-buffered distilled water rather than wastewater effluent because changes in cellular constituents following irradiation are dependent primarily upon their interaction with the products of irradiated water and to a lesser, but by no means insignificant, extent upon the direct interaction of the constituents with the radiation (11). If the combination of chloramine and gamma radiation did not result in a synergistic microbicidal effect in a phosphate-buffered distilled water, then it is assumed that the combination would be even less effective in wastewater because of the presence of free radical scavengers in the form of organic matter.
Room temperature (25 ± 3 C) prevailed throughout all experiments. Temperature fluctuations within the range indicated were due to the lack of adequate insulation of the laboratory wherein the cobalt-60 well was located. However, it should be stressed that these variations, although reflected in the data from experiment to experiment, did not affect the validity of the data reported because all test containers in individual experiments were exposed in parallel to identical environmental conditions.

The results indicate that combining chloramine and gamma radiation in any manner results in an additive to less than additive bactericidal effect on E. coli in pure water. When the organisms are subjected to the action of both germicides simultaneously, the rate of decomposition of chloramine is greater than the rate of inactivation of the microorganisms at the dosages and concentrations studied (Fig. 6 and 7). The explanation for this is beyond the scope of this paper. However, Julien and Pucheault (8) reported that neutral solutions of hypochlorites irradiated by cobalt-60 gamma rays were partly reduced to chloride and partly oxidized to chlorate, with simultaneous formation of hydrogen and oxygen. It is possible that such a mechanism may have been responsible for the destruction of chloramines in this study.

When the organisms were exposed first to radiation and then to chloramine, the resultant inactivation curve paralleled that of the baseline kill curve for chloramine (Fig. 8), although the initial lag phase (shoulder) of the former after chloramine was added was somewhat shorter than that of the latter. The decreased shoulder is presumably due to sublethal injury to a small portion of the pre-irradiated population of cells and subsequent death, as evidenced by the slight drop-off in survival of the cells in irradiated, but not chlorinated, water (Fig. 8).

When the organisms were first treated with chloramine and then irradiated, the resultant curve exhibited a steeper slope than that of either base line disinfection curve. This increased death rate, however, was still virtually identical to the calculated theoretical rate that would have resulted if the disinfecting agents were acting independently. This again indicates that the free radicals produced by radiolysis apparently react with the chloramine molecules faster than they react with the bacterial suspension. The observation was also made by Vajdic (15) who found that 1.0 µg of free chlorine per ml was decreased to undetectable levels by 4.1 × 10⁴ rad.

The data presented above suggest that disinfection of water and wastewater effluents by combining chlorine with ionizing radiation would probably not be economically feasible, since the effect is at most additive in pure water. The work of Sidorenko et al. (13) leaves the impression that combining the two germicides results in a substantially greater effect than the separate action of either alone. For example, it was stated that a radiation dosage of one-tenth that necessary for water sterilization resulted in a reduction in the coliform index from 10⁴ cells/ml to 10 cells/ml. Under the additional influence of “active chlorine” (0.5 to 3.0 µg/ml), sterilization was achieved. It can be seen, however, that if a seven log reduction in the coliform index is achieved with radiation alone, it would not take much chlorine to destroy the remaining organisms. Accordingly, it seems likely that this effect would be at most additive and thus predictable. Therefore, it is reasonable to conclude that combining gamma radiation and chlorination to achieve adequate disinfection of wastewater effluents would be impractical both economically and bacteriologically.
Fig. 8. Effect of exposing E. coli ATCC 11229 to gamma radiation followed by chloramine. At arrows chloramine (0.35 μg/ml final concentration) was added to samples indicated. Symbols: O---O, survival control (no additions); O---O, chloramine; ---, gamma radiation; --*, gamma radiation, repeated from Fig. 4 for comparison; ●, chloramine added following irradiation; Δ, no additions following radiation.
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