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Inactivation of Human and Simian Rotaviruses by Chlorine

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The inactivation of simian rotavirus SA-11 and human rotavirus type 2 (Wa) by chlorine was compared at 4°C by using single-particle virus stocks. Both virus types were usually more readily inactivated at pH 6.0 than at pH 8.0 when low chlorine concentrations (0.05 to 0.2 mg/liter) were used. A complete (5 log) reduction of both was obtained within 20 s at all pH levels when chlorine concentrations were increased to 0.3 mg/liter. Slight differences in the chlorine sensitivities of SA-11 and human rotavirus type 2 were noted but were not considered to be significant.

Human rotaviruses (HRV), members of the family *Reoviridae*, comprise a unique homogenous group of 70-nm, icosehedral particles consisting of a double-layered capsid and double-stranded RNA (8). HRV are thought to be distributed worldwide, with no isolated population devoid of rotavirus antibody (8). Members of this group are responsible for many of the reported cases of acute, epidemic, or endemic diarrhea (2), affecting both children and adults (6, 7, 14, 25, 28). Owing to their excretion in large numbers during the acute phase of a disease (13), their reported inefficient adsorption to and subsequent easy removal from sludge solids (4), and their apparent stability in aquatic systems (9), HRV have long been suspected in waterborne outbreaks. Recent reports of waterborne gastroenteritis implicating an HRV etiology have confirmed these suspicions (12, 23, 27) and stimulated considerable interest in studies of HRV inactivation by commonly used water disinfectants such as chlorine.

Initial published findings suggested a high chlorine tolerance in simian (SA-11) and lamb rotavirus preparations (20, 24). Since the virus stocks used in these experiments were not purified and since the chlorine demand of the experimental systems was not controlled, the findings were subject to some doubt (1). In a more recent study, Berman and Hoff (1) demonstrated efficient SA-11 inactivation by chlorine and chlorine dioxide. In their experiments, highly purified, single-particle virus stocks reacted with chlorine in a chlorine-demand-free (CDF) buffer system. They also observed that inactivation proceeded more rapidly at pH 6 than at pH 10.

In the present study, the chlorine-induced inactivation rates of purified simian (SA-11) rotavirus and purified HRV type 2 (Wa) rotavirus were compared over a range of disinfectant concentrations and pH levels.

MATERIALS AND METHODS

Stock virus preparation and purification. Simian rotavirus SA-11 was obtained from Charles Gerba, University of Arizona, Tucson. HRV type 2 (Wa) was obtained from Biotech Research Labs, Rockville, Md. Methods for the preparation of both virus stocks were identical. Host cell monolayers (MA-104; Microbiological Associates, Bethesda, Md.) were propagated in 800-cm^2 roller bottles with a growth medium containing Eagle minimum essential medium, glutamine, nonessential amino acids, penicillin-streptomycin-gentamicin, and 4 to 8% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). After three washes with Hanks balanced salt solution, monolayers were inoculated with rotavirus at a multiplicity of infection of 10 and then incubated at 36°C for 1 h at a roller speed of 0.1 rpm (Cell-Production Roller Apparatus; Bellco Glass, Inc., Vineland, N.J.). Prewarmed Eagle minimum essential medium containing antibiotics, glutamine, nonessential amino acids, DEAE-dextran (100 μg/ml), and trypsin (15 μg/ml) was then added, and incubation was resumed at a roller speed of 0.5 rpm. After 18 to 20 h of incubation, cells (most of which had already come off the glass) were harvested, chilled to 4°C, pelleted (4,000 × *g* for 10 min), washed twice with phosphate-buffered saline, resuspended in 5 ml of phosphate-buffered saline, and subjected to two freeze-thaw cycles in ethanol-dry ice. An additional 10 ml of phosphate-buffered saline was then added, and the cells were homogenized for 2 min at half speed (Eberbach Micro-container fitted to a Waring blender). Homogenates were supplemented with 7 ml of Freon 113 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), homogenized for an additional 2 min, and centrifuged (4,000 × *g* for 10 min). The upper aqueous phase was removed, and the cells were extracted a second time. The combined aqueous phases were sonicated (20 Hz for 10 to 15 s; Kontes Microultrasonic Cell Disruptor), and 2 ml was layered on sucrose cushions composed of 1 ml of 50% sucrose and 2 ml of 20% sucrose. After centrifugation for 20 min at 20,000 rpm (20°C) in a Beckman SW50.1 rotor, the top 2 ml of each tube containing single virus particles (5) was pooled and stored at 4°C. Before each experiment, this virus stock was dialyzed twice against CDF phosphate-buffered saline (pH 7.0) to remove residual sucrose.

The above procedure routinely yielded 20 to 30 ml of stock containing 10^5 to 10^7 virus PFU/ml. Single-particle SA-11 stocks maintained titers for only 3 to 5 weeks, whereas HRV stocks were stable for 2 to 3 months.

Rotavirus assay. SA-11 and HRV were assayed on monolayers of MA-104 cells propagated as described above. Virus-containing samples were diluted in prewarmed Tris-buffered saline and inoculated (0.5 ml per well) onto Hanks balanced salt solution-washed cell monolayers grown in six-well culture dishes (9.6 cm^2 per well; Nunc, Roskilde, Denmark). After 1 h of adsorption, the inoculum was aspirated and replaced with 3 ml of overlay medium containing Eagle minimum essential medium, glutamine, antibiotics, nonessential amino acids, DEAE-dextran (100 μg/ml), pancreatin (330 μg/ml; GIBCO), and agar. A second overlay

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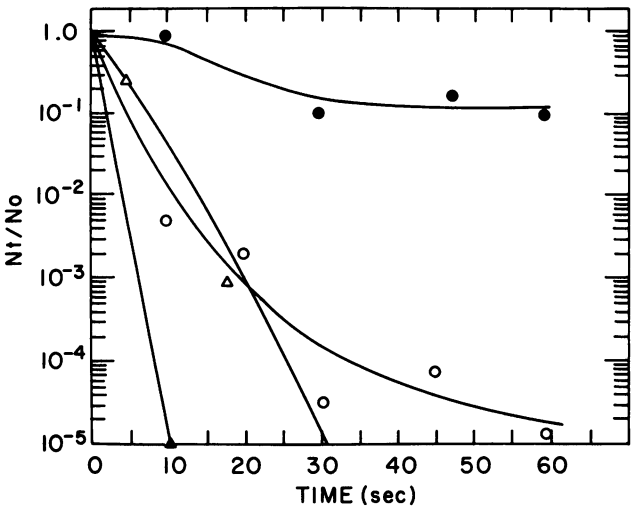

FIG. 1. Inactivation of SA-11 by chlorine at pH 6.0. Chlorine concentrations (in milligrams per liter) were as follows: <0.03 (●), 0.05 (○), 0.17 (△), and 0.25 (▲). Nt/N0, number of viruses at a given time/number of viruses at zero time.

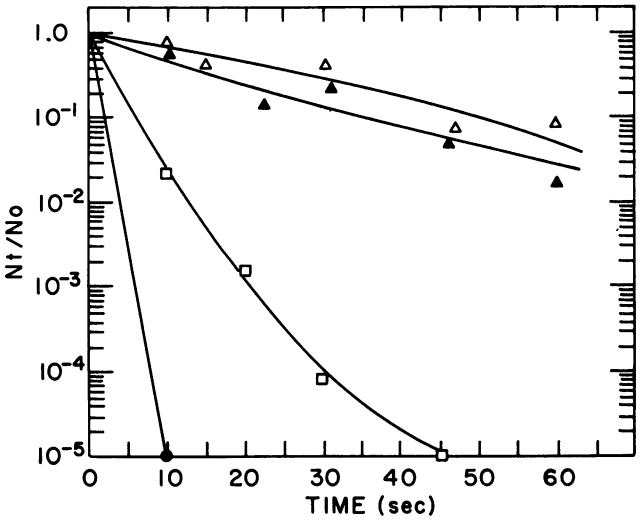

FIG. 2. Inactivation of SA-11 by chlorine at pH 7.0. Chlorine concentrations (in milligrams per liter) were as follows: 0.03 (△), 0.05 (▲), 0.10 (□), and 0.20 (●). See the legend to Fig. 1 for an explanation of Nt/N0.

containing agar and 0.1% neutral red was applied 2 days later, and plaque formation was observed over the next 6 days. During the course of the project, it was determined that the nutrient overlay for SA-11 could be further supplemented with sterile skimmed milk (3 ml/100 ml) and 50% $MgCl_2$ (1 ml/100 ml) without a loss in plaquing efficiency. Use of these supplements tended to extend monolayer life and provided greater contrast for reading plaques.

Inactivation experiments. Stock chlorine solutions (5% sodium hypochlorite; Fisher Scientific Co., Pittsburgh, Pa.) were prepared in CDF distilled water. Fresh solutions were prepared for each experiment. Chlorine concentrations were measured amperometrically in a chlorine Titrimeter (Fisher).

Inactivation experiments were conducted in phosphate-carbonate buffer prepared at the desired pH and ionic strength by the method of Sharp et al. (19). Buffer and glassware were made CDF as described elsewhere (3) and stored at 4°C. All experiments were carried out in a 4°C cold room. Experimental procedures were similar for both virus types, with the exception of the buffer volume in the reaction vessels (400 ml for SA-11 and 100 ml for HRV). Appropriate volumes of CDF buffer at the desired pH were inoculated with 1 ml of dialyzed single-particle virus stock and mixed with a magnetic stirrer. After collection of a zero-time control sample (10 ml), dilute sodium hypochlorite was added to the reaction chamber. Test samples (10 ml) were collected at 10, 20, 30, 45, and 60 s and placed in test tubes containing 0.1 ml of 0.5 M sodium thiosulfate to reduce the chlorine. Free chlorine concentrations were measured before and after each experiment. (In HRV inactivation experiments, chlorine concentrations were determined before the addition of a virus sample to the chlorinated buffer. A zero-time control sample was placed in a separate vessel with CDF buffer at the same pH.) Samples were then treated with 0.5 ml of chloroform to eliminate microbial contamination, diluted in Tris-buffered saline, and assayed as described above.

Positive and negative virus controls were used in each experiment to verify that the cells were both susceptible and virus contaminant free. Chlorine Titrimeter accuracy was frequently checked with chlorine control samples obtained from the U.S. Environmental Protection Agency. Experiments were usually conducted in duplicate and repeated several times to assure the consistency of results. Data were statistically analyzed by methods described by Sokal and Rohlf (21) and Steel and Torrie (22). Statistical analyses were performed on a Hewlett-Packard HP9845B computer with preprogrammed statistical software.

RESULTS

Titrations performed at the end of each experiment revealed reductions in free chlorine concentrations ranging from 0.03 to 0.07 mg/liter. Chlorine levels reported below represent those recorded at the beginning of each experiment.

The results of SA-11 inactivation experiments conducted

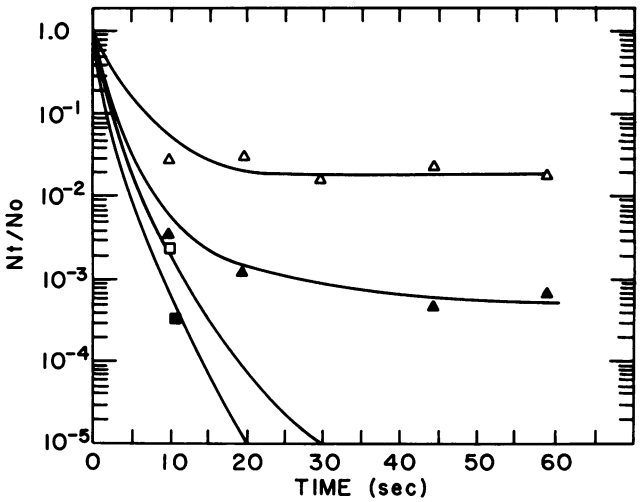

FIG. 3. Inactivation of SA-11 by chlorine at pH 8.0. Chlorine concentrations (in milligrams per liter) were as follows: 0.03 (△), 0.10 (▲), 0.30 (□), and 0.50 (■). See the legend to Fig. 1 for an explanation of Nt/N0.

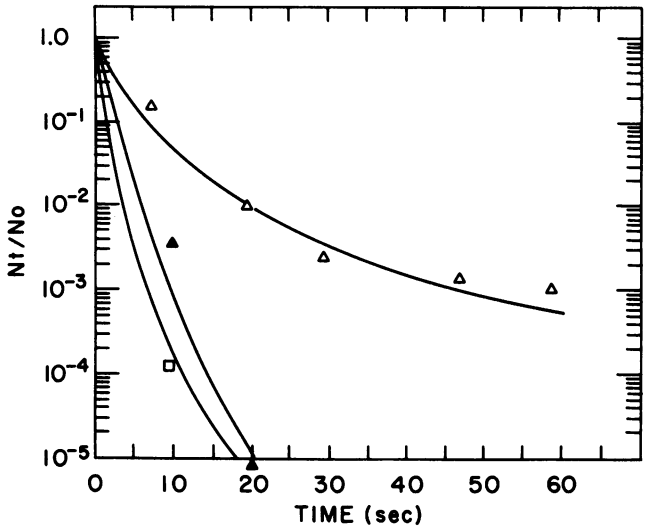

FIG. 4. Inactivation of HRV by chlorine at pH 6.0. Chlorine concentrations (in milligrams per liter) were as follows: 0.10 (△), 0.20 (▲), and 0.30 (□). See the legend to Fig. 1 for an explanation of Nt/N0.

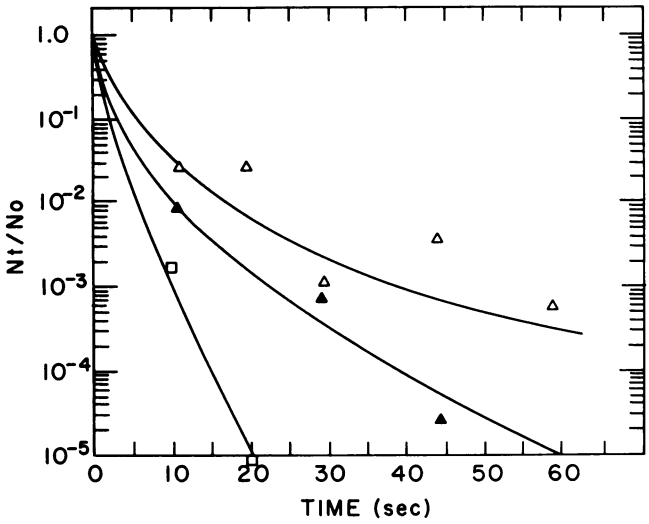

FIG. 6. Inactivation of HRV by chlorine at pH 8.0. Chlorine concentrations were as in Fig. 4. See the legend to Fig. 1 for an explanation of Nt/N0.

at pH 6 to 8 are shown in Fig. 1 to 3. Datum points in each curve represent median values from several (five to seven) experimental runs. Inactivation was quite rapid when chlorine concentrations of 0.1 mg/liter or greater were applied at an acid or a neutral pH (Fig. 1 and 2, respectively). pH-related effects were noted, with considerable reductions in virucidal activity occurring at an alkaline pH (Fig. 3). Virus survival was usually enhanced when chlorine concentrations of less than 0.1 mg/liter were applied, a notable exception occurring at pH 6 with 0.05 mg of free chlorine per liter (Fig. 1). Complete virus inactivation (5 logs) was obtained in less than 20 s at free chlorine concentrations of ≥20mg/liter (pH 6 and 7) and 0.5 mg/liter (pH 8).

HRV inactivation proceeded in a similar manner (Fig. 4 to 6). No pH-related effect was observed at a chlorine concentration of 0.1 mg/liter; however, virus survival was slightly enhanced at an alkaline or a neutral pH (Fig. 6 and 5, respectively) when chlorine concentrations were 0.2 mg/liter. Overall, the resistance of HRV to chlorine treatment appeared to be somewhat greater than that of SA-11, as illustrated in Table 1, in which the approximate times for 99.9% inactivation (as extrapolated from Fig. 1 to 6) of SA-11 and HRV are compared. Discrete differences aside, both virus types were rapidly inactivated at chlorine concentrations as low as 0.3 mg/liter.

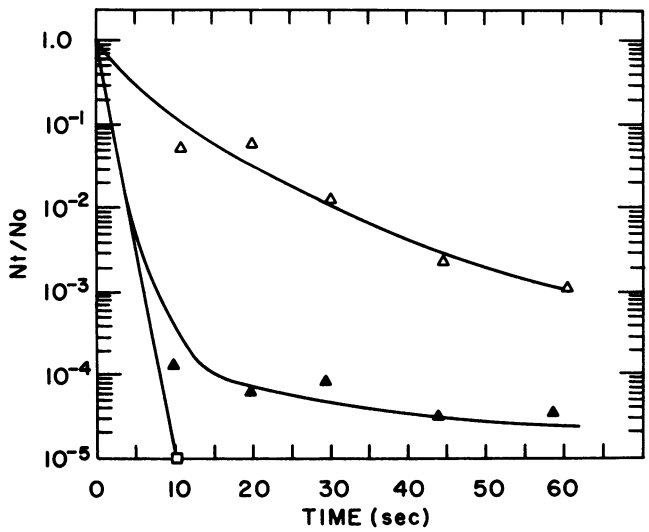

FIG. 5. Inactivation of HRV by chlorine at pH 7.0. Chlorine concentrations were as in Fig. 4. See the legend to Fig. 1 for an explanation of Nt/N0.

TABLE 1. Approximate times for 99.9% inactivation[a] of SA-11 and HRV by chlorine at 4°C

pH	Chlorine concn (mg/liter)	Time(s) required for 99.9% inactivation of:	
		SA-11	HRV
6.0	0.03	>60[b]	NT[c]
	0.05	19	NT
	0.10	NT	46.0
	0.17	20	NT
	0.20	NT	10.0
	0.25	6.0	NT
	0.30	NT	7.0
7.0	0.03	>60[d]	NT
	0.05	>60[e]	NT
	0.10	21	60.0
	0.20	6.0	8.0
	0.30	NT	6.0
8.0	0.03	>60[f]	NT
	0.10	28.5	39.0
	0.20	NT	22.0
	0.30	12	10.0
	0.50	8	NT

[a] As extrapolated from Fig. 1 to 6.
[b] 90% reduction at ~60 s.
[c] NT, Not tested.
[d] 90% reduction at 50 s.
[e] 90% reduction at 35 s.
[f] 90% reduction at 10 s.

DISCUSSION

As the major mode of drinking and wastewater disinfection used in the United States, chlorine has been used in a variety of virus inactivation studies. Experiments have usually dealt with the inactivation dynamics of enteric viruses and bacteriophages. The principal factors affecting enterovirus disinfection by chlorine include pH (as related to the predominant chlorine species hypochlorous acid [HOCl] and hypochlorite ion [OCl^-]), the ionic strength of the suspending medium, exposure time, and virus type (3, 10, 11, 15–18, 26).

Mounting evidence for the role of human rotaviruses in waterborne disease outbreaks (12, 23) has underscored the need for an evaluation of their inactivation by principal disinfectants such as chlorine. Recent work with single-particle suspensions of simian rotavirus SA-11 revealed rapid inactivation by free chlorine, chlorine dioxide, and monochloramine (1; R. Floyd, unpublished data). Virus inactivation rates were shown to be pH dependent, with a marked reduction in inactivation occurring at pH levels above 8.0, at which hypochlorite ion (OCl^-) becomes the predominant chlorine species. A notable exception occurred with chlorine dioxide, with which inactivation appeared to be more efficient at an alkaline pH (1).

In presenting their findings on SA-11 inactivation, Berman and Hoff (1) cautioned against an inappropriate extrapolation of results from their model system and suggested the need to pursue such studies with a strain of human rotavirus. Data from our study of HRV type 2 (Wa) correlate well with those from SA-11 studies. HRV were rapidly inactivated at free chlorine concentrations as low as 0.3 mg/liter. A reduction in disinfection efficiency was noted with increasing pH (i.e., increasing OCl^-) but was only appreciable at extremely low free chlorine levels (i.e., <0.3 mg/liter). Results from HRV studies support the conclusions of Berman and Hoff (1) which predict little difficulty in inactivating rotaviruses in water treatment facilities which maintain chlorine concentrations of 1 to 3 mg/liter. Our data also indicate that the simian rotavirus strain is a satisfactory model for human rotavirus inactivation studies in chlorinated systems.

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