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Inactivation of *Naegleria gruberi* Cysts by Chlorinated Cyanurates

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The resistance of *Naegleria gruberi* cysts to chlorine in the presence of cyanuric acid was compared at pH 5 and 7. An amperometric membrane electrode was used to measure HOCl concentrations independently of the chlorinated cyanurate species, thus permitting an analysis of the role of free chlorine versus chlorinated cyanurates in cyst inactivation. In the presence of cyanuric acid, the products of the HOCl residual and the contact time required for 99% cyst inactivation were 8.5 mg · min/liter and 13.9 mg · min/liter at pH 5 and 7, respectively. The Watson's Law coefficients of dilution (n) were 1.3 and 1.6 at pH 5 and 7, respectively. The results strongly suggest that HOCl is the predominant cysticide with no measurable cysticidal effect of the chlorinated cyanurate species.

The free-living protozoan *Naegleria fowleri* and at least one species of *Acanthamoeba* have been shown to be the causative agents of primary amoebic meningoencephalitis (2, 7). The disease has been found to result from contact made by swimming in waters contaminated with trophozoites of these species. One outbreak of primary amoebic meningoencephalitis was associated with the use of an indoor chlorinated swimming pool (13), indicating the resistance of these organisms to chlorine.

Chlorine is the most commonly used chemical for the disinfection of water. In the absence of ammonia, amines, or reducing agents, chlorine hydrolyzes to hypochlorous acid, HOCl, with subsequent dissociation to the hypochlorite ion OCl^-. The latter reaction, and thus the relative distribution of the two free chlorine species, is pH dependent (14). Chlorine, specifically HOCl, is an extremely effective disinfectant for bacteria and many viruses; however, the cysts of protozoans are more resistant (18). Moreover, free chlorine is subject to photolytic decomposition by sunlight. Consequently, in swimming pools and other applications, cyanuric acid (2,4,6-trihydroxytriazine) is used as a chlorine stabilizer. Because of the limited hydrolysis of chlorinated cyanurates, they also serve as a protected reservoir to maintain a relatively constant level of germicidally potent free chlorine (3, 11, 15, 16).

De Jonckheere and Van De Voorde (6) found that cysts of *N. fowleri* HB-1 are inactivated by a 15-min exposure to chlorine concentrations of 2.0 mg/liter at pH 7.3 to 7.4 and 25°C. Under the same conditions, 30 min were required to inactivate cysts of *Naegleria gruberi* 1518/1e. Chang (4) used a quantitative plaque-forming technique to measure the rates of inactivation of pathogenic *Naegleria* cysts to chlorine and iodine. A residual chlorine concentration of 4 mg/liter required 10 min of contact to obtain 99.9% cyst inactivation at pH 7.2 to 7.3 and 25°C. Cursons et al. (5) examined the effect of chlorine, chlorine dioxide, ozone, and Deciquam 222 on the trophozoites of *N. gruberi*, *N. fowleri*, *Acanthamoeba castellani*, and *Acanthamoeba culbertsoni*.

To our knowledge, there are no reports in the literature on the inactivation of protozoan cysts by chlorinated cyanurates. It has been shown that cyanuric acid inhibits the bactericidal effect of free chlorine (8, 9, 17; J. R. Anderson, Ph.D. thesis, University of Wisconsin, Madison, 1963). The probable explanation is that some of the chlorine is converted to chlorinated cyanurate species, which are much less effective disinfectants than free chlorine.

These studies were conducted with *N. gruberi* to provide additional data on the inactivation of protozoan cysts by chlorine and to elucidate the role of chlorinated cyanurates. An amperometric membrane electrode was used to independently measure HOCl concentrations in the presence of chlorinated cyanurate solutions. The results thus show the relative importance of free chlorine and chlorinated cyanurates in cyst inactivation.

MATERIALS AND METHODS

Cultures, solutions, and analyses. Cultures of *N. gruberi* NEG were maintained and cysts were produced in association with *Escherichia coli* on PM medium as described previously by Fulton (10). This medium was formulated from 4.0 g of Bacto-Peptone, 2.0 g of dextrose, 1.5 g of K_2HPO_4, 1.0 g of KH_2PO_4, and 20 g of Bacto-Agar per liter. The bacteria were maintained on stock slants of tryptic soy agar, and suspensions were prepared by inoculating tubes of tryptic soy broth and culturing at 28°C for 24 h. Stocks of *N. gruberi* were grown by spreading several agar plates with 0.2 ml of the *E. coli* broth. The plates were then inoculated on one edge with a loop of cysts or trophozoites, or both, and cultured at 28°C for 5 to 7 days. Cysts were recovered by washing and scraping the plates with 5 to 10 ml of sterile 0.2% sodium *N*-lauroyl sarcosine, a procedure which lysed any remaining trophozoites. The cysts were purified by centrifugation, followed by washing five times in sterile distilled water. The suspension was then passed through a 28-μm nylon mesh filter to eliminate large clumps. The cyst concentration of the filtrate, which contained mostly single cysts, was determined by counting in a hemacytometer.

All solutions were prepared with chlorine demand-free water prepared from deionized water to which at least 4 mg of sodium hypochlorite per liter had been added. After standing protected from sunlight for 24 h or more, the remaining chlorine was removed by exposing the solution to UV light.

Stock buffer solutions were prepared so that the final diluted total concentration was 5×10^{-3} M. The pH 5.0 buffer was 0.18 M in acetic acid and 0.32 M in sodium acetate; the pH 7.0 buffer was 0.279 M in potassium dihydrogen phosphate and 0.221 M in dipotassium hydrogen phosphate; and the pH 9.0 buffer was 0.478 M in sodium bicarbonate and 0.224 M in sodium carbonate. If necessary, the final experimental pH was adjusted with a few drops of either 2.33 M HCl or 3.0 M NaOH. Over the course of an experiment, the pH did not change more than 0.02 U as measured by a Sargent-Welch model LS pH meter and combination pH electrode.

A stock solution of ca. 1,000 mg of chlorine per liter was prepared by diluting commercial sodium hypochlorite (Clorox). This solution was standardized and used to prepare dilute chlorine solutions. A 1,000-mg/liter stock solution of cyanurate was prepared by dissolving 510.2 mg of 98% cyanuric acid (Aldrich Chemical Co.) in 300 ml of chlorine demand-free water with the aid of 10 ml of 0.3 N NaOH, followed by dilution to 500 ml.

Total chlorine and free chlorine concentrations in the absence of cyanurate were determined by amperometric titration with phenylarsine oxide (1). Free chlorine concentrations in the presence of cyanurate could not be measured by this method because of the rapid equilibria between chlorinated cyanurate and free chlorine species.

An HOCl membrane electrode described by Johnson et al. (12) was used to measure the equilibrium HOCl concentrations in the presence of cyanurate. The electrode was a prototype manufactured by Orion Research, Inc. It consisted of a platinum cathode and a silver-silver chloride internal reference anode. The internal electrolyte solution was 0.2 M KCl buffered to pH 6.4 with 0.2 M phosphate buffer, which was prepared from 14.9 g of KCl, 16.7 g of KH_2PO_4, and 13.4 g of K_2HPO_4 per liter. The cathode was covered with a polyfluoroethylene 0.2-μm microporous membrane reinforced with a nylon mesh (Fluoropore, catalog no. FGLP 047 00; Millipore Corp.) A polarographic circuit was designed to apply a cell voltage of +0.33 V to the cathode and to measure the current response of the electrode. The voltage source was a 1.4-V mercury dry cell, and the applied potential was controlled by 10^5-ohm and 10^3-ohm potentiometers used as coarse and fine controls, respectively. The reduction current produced by the electrode was determined by measuring the voltage drop across a 10^5-ohm precision resistor with either a model 160 or model 177 digital multimeter (Keithley Instruments). The electrode was calibrated daily with standardized chlorine solutions. For most experiments, the electrode was calibrated at the same pH as the experimental pH used on that day. Since the electrode responds only to the HOCl fraction of free chlorine, calibration should be more accurate at pH 5. However, the electrode responses to the same HOCl concentration at pH 7 and especially at pH 9 were significantly greater than at pH 5, thus requiring independent calibration at each pH.

Cyst inactivation procedures. The cyst inactivation studies were carried out in a 3-liter, wide-mouth amber glass bottle. A thermometer and a 50-W aquarium heater were fitted to the bottle with a rubber stopper. The rubber stopper contained ports for sampling and for the introduction of cysts. The reactor, which was insulated by several asbestos pads, was placed on a magnetic stirrer and stirred at ca. 120 rpm. Two liters of the buffered solution containing the disinfecting agents were added to the reactor. The heater was used to maintain an average temperature of 25.0 ± 0.5°C. The pH was measured and, if necessary, adjusted with either 2.33 M HCl or 3.0 M NaOH. Once the desired pH was obtained, a sample was removed and titrated for free and combined chlorine. Samples were removed at the beginning and end of the experiment and frequently at 5- to 10-min intervals for chlorine residual measurements with the electrode.

At the beginning of the contact time, a sample of cyst suspension not greater than 3.0 ml was injected into the reactor with a syringe, resulting in a final concentration of ca. 1,000 cysts per ml. At suitable intervals, 20-ml samples were withdrawn with a 20-ml syringe and injected into 25-ml screw-capped test tubes containing 0.3 to 1.5 ml of sterile 0.04 N sodium thiosulfate. This procedure required a minimum sampling interval of 30 s and provided for the rapid neutralization of the chlorine. Cyst survival was determined from these samples after the completion of the experiment. Temperature readings were taken several times during the course of an experiment, and solution pH was measured again at its end.

An identical control reactor was set up as described above but without disinfectants. After the reactor volume was adjusted, the same-sized sample of cysts was added, and 20-ml samples were taken after 1 min and at the longest contact time used in that experiment.

The experimental samples were cultured to determine cyst survival. A small portion of the sample was spread with 0.15 ml of *E. coli* broth on PM agar and

incubated at 28°C for ca. 40 h. At this time, amoebic colonies or plaques were visible as clearings in the bacterial lawn. The plates were counted and examined again at ca. 48 and 64 h to determine the plate total. Portions (0.1 ml) of the control samples were plated in triplicate. In the experimental samples, portions of either 0.1 or 0.5 ml were plated in triplicate, depending on the expected survival. The mean of each set of triplicates was used to determine the number of viable cysts. As survival decreased, the 0.5-ml sample was inadequate because of the low numbers of viable cysts. Therefore, at longer contact times, 10-ml samples were placed in 10-ml conical centrifuge tubes and centrifuged for at least 10 min. The supernatant was withdrawn, leaving a 0.8-ml volume. After being mixed, the sample was divided onto two separate plates. The tube was rinsed with an additional 0.4 ml of sterile distilled water and added to a third plate. The results of cultures from these three plates were added to give the number of viable cysts in 10 ml of sample. If more than one dilution gave reliable plaque counts, the numbers of cysts per milliliter were averaged for those two dilutions. The percent cyst survival was determined by designating the controls as 100% and expressing the numbers of viable cysts in the chlorinated samples relative to the controls.

RESULTS

The HOCl membrane electrode and polarograph responded linearly to the concentration of HOCl. Measurements were made while stirring the solution at a constant speed of approximately 100 rpm and a temperature of 25.0 ± 0.5°C. The calibration curves (measured daily) indicated an electrode response of approximately 55 nA/mg per liter as Cl_2 of HOCl. The suitability of using the electrode to measure free chlorine in solutions of cyanurate was determined by measuring the electrode response in solutions of dichlorocyanurate ranging from 1 to 80 μM. The free chlorine concentrations determined by the electrode were then compared with the calculated equilibrium free chlorine concentrations by using the equilibrium constants of O'Brien et al. (15). The close agreement between the two sets of values suggested that the electrode responded only to HOCl and therefore was used for that purpose in these studies.

Free chlorine standard concentrations and total chlorine (i.e., the sum of free chlorine and chlorinated cyanurate species) was measured by amperometric titration. The studies at pH 5 were carried out after calibrating the electrode at pH 5. The studies at pH 7 were carried out after calibrating the electrode at either pH 5 or 7 since the electrode gave essentially the same response at these two pH values.

The electrode was more difficult to operate at pH 9. Since the electrode responds to HOCl but not OCl^-, changes in pH had a great effect on the electrode response at the higher pH. There also appeared to be other factors, such as ionic strength, which caused the electrode response to be more variable at pH 9. The best results were obtained when the electrode was calibrated at pH 9, and very careful checks were made on the pH. The problems described, however, resulted in fewer data being reported for HOCl concentrations in cyanuric acid solutions at pH 9.

The rates of cyst inactivation by solutions of chlorinated cyanurates were determined at pH values of 5, 7, and 9. The HOCl concentrations were measured by the membrane electrode, and the total chlorine concentrations were determined by amperometric titration. Total chlorine in this context refers to the sum of the free chlorine and the chlorinated cyanurate species. The term cyanurate is used here to mean all cyanurate and cyanuric acid species in solution. The experiments were carried out with chlorine-to-cyanurate ratios of either 1, 2, or 3. This corresponds to the stoichiometry of mono-, di-, and trichlorocyanurate, respectively.

The rate curves for cyst inactivation by dichlorocyanurate at pH 5 are shown in Fig. 1. These are typical survival curves at all pH values for inactivation experiments with chlorinated cyanurates. All curves had an initial lag period of relatively slow dying off of cysts, followed by a period of exponential dying off. A straight line was usually fitted to the linear portion of the semilog survival curves. If the semilog survival rate was still increasing, a curve was used to fit the data. The data for all the inactivation experiments are summarized in

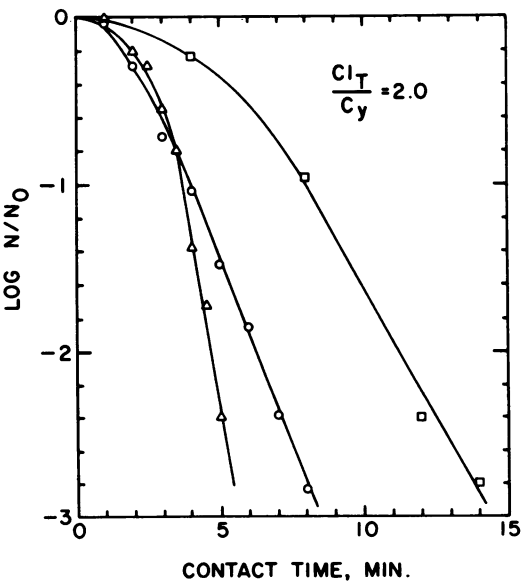

FIG. 1. Cyst inactivation by dichlorocyanurate at pH 5. Residual HOCl concentrations were 0.88 mg/liter (□), 1.30 mg/liter (○), and 1.72 mg/liter (△).

TABLE 1. Summary of cyst inactivation data by chlorinated cyanurates

Cy[a] (M × 10^5)	Cl_T[b] (M × 10^5)	Cl_T/Cy ratio	Titrable Cl_2 (mg/liter as Cl_2)		Time (min)[c]	Residual HOCl (mg/liter Cl_2)	99% Kill contact time (min)	Measured final pH
			Initial	Residual				
pH 5								
0.75	1.5	2.0	1.16	1.06	28	0.88	11.4	5.00
1.25	2.5	2.0	1.86	1.77	10	1.30	6.2	5.00
2.0	4.0	2.0	2.82	2.71	7.0	1.72	4.71	5.00
0.83	2.5	3.0	1.74	1.66	9.0	1.12	6.05	4.99
2.5	2.5	1.0	1.77	1.68	12	1.06	9.01	5.00
pH 7								
1.25	2.5	2.0	1.80	1.73	26	0.86	21.4	6.99
3.5	7.1	2.0	4.92	4.81	10	1.43	7.19	6.99
7.0	14.0	2.0	9.85	9.76	7.0	1.88	6.11	7.01
2.3	7.0	3.0	4.99	4.86	5.0	2.00	5.50	7.01
7.1	7.1	1.0	5.24	5.13	20	0.78	23.4	7.01
pH 9								
80	80	1.0	56.5	56.1	30	0.43	22.4	8.98
40	80	2.0	49.7	50.9	20		9.5	9.02
27	80	3.0	56.5	55.5	10		7.4	8.99

[a] Cy, The total concentration of cyanurate species.
[b] Cl_T, Total chlorine (sum of the free chlorine and the chlorinated cyanurate species).
[c] Time of residual measurements.

Table 1. The first three columns show the established experimental conditions. Columns four and five show the total chlorine concentrations measured by amperometric titration immediately before and after the experiments. The HOCl concentrations shown in column six were determined from the electrode response. The 99% kill times in the next column were interpolated from the semilog rate curves such as those in Fig. 1. The pH values in the last column were determined at the end of the experiment.

The effect of pH on the chlorinated cyanurate disinfection process was similar to the results of the free chlorine experiments. To maintain the same level of cyst inactivation, much higher chlorine concentrations were required at pH 9 to overcome both the dissociation of HOCl and the formation of chlorinated cyanurates. The data also show that although the total chlorine concentrations remained constant, as the cyanuric acid concentration was increased, less HOCl became available, and therefore a longer contact time was required to effect 99% cyst inactivation. For example, at pH 5 a total chlorination concentration of 2.5×10^{-5} M required 99% kill times of 6.05 and 9.01 min for cyanuric acid concentrations of 8.3×10^{-6} M and 2.5×10^{-5} M, respectively. Similarly, at pH 7 a total chlorine concentration of 7.1×10^{-5} M required 99% inactivation times of 7.19 and 23.4 min for cyanuric acid concentrations of 3.5×10^{-5} M and 7.1×10^{-5} M, respectively.

At the fixed chlorine-to-cyanurate ratio of 2.0, the 99% kill times decreased with increasing concentration at a given pH. It is also apparent from the data in Table 1 that as the total concentration of dichlorocyanurate increased, a smaller fraction of the total chlorine was present as free chlorine, even though more total chlorine was present in solution. For example, at pH 5, when the dichlorocyanurate concentration increased from 7.5×10^{-6} M to 2.0×10^{-5} M, the residual HOCl-to-total chlorine ratio decreased from 0.83 to 0.63. Consequently, the 99% kill time decreased less dramatically, from 11.4 to 4.7 min, than would be expected from the increase in total chlorine concentration from 1.5×10^{-5} M to 4.0×10^{-5} M.

Table 2 summarizes the $C \cdot t$ products calculated from the data at pH 5 and 7 shown in Table 1. The $C \cdot t$ products are the multiples of the residual equilibrium HOCl concentrations and the contact time required to effect 99% cyst inactivation. The initial chlorine concentrations in column one are the titrable total chlorine concentrations corresponding to the data in column four of Table 1. The $C \cdot t$ products are described by the Watson's Law equation: $C^n t$ = constant, where C = concentration, t = contact time, and n = coefficient of dilution. The mean $C \cdot t$ product is shown for each pH. This is not an adequate descriptor of the cyanurate system, however, because the coefficients of dilution are significantly different from 1.0 (Table 2).

DISCUSSION

The results presented show that cyanuric acid effectively inhibits the effect of chlorine on *N.*

TABLE 2. HOCl $C \cdot t$ products and coefficients of dilution for 99% cyst inactivation by chlorinated cyanurates[a]

Initial chlorine (mg/liter as Cl_2)	$C \cdot t$ (mg · min/liter)[b]
pH 5	
1.16	10.0
1.86	8.07
2.82	8.10
1.74	6.78
1.77	9.55
pH 7	
1.80	18.4
4.92	10.3
9.85	11.5
4.99	11.0
5.24	18.3

[a] The coefficient of dilution was 1.29 at pH 5 and 1.60 at pH 7.

[b] The mean $C \cdot t$ was 8.50 ($r^2 = 0.845$) at pH 5 and 13.9 at pH 7 ($r^2 = 0.972$). r^2, Coefficient of determination.

gruberi cysts. This was the result predicted from the reports of bacterial inactivation by chlorinated cyanurates (8, 9, 17; Anderson, Ph.D. thesis). However, this was the first time that an attempt was made to measure the equilibrium free chlorine independently from the chlorinated cyanurate species with a membrane electrode. These results support the assumption that the free chlorine is the agent primarily responsible for cyst inactivation and that the chlorinated cyanurate species have no measurable cysticidal effect.

At pH 5, the mean 99% kill $C \cdot t$ product for HOCl was 8.5 mg · min/liter in the presence of cyanuric acid. The mean 99% kill $C \cdot t$ product for HOCl at pH 5 in the absence of cyanuric acid reported by Rubin et al. (18) was 7.7 mg · min/liter. These values are sufficiently close to conclude that the effect of HOCl at this pH is the same with or without cyanurate. That is, HOCl is the far superior cysticide, and its concentration appears to control the rate of cyst inactivation. Similarly, at pH 7 the corresponding $C \cdot t$ product for HOCl in the presence of cyanuric acid was 13.9 mg · min/liter. The value reported by Rubin et al. (18) at pH 7 was 9.3 mg · min/liter. Although these values are still in the same range, their difference suggests that the cysts may be responding differently to HOCl in the presence of cyanuric acid. Since the mean HOCl $C \cdot t$ products are higher in the presence of cyanuric acid than in the absence of cyanuric acid, it is clear that the chlorinated cyanurate species have no apparent cysticidal effect.

The higher HOCl $C \cdot t$ products in the chlorinated cyanurate system suggest two possible explanations. Either the *N. gruberi* cysts are more resistant to HOCl in the presence of cyanurate or the higher values represent an error in the measurement of HOCl by the membrane electrode. The former conclusion is supported by the observed changes in the Watson's Law coefficient of dilution (n) between free chlorine alone (18) and the results reported here for chlorinated cyanurates. Rubin et al. (18) reported values of n for HOCl of 0.96 and 1.19 at pH 5 and 7, respectively. The experiments reported here, however, resulted in values of n for HOCl (in the presence of cyanurate) of 1.29 and 1.60 at pH 5 and 7, respectively. At both pH values, the values of n were 1.34 times greater in the cyanurate system than in the free chlorine system (Fig. 2). Our results indicate a quantitative difference in the response of the cysts to HOCl in the two different systems. It is suggested, therefore, that the chlorinated cyanurate species in solution may react with the *N. gruberi* cysts to induce a qualitative change which is reflected in the greater resistance to HOCl and the increased values for the coefficients of dilution. This conclusion is supported most strongly by the change in the coefficients of dilution (n) for HOCl, since this value is usually associated with the response of microorganisms to disinfectants (19).

Our results clearly show the role of HOCl in the inactivation of *N. gruberi* cysts by chlorinated cyanurates. The data at pH 9 were insufficient to determine HOCl concentrations. However, it is clear that much higher total chlorine concentrations were required to at least overcome the dissociation of HOCl to OCl^-. The rate of cyst inactivation at pH 5 and 7 is clearly dependent on the equilibrium HOCl concentration. Cyanurates, therefore, have the effect of inhibiting free chlorine by the formation of

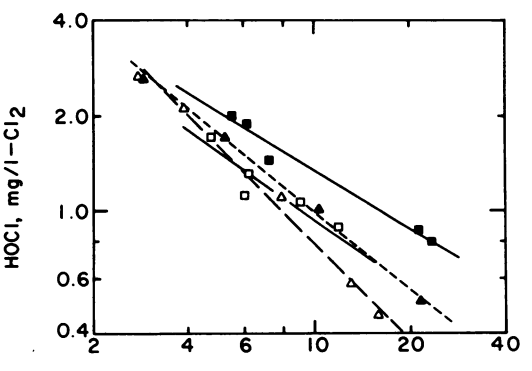

FIG. 2. Watson's Law plots for 99% *N. gruberi* cyst inactivation by chlorine at pH 5 (△) and pH 7 (▲) and chlorinated cyanurates at pH 5 (□) and pH 7 (■). (Chlorine results after Rubin et al. [18]).

chlorinated cyanurate species, which have no measurable cysticidal effect. In addition, chlorinated cyanurates may further inhibit the disinfection process by free chlorine by reacting with the cysts in such a way as to induce a qualitative change, making the cysts less vulnerable to HOCl.

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