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NEW DISINFECTION AGENTS FOR WATER

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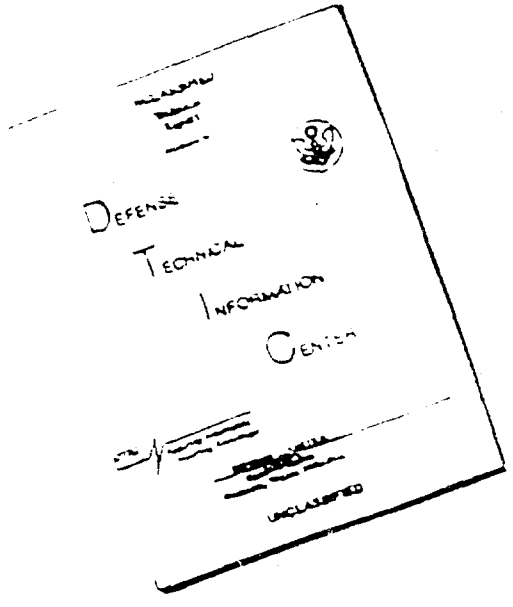

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The purpose of this work was to determine the feasibility of using organic N-chloramine agents for water disinfection in the field to possibly replace or supplement the free chlorine agent HTH which is currently used. The model N-chloramine agent chosen for this work was 3-chloro-4,4-dimethyl-2-oxazolidinone (Agent I). A variety of comparisons of Agent I and HTH were made which included their actions on bacteria, viruses, fungi, protozoa, organic impurities, and water treatment materials and their stabilities in solution and the solid state.

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PREFACE

This report was prepared by the Water Resources Research Institute at Auburn University, Auburn, Alabama 36849, sponsored jointly by the U.S. Army Research and Development Command, Fort Detrick, Frederick, MD 21701 and the Air Force Engineering and Services Center, Air Force Engineering and Services Laboratory, under Contract Number DAMD17-82-C-2257.

This report covers work performed between September 1982 and August 1983. The Principal Investigator for the project was Dr. S.D. Worley of the Department of Chemistry at Auburn University. Project officers were Mr. James C. Eaton of USAMRDC, Ft Detrick, and 1Lt Jack H. Jeter of HQ AFESC/RDVW, Tyndall AFB, FL.

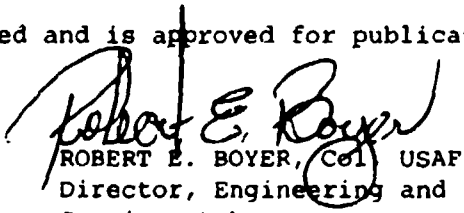
This report discusses a direct comparison of a new N-chloramine compound with calcium hypochlorite (HTH) as a water disinfectant for military field use. Citations of commercial organizations and trade names in this report do not constitute an official Department of Defense endorsement, approval, or rejection of the products or services of these organizations.

The authors wish to thank J. Price of the Alabama Department of Environmental Management for his assistance in obtaining gas chromatographs for the trihalomethane studies, E.C. Mora of the Department of Poultry Science at Auburn University for obtaining electron microscope photographs of damaged materials, B.Z. Jang of the Department of Mechanical Engineering at Auburn University for his help in tensile-strength testing, J. Aull of the Department of Chemistry at Auburn University for the loan of high-quality distilled water, and W. Keown and B. Lewis of Academic Computing Services at Auburn University for their help with the statistics program and computer-generated plots. We thank A. Riedinger of UOP, Inc., for performing the testing of agent I in a membrane filter test cell. Finally, we would especially like to thank Ms. Nancy Dickinson and Ms. Nelda Ruff for typing this document and all monthly reports.

This report has been reviewed and approved for publication by the Public Affairs Office (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

JACK H. JETER, 1Lt, USAF, BSC
Project Officer



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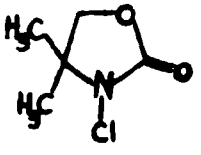
INTRODUCTION

The agent currently employed by the U.S. Army and U.S. Air Force for disinfection of field water supplies is HTH which is composed of 65 percent calcium hypochlorite and 35 percent "inert ingredients." While HTH is reasonably bactericidal, it is not particularly stable either in water solution or in the solid state. In fact improperly stored calcium hypochlorite can spontaneously ignite packaging materials (1). Furthermore, it is well known that "free chlorine" agents such as HTH react with organic impurities in water to produce toxic trihalomethanes, which have been shown to be carcinogenic in laboratory animals (2). It has been stated that N-chloramines do not react appreciably with organic material to produce toxic trihalomethanes (2). However, it has also been suggested that chloramines are much weaker bactericides than are free chlorine agents (3). We believe that there are N-chloramine agents which are adequate disinfectants and which possess other attributes which render them of possible use to the military as all-purpose water disinfectants.

One such N-chloramine agent (3-chloro-4,4-dimethyl-2-oxazolidinone, henceforth referred to as Agent I) has been studied extensively in these laboratories. Agent I was first prepared and



Agent I

and shown to be bactericidal by Kaminski and co-workers (4,5). Recent experiments in these laboratories have demonstrated that Agent I is an effective bactericide in a laboratory water treatment plant (6), that Agent I is exceptionally stable in water and in dry storage (7), that Agent I eradicates a broad spectrum of bacteria in water (8), and that Agent I is apparently nontoxic to chickens drinking water containing the agent at the 200 ppm level (9), and that it detoxifies aflatoxin (9). The cellular mechanisms of action of Agent I in inhibiting bacterial DNA, RNA, and protein synthesis have been addressed in these laboratories in a preliminary manner also (10). A general summary of the chemical and biological properties of Agent I was presented at the Fourth International Conference on Water Chlorination (11). All of this preliminary work led us to believe that Agent I, or some other chloramine with similar molecular structure, might be a better water disinfectant than HTH for military use.

The goals for the first year of contract DAMD17-82-C-2257 were to prepare sufficient quantities of Agent I for testing purposes (Task I), to compare Agent I and HTH as to their efficacies in inactivating

microgenic organisms (Task II), to compare the long-term stabilities of Agent I and HTH (Task III), to compare the corrosive properties of the two agents against DOD materials (Task IV), and to compare the two agents as to their tendencies to react with organic material in water to produce toxic trihalomethanes (Task V).

In this report each task will be addressed in turn with experimental protocol, results, and discussion being presented. This material will be followed by a section giving the primary conclusions, and finally our recommendations.

SECTION II

TASK I (PREPARATION OF MATERIALS)

The goal of Task I was to prepare sufficient quantities of Agent I for use in the other tasks and for use in military laboratories. In April 1983, 12 grams of the compound was sent to Alan Riedinger of UOP, Inc., for his use in testing the action of the material in a laboratory-scale membrane filtration unit. Also, 0.8 grams of Agent I was sent to Marilyn George of Wright-Patterson AFB in May 1983 to be used in experiments designed to evaluate its mutagenicity.

The reaction scheme used in these laboratories for preparation of Agent I is shown below. The procedure followed is to place about 100 grams

of 2-amino-2-methyl-1-propanol, about 175 mL of diethyl carbonate, and about 1 gram of sodium methoxide, all of which can be obtained commercially (e.g., from Aldrich Chemical Co.), in a dry 1000 mL round-bottom flask. Using a specially designed distillation head, the mixture is stirred and heated by means of an oil bath at 110-115°C until all of the ethanol product is removed by distillation; this generally requires 24-36 hours. Then the excess diethyl carbonate is removed by vacuum distillation (water aspirator vacuum) at 135°C. The product 4,4-dimethyl-2-oxazolidinone remains in the round-bottom distillation flask. It should be noted that we have prepared this compound also by direct reaction of the amino alcohol and phosgene gas. The product is passed through a gravity filter while warm (90°C) and crystallized from a 3:1 ethanol/hexane solution over several hours at 0°C. The product crystals are removed by suction filtration and dried under vacuum. Then about 50 grams of the 4,4-dimethyl-2-oxazolidinone are dissolved in 200 mL of water, and the mixture is placed in a specially designed Pyrex® chlorination apparatus. The mixture is allowed to stand in the presence of 20 psi of chlorine gas while being stirred at 0°C for about 30 minutes after the first crystals of Agent I form. The crystals are removed by suction filtration, and the solution is replaced in the chlorination apparatus. The chlorination and filtration procedure is repeated twice. The crystals are then dried under vacuum. If desired, purification can be accomplished by recrystallization from a carbon tetrachloride/water mixture. The yield of Agent I varies between 50 and 70 percent; the purity can be obtained as high as 99 percent. Purity is routinely verified by ^1H NMR (two sharp singlets are obtained separated by about 2.76 ppm). Some of the properties of Agent I include white, odorless, tasteless crystalline solid, m.p. 71-72.5°C, solubility in water 1200 mg/100 mL at 28°C and 800 mg/100 mL at 2°C. Its solutions have a pH of 5.2 in unbuffered water at 42 mg/L, and less than 1 percent dissociation in water to form free chlorine at room temperature.

SECTION III

TASK II (COMPARISON OF DISINFECTION EFFICACY OF AGENT I AND HTH)

The goal of Task II was to compare the efficacies of Agent I and HTH as disinfectants in water against a variety of organisms to include bacteria (Staphylococcus aureus, Pseudomonas aeruginosa, and Shigella boydii), viruses (Poliovirus type 1 and rotavirus), protozoa (Entamoeba invadens and Giardia lamblia), and fungi (Rhodotorula rubra and Candida albicans). Variables in the study were concentration, contact time, pH, and temperature.

GENERAL

The buffer salts employed in this work were reagent grade chemicals and were used without further purification. Distilled, deionized water (15 megaohm or greater) was used for the buffer solutions. A 0.05 M sodium phosphate buffer was used for pH 7.0 solutions; 0.05 M sodium acetate and 0.05 M borate-sodium hydroxide were employed for pH 4.5 and pH 9.5 studies, respectively. Buffer pH was maintained within 0.05 pH units of the target value throughout the study. The buffer solution was rendered "chlorine-demand-free" in a manner similar to that used at the U.S. Army Medical Bioengineering Research and Development Laboratory (12) by the addition of sodium hypochlorite (Clorox) at a concentration of 1-2 ppm Cl^+ followed by standing for 24 hours and then dechlorination by exposure to direct sunlight for 8 to 14 hours. The buffer solutions were checked for residual chlorine before use and were autoclaved at 121°C for 15 minutes for sterilization immediately before use. The pH and chlorine contents were monitored again after autoclaving. All glassware used for experiments involving bacteria or stability studies was made "chlorine-demand-free" by soaking in a 3-5 ppm Clorox solution for 24 hours, followed by rinsing in demand-free distilled, deionized water. Ionizable chlorine concentrations (Cl^+) were monitored by standard titrations, generally amperometric, (Wallace-Tiernan) but occasionally iodometric as a check (13).

For some experiments a synthetic "worst-case water" (henceforth referred to as WCW) was employed. The WCW consisted of 375 mg/L of each of the following inorganic salts: calcium chloride, magnesium chloride, potassium chloride, and sodium chloride, 50 mg/L bentonite clay, 30 mg/L humic acid, 0.01 percent final concentration heat treated horse serum (collected by the Auburn University School of Veterinary Medicine and heated to 60°C for 1 hour), and 5×10^5 cells/mL heat-killed (100°C for 20 minutes) Saccharomyces cerevisiae. The inorganic salts, bentonite clay, and humic acid were dissolved (or suspended) in pH 9.5 buffer and autoclaved. In a few experiments pH 7.0 buffer was used for comparison. The dead yeast cells and horse serum were added to the treatment vessel at the time the experiments were performed.

It was found that stock solutions of both chlorinating agents

could be stored without appreciable decomposition in sealed flasks in a refrigerator at a temperature near 0°C.

BACTERIA STUDIES

The strains of bacteria employed were grown from either Bactrol discs (Difco Co.) or directly from stocks supplied by the American Type Culture Collection (ATCC). The species used were Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, and Shigella boydii ATCC E9207. All bacteria were initially grown in nutrient broth and checked for authenticity by differential growth requirements, gram stain, and biochemical differentiation with the aid of API diagnostic kits. The strains were maintained on the media recommended by ATCC (trypticase soy agar for Staphylococcus and nutrient agar for Pseudomonas and Shigella). A fresh plate of the organism under study was streaked the day before use, and cells were removed from the surface and suspended in sterile saline to a cell density of $1\text{-}2\times10^8$ cfu/mL. Cell density determinations were made using a Klett-Summerson colorimeter equipped with a green filter. The instrument was calibrated by making a series of suspensions of cells in saline medium (5×10^7-1×10^{10} cells/mL) and determining readings in Klett units. Direct counts using a Petroff-Hauser counting chamber were made for each suspension. Then aliquots of serial dilutions of each suspension were plated on appropriate media, and cfu/mL were determined by counting colonies present. This calibration was done for each species of bacteria to be studied.

In a typical experiment 50 mL of buffer solution, or in some cases WCW, were placed in a 125 mL flask and inoculated with 0.5 mL of cell suspension such that the final cell density was about 1×10^6 cfu/mL. This solution of bacteria was allowed to equilibrate at a given temperature (4 or 22°C) by immersion in a thermostated water bath (±1°C) for 15 minutes under continued stirring. Then an appropriate amount of solution containing one of the chlorinating agents held at the same temperature was added so as to bring the total ionizable chlorine (Cl^+) concentration in the mixture to a specified level (either 10, 5, 2.5, or 1 ppm). The stock solution of the agent was checked by amperometric titration for concentration of Cl^+ shortly before each experiment. Residual chlorine was determined in the treatment solutions following each experiment. One mL aliquots were removed from the stirred mixture using Gilson digital pipets at various predetermined time points and quenched by one mL portions of sterile 0.02 N sodium thiosulfate. Serial dilutions of the aliquots were made into sterile saline. Then three 25 uL aliquots of each of the resulting dilutions were applied to the dried surface of a Petri dish containing the appropriate media for plating the organism under study. Using this procedure it was possible to plate four dilutions in quadrants on one Petri plate. The three replicates for each dilution were counted and averaged, and this average was used in computing the cfu/mL for that particular aliquot. Plates were read at 24- and 48-hour incubation times (at 37°C) to allow growth of weak colonies. Inactivation was considered to be at least 99.999 percent when no

colonies were detected in the thiosulfate quenched aliquots. In these experiments one colony per 25 µL of thiosulfate quenched aliquot of sample is equivalent to 80 cfu/mL in the original reaction flask.

Assay conditions consisted of the following combinations of pH, temperature, and organic load: pH 7, 22°C; pH 7, 4°C; pH 4.5, 22°C; pH 9.5, 22°C; pH 9.5, 4°C; WCW, pH 9.5, 4°C. For each assay condition listed, except pH 9.5, 4°C and WCW, all four concentrations of Cl^+ for both agents were run. A lesser number of concentrations were generally employed in the latter two cases. Triplicate runs were performed also for most experiments, and at least duplicate runs were made for all experiments.

Data analyses were conducted using the Statistical Analysis System (SAS, Cary, N.C.), operating on an IBM 3032 computer at Auburn University. The General Linear Model procedure was used for fitting a regression equation to predict inactivation time and for performing the analysis of variance.

The data for the experiments pertaining to the actions of Agent I and HTH against Staphylococcus aureus in buffered, demand-free water are presented in Table 1 and in Figures 1 and 2 (additional figures are in the appendix). The straight-line curves generated in the computer-drawn plots for Staphylococcus probably do not realistically represent the behavior of Agent I for this organism (see Figure 2). The data in the tables are better indicators of the action of Agent I. As can be seen, HTH is the superior disinfectant for this organism in demand-free water. However, Agent I will eradicate the organism, given sufficient contact time. It should be noted that Agent I is more active at pH 9.5, while HTH is less active at this pH, relative to pH 7.0 and pH 4.5. An obvious conclusion is that "free chlorine" is more effective against Staphylococcus than "combined chlorine." Agent I is completely undissociated at pH 4.5 and 7.0, but it is somewhat less stable at pH 9.5 (see results in Task III also). Thus a higher concentration of "free chlorine" at pH 9.5 makes Agent I a better disinfectant at this pH, although it is still not extremely impressive because "free chlorine" exists primarily as hypochlorite rather than hypochlorous acid at that pH; hypochlorous acid is a much better bactericide than hypochlorite (14).

Although HTH is clearly a better disinfectant against S. aureus in demand-free water, this is not necessarily the case when organic demand is introduced in synthetic WCW. The data for WCW and Staphylococcus aureus are presented in Table 2 and Figure 2. Neither agent perfoms well under these circumstances. The organic load in WCW obviously rapidly eliminates the free chlorine from HTH. The resulting combined chlorine is probably less efficient against Staphylococcus than is the combined chlorine in Agent I (see 5.0 ppm Cl^+ data), because Agent I eventually inactivates all of the organisms, while HTH could not under these conditions. The statistical analyses here were not reliable, because in general, the organisms were not killed by either agent within the contact time considered. At a high concentration of HTH

TABLE 1. ACTION OF AGENT I AND HTH AGAINST STAPHYLOCOCCUS AUREUS IN BUFFER ED DEMAND-FREE WATER

Agent and Conditions[a]	Concentration of Cl^+ (ppm)	Log_{10} cfu/mL at t_0	Time Predicted for 0 cfu/mL (min)[b]	Time Points Considered (min)	Time Interval for 0 cfu/mL obs.
I, pH 7, 22C	10.0	6.27	31.6	0,1,2,5,10,30,60	10-30
	5.0	6.33	41.4	0,1,2,5,10,20,30,60	20-30
	2.5	5.88	291.1	0,5,10,30,60,120	>120
	1.0	5.92	276.8	0,5,10,30,60,120,240	120-240
I, pH 4.5, 22C	10.0	6.06	57.0	0,2,5,10,20,30,60,90	30-60
	5.0	6.67	65.3	0,2,5,10,20,30,60,90	30-60
	2.5	6.03	231.2	0.2,5,10,20,30,60,90	>90
	1.0	6.04	250.8	0,2,5,10,30,60,120,240	120-240
I, pH 9.5, 22C	10.0	6.51	21.5	0,1,2,5,10,20,30	10-20
	5.0	6.73	23.2	0,1,2,5,10,20,30,60	10-20
	2.5	6.20	55.8	0,1,2,5,10,20,30,60	30-60
	1.0	6.13	133.0	0,1,2,5,10,20,30,60	>60
HTH, pH 9.5, 22C	2.5	6.03	1.9	0,1,2,5,10,20,30,60	1-2
	1.0	5.77	4.7	0,1,2,5,10,20,30,60	2-5
I, pH 9.5, 4C	10.0	6.85	67.2	0,1,2,5,10,30,60,120,240	30-60
	5.0	6.48	132.3	0,1,2,5,10,30,60,120,240	60-120
	2.5	6.44	257.5	0,1,2,5,10,30,60,120,240	120-240
	1.0	6.21	3076	0,1,2,5,10,30,60,120,240	>240
HTH, pH 9.5, 4C	10.0	6.27	1.9	0,1,2,5,10,30,60,120,240	1-2
	5.0	6.40	4.9	0,1,2,5,10,30,60,120,240	2-5
	2.5	6.39	9.4	0,1,2,5,10,30,60,120,240	5-10
	1.0	6.04	27.7	0,1,2,5,10,30,60,120,240	10-30

a. If HTH is not listed, all cfu/mL were eradicated in less than 1 minute; all data are an average of three replicates.

b. Statistical analysis (see text).

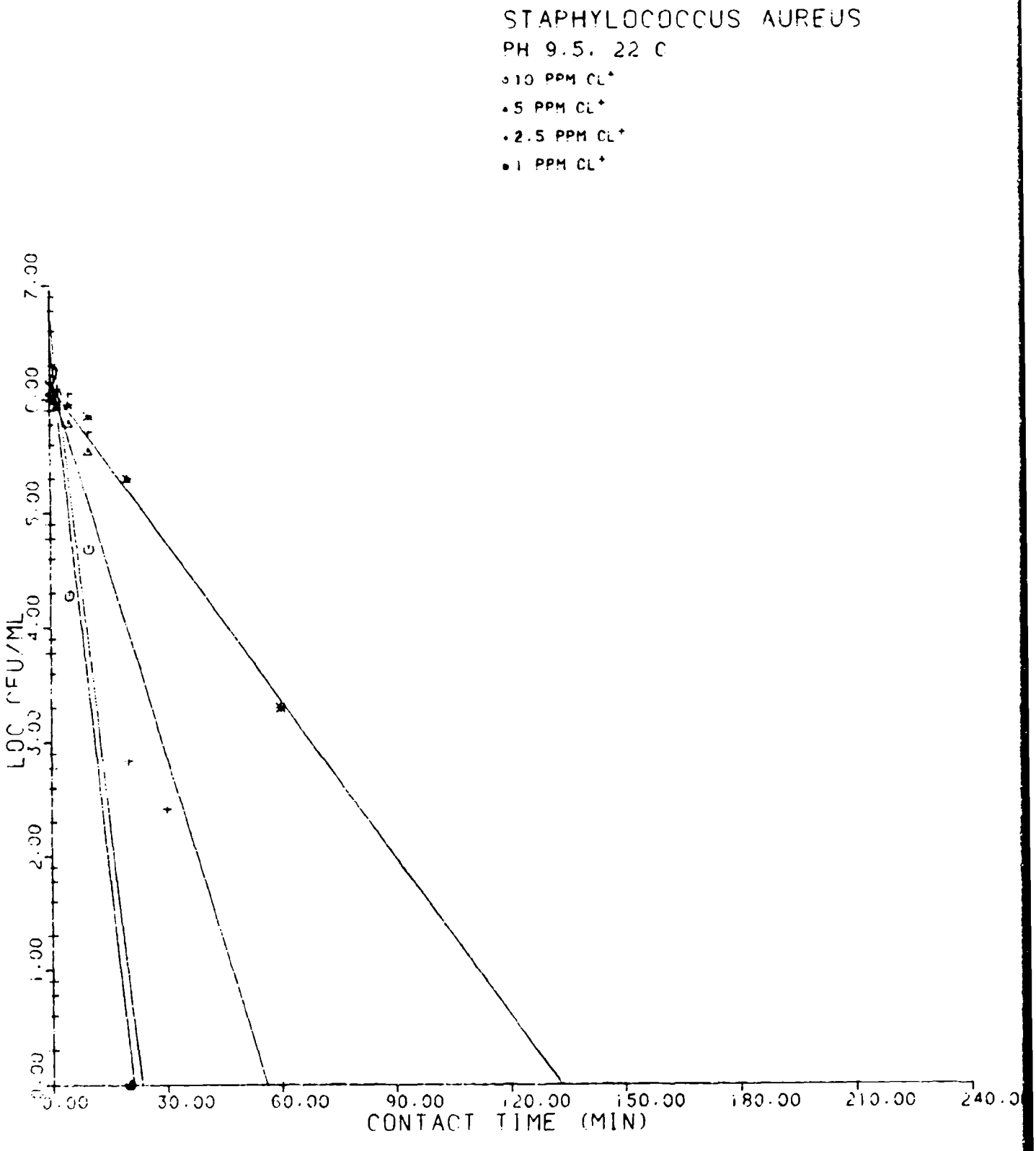


Figure 1. Disinfection of Staphylococcus aureus by Agent I at pH 9.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

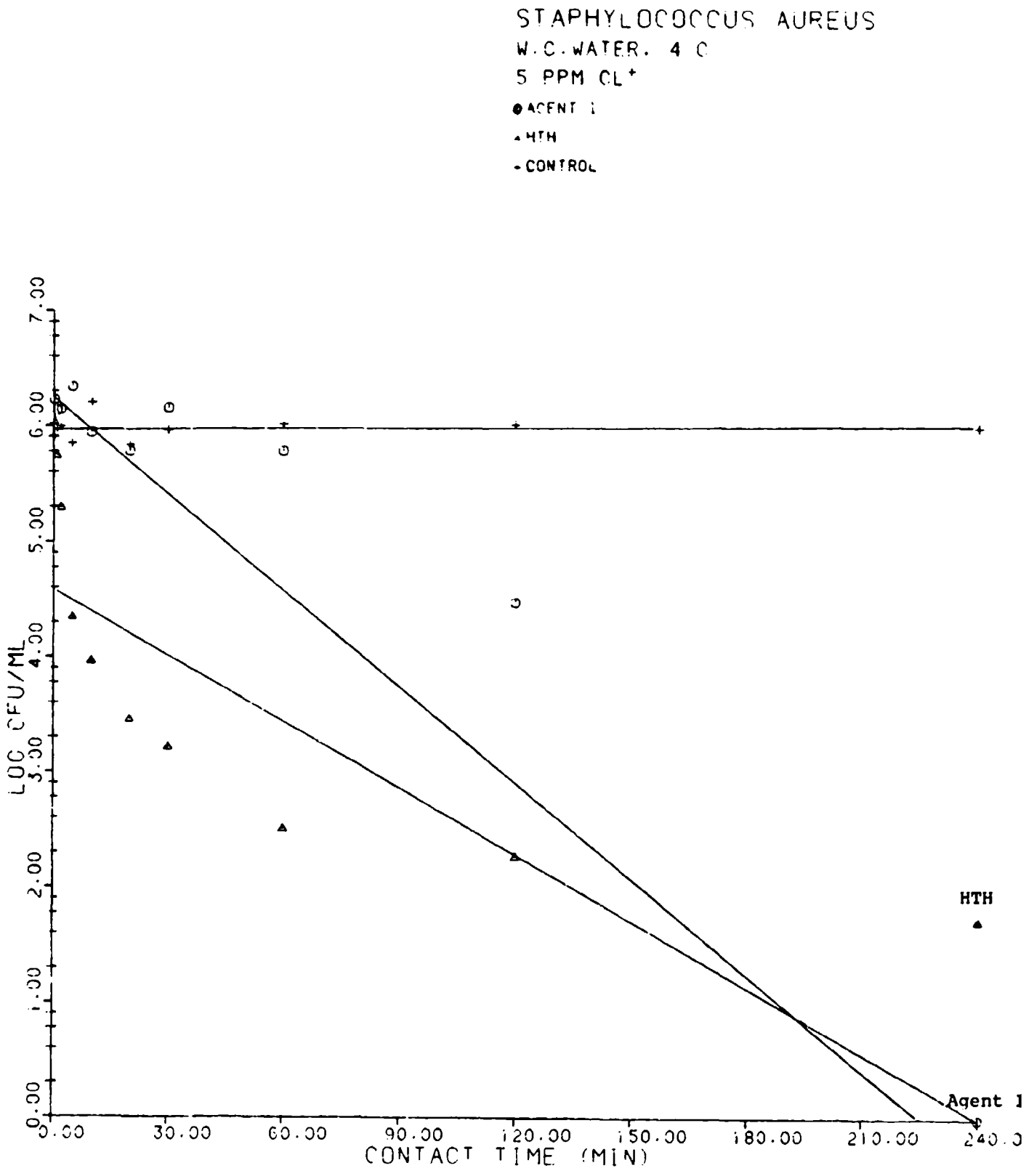


Figure 2. Disinfection of Staphylococcus aureus by Agent I and HTH in WCW at 5 ppm Potentially Ionizable Chlorine.

TABLE 2. ACTION OF AGENT I AND HTH AGAINST STAPHYLOCOCCUS AUREUS IN WCW

Agent and Conditions[a]	Concentration of Cl^+ (ppm)	Log_{10} cfu/mL at t_0	Time Predicted for 0 cfu/mL (min)[b]	Time Points Considered (min)	Time Interval for 0 cfu/mL obs.
I, pH 9.5, 4C	10.0	6.34	264.3	0,1,2,5,10,20,30,60,120,240	120-240
	5.0	6.25	225.0	0,1,2,5,10,20,30,60,120,240	120-240
	2.5	6.20	849.0	0,1,2,5,10,20,30,60,120,240	>240
	1.0	5.85	3076	0,5,10,30,60,120,240	>240
HTH, pH 9.5, 4C	10.0	6.52	4.6	0,1,2,5,10,20,30,60,120,240	2-5
	5.0	4.59	306.0	0,1,2,5,10,20,30,60,120,240	>240
	2.5	5.77	586.3	0,1,2,5,10,20,30,60,120,240	>240
	1.0	5.98	1596	0,5,10,30,60,120,240	>240

a. See Table 1 for explanation of terms.
b. Statistical analysis (see text).

(10.0 ppm Cl^+) the organisms are overwhelmed before the organic demand completely consumes HTH, but at lower concentrations HTH is not an adequate disinfectant.

It should be noted that all of the data for Staphylococcus aureus are considered slightly less reliable than those for the other two bacteria in this study because S. aureus was studied first while protocols were still being developed and perfected.

The data for Pseudomonas aeruginosa are presented in Table 3, Figures 3 and 4, and several figures in the appendix. Clearly, Agent I is a better bactericide against this organism than against S. aureus. HTH remains the better disinfectant, except in WCW, but by a considerably lesser margin. Again, both agents perform less satisfactorily at lower temperatures. Agent I would be an adequate disinfectant for Pseudomonas in the field. Since Pseudomonas slowly is inactivated by the pH 4.5 and pH 9.5 buffer solutions (controls), future experiments should be performed at pH 5 and pH 9.

The data for the organism Shigella boydii are summarized in Table 4, in Figures 5 and 6, and in several figures in the appendix. Shigella is slowly killed by the two extreme buffer (pH 4.5 and pH 9.5) controls, but, of course, much more rapidly when either agent is present. In fact, Agent I is more effective against Shigella than against Pseudomonas. Both agents kill Shigella rapidly in WCW. There seems to be no temperature effect for Agent I action on Shigella at pH 7.0. Agent I could certainly be used as a disinfectant against this organism in the field.

In summary, Agent I killed the three bacteria tested in this study, although, in general, longer contact times were necessary than for HTH, except for WCW for which Agent I was, in general, the better disinfectant. Prior studies in these laboratories (8) had established that Agent I is more effective against some strains of bacteria (eg. Klebsiella pneumoniae, Proteus vulgaris, and Serratia marcescens) than is HTH. This provides circumstantial evidence that Agent I itself is the active bactericide rather than the small amount of "free chlorine" produced from it upon hydrolysis.

VIRUS STUDIES

The research protocol called for the testing of Agent I and HTH against poliovirus type 1 and a rotavirus. Studies with the rotavirus have not been completed; they will be included in a later report.

Poliovirus type 1, attenuated Chat strain, was obtained from the American Type Culture Collection (ATCC). It was propagated and assayed in a rhesus monkey kidney cell line, LLC-MK_2 Derivative, CCL 7.1, which was also obtained from ATCC. The stock preparation of poliovirus was harvested from infected cells by three cycles of rapid freezing and thawing, followed by centrifugation at 5000 x g to remove particulate cell debris from the suspension of the virus in cell culture fluids.

TABLE 3. ACTION OF AGENT I AND HTH AGAINST PSEUDOMONAS AERUGINOSA

Agent and Conditions[a,b]	Concentration of Cl^+ (ppm)	Log_{10} cfu/mL at t_0	Time Predicted for 0 cfu/mL (min)	Time Points Considered (min)	Time Interval for 0 cfu/mL obs. (min)
I, pH 7, 22C	10.0	6.08	2.1	0,1,2,5,10,20	1-2
	5.0	6.44	9.4	0,1,2,5,10	5-10
	2.5	6.47	10.4	0,1,2,5,10,20	5-10
	1.0	6.38	25.6	0,1,2,5,10,20	>20
I, pH 4.5, 22C	10.0	4.79	4.4	0,1,2,5,10	2-5
	5.0	6.36	5.0	0,1,2,5,10	2-5
	2.5	6.21	9.6	0,1,2,5,10	5-10
	1.0	5.90	9.5	0,1,2,5,10	5-10
I, pH 9.5 22C	10.0	6.73	1.0	0,1,2,5,10	0-1
	5.0	6.10	2.1	0,1,2,5,10	1-2
	2.5	5.41	5.0	0,1,2,5,10	2-5
	1.0	5.38	17.4	0,1,2,5,10	>10
I, pH 7, 4C	10.0	4.95	9.2	0,1,2,5,10,20	5-10
	5.0	5.63	9.8	0,1,2,5,10,20	5-10
	2.5	5.70	18.4	0,1,2,5,10,20	10-20
	1.0	5.78	38.6	0,1,2,5,10,20	>20
I, pH 9.5, 4C	2.5	3.61	5.0	0,1,2,5,10	2-5
I, pH 9.5, 4C, WCW	2.5	6.04	56.6	0,1,2,5,10,20,30,60	30-60
HTH, pH 9.5, 4C, WCW	2.5	5.63	96.1	0,1,2,5,10,20,30,60	>60

a. See Table 1 for explanation of terms.
b. If HTH is not listed, all cfu/mL were eradicated in less than 1 minute.

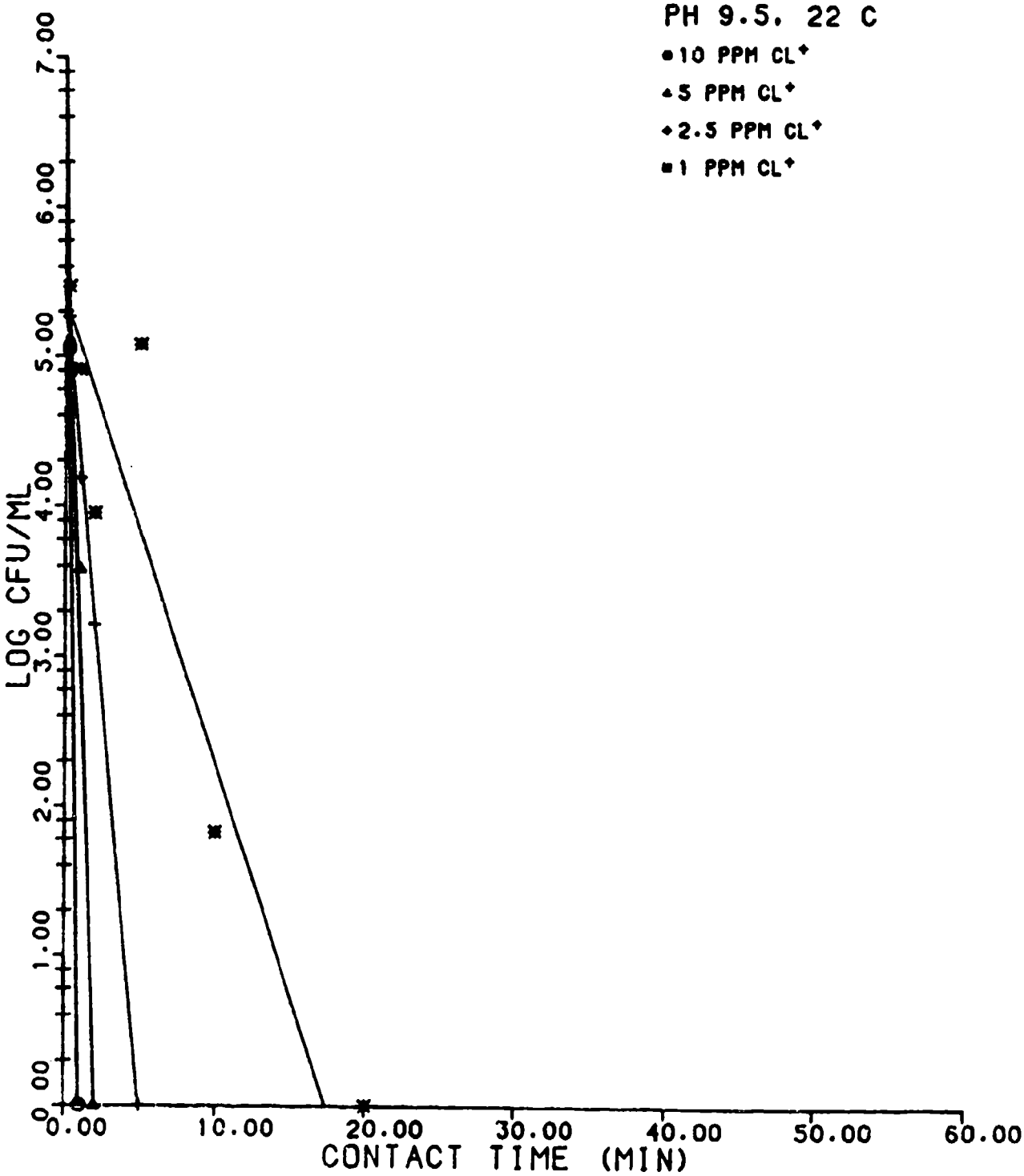


Figure 3. Disinfection of Pseudomonas aeruginosa by Agent I at pH 9.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

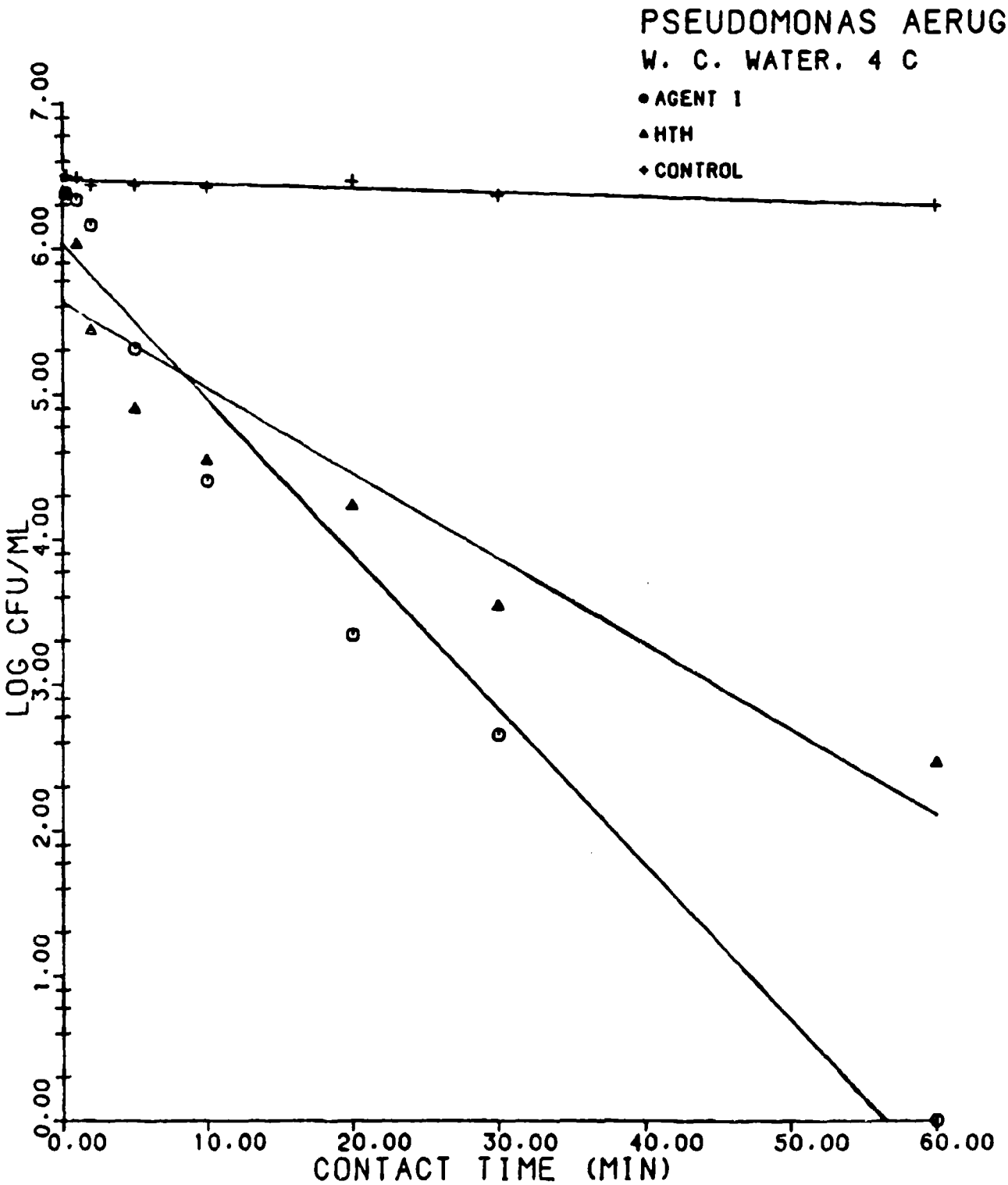


Figure 4. Disinfection of Pseudomonas aeruginosa by Agent I and HTH in WCW at 2.5 ppm Potentially Ionizable Chlorin..

TABLE 4. ACTION OF AGENT I AND HTH AGAINST *SHIGELLA BOYDII*

Agent and Conditions[a,b]	Concentration of Cl^+ (ppm)	Log_{10} cfu/mL at t_0	Time Predicted for 0 cfu/mL (min)	Time Points Considered (min)	Time Interval for 0 cfu/mL obs. (min)
I, pH 7, 22C	10.0	5.30	4.6	0,1,2,5,10,20	2-5
	5.0	6.75	5.0	0,1,2,5,10,20	2-5
	2.5	7.02	10.4	0,1,2,5,10,20	5-10
	1.0	6.82	20.9	0,1,2,5,10,20	10-20
I, pH 4.5, 22C	10.0	6.50	2.0	0,1,2,5,10	1-2
	5.0	7.88	2.2	0,1,2,5,10	1-2
	2.5	6.30	4.9	0,1,2,5,10	2-5
	1.0	7.02	5.2	0,1,2,5,10	2-5
I, pH 9.5, 22C	10.0	4.36	<1.0	0,1,2,5,10	0-1
	5.0	4.84	1.9	0,1,2,5,10	1-2
	2.5	5.11	5.1	0,1,2,5,10	2-5
	1.0	4.80	10.0	0,1,2,5,10	5-10
I, pH 7, 4C	10.0	5.21	4.6	0,1,2,5,10,20	2-5
	5.0	6.50	5.0	0,1,2,5,10,20	2-5
	2.5	6.14	9.4	0,1,2,5,10,20	5-10
	1.0	6.28	19.6	0,1,2,5,10,20	10-20
I, pH 9.5, 4C, WCW[c]	2.5	5.08	4.8	0,1,2,5,10	2-5

a. See Table 1 for explanation of terms.
b. If HTH is not listed, all cfu/mL were eradicated in less than 1 minute.
c. The *Shigella boydii* controls do not survive at pH 9.5, 4C. The WCW seems to prolong their lifetimes.

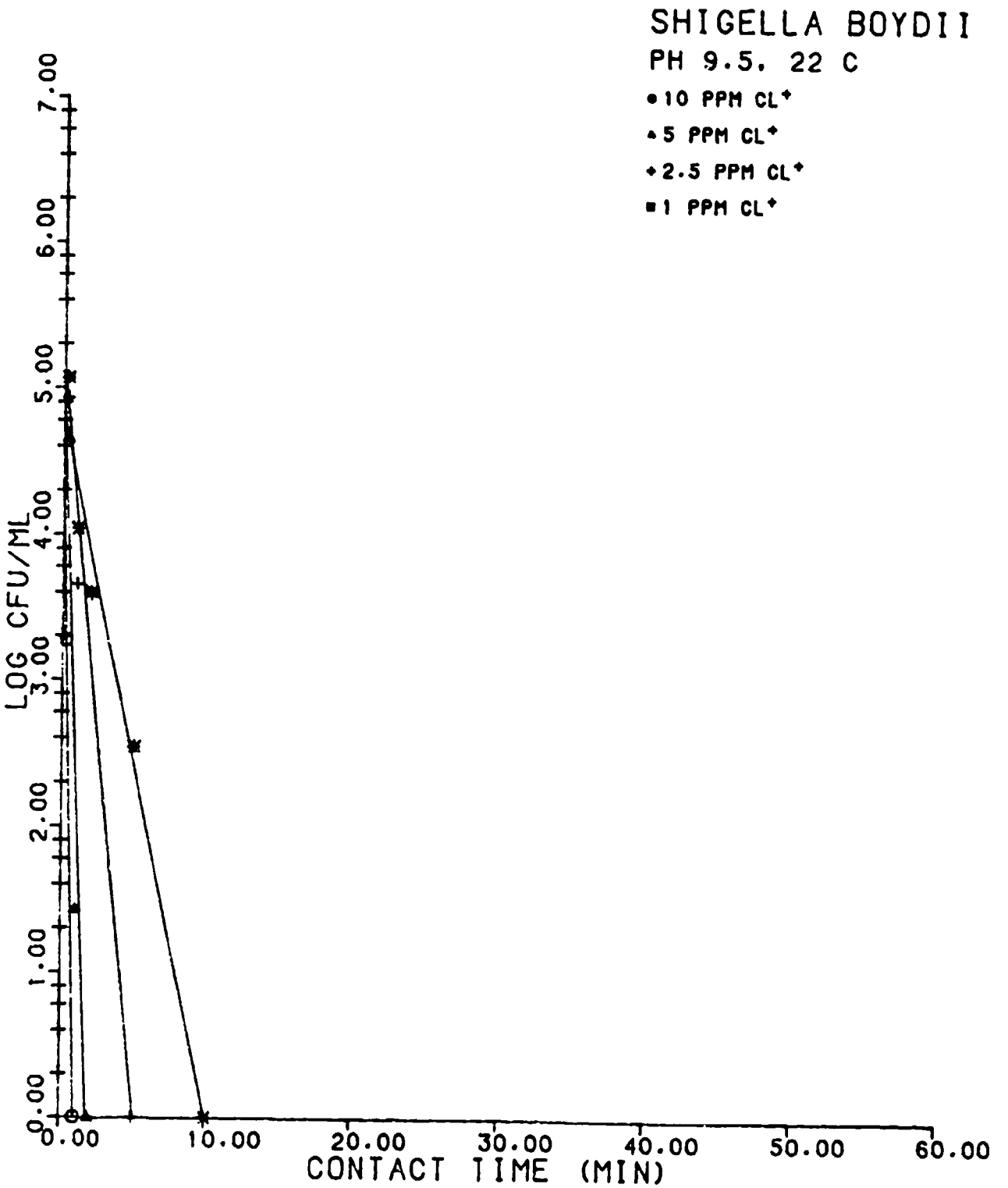


Figure 5. Disinfection of Shigella boydii by Agent I at pH 9.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

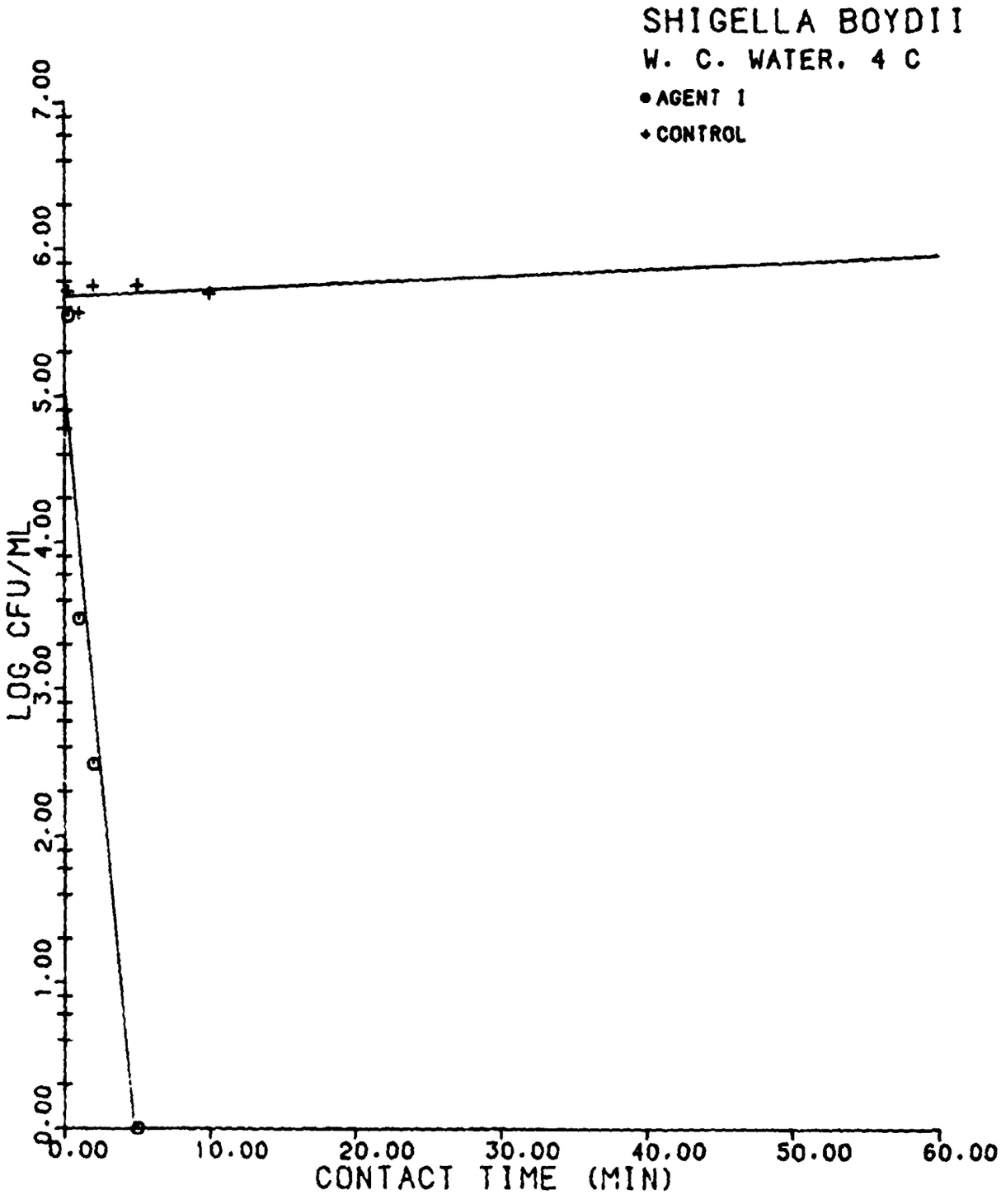


Figure 6. Disinfection of Shigella boydii by Agent I in WCW at 2.5 ppm Potentially Ionizable Chlorine.

The virus-containing supernatant fluids were stored at -70°C in heat-sealed ampules. Titrations were done in 96-well microtiter tissue culture plates using log dilutions and inoculating 0.2 mL in each of five replicate wells of cells per dilution.

For virucidal assays, the poliovirus stock was diluted to approximately 10^6 $TCID_{50}$ (tissue culture infective dose at 50 percent endpoint) in buffer or WCW at an appropriate pH. Viral aggregation was checked by filtration through 100 and 50 nm filters of virus diluted in buffer. Aggregation was quite prominent at pH 4.5 with the 100 and 50 nm filters reducing the titer by more than 2 logs and 4 logs, respectively. At pH 7.0 the titers were decreased by 1.25 logs and 4 logs after filtration through the 100 and 50 nm filters, respectively. At pH 9.5 the titer was not significantly reduced by the 100 nm filter, and the 50 nm filter removed less than 2 logs of virus. Other workers have reported that poliovirus virions commonly aggregate at pH 7.0 and lower; whereas aggregation does not occur at alkaline pH (15).

Assays for virucidal effectiveness of Agent I and HTH were done in buffers at pH 4.5, 7.0, 9.5, and in WCW at pH 9.5 prepared as described above. Temperature was controlled at 4°C or 22°C with water baths in a walk-in refrigerator. The chlorinating agents were diluted in the appropriate buffer at 2X the concentration for the assay, generally four different concentrations were assayed in the same experiment. The virus was diluted in the appropriate buffer to a concentration of about 2×10^6 $TCID_{50}$ per 0.2 mL. The 2X buffer and diluted virus were equilibrated to proper temperature in the water bath. The equilibration time was 15 minutes for the buffers and 60 minutes for WCW. After equilibration, an equal volume of virus and 2X chlorinating agent were combined, mixed by vortexing, and returned to the water bath. Virus controls consisted of an equal volume of buffers at the appropriate pH, mixed with the virus diluted in the buffer. At times of 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours the reaction mixtures were vortexed, and a 1.0 mL sample was removed and immediately added to 1.0 mL of sterile 0.02 N sodium thiosulfate as a quencher for the chlorine. Serial log dilutions were made immediately in cell culture medium, and five replicate microtiter cell cultures were inoculated for each dilution at 0.2 mL per well. Inoculated cell cultures were incubated at 37°C in a humidified incubator in the presence of 5 percent CO_2. Cultures were observed periodically for viral cytopathic effects, and final observations were recorded 7 days after inoculation. The titer of virus in each assay was calculated by the Reed-Muench method (16) and expressed as $TCID_{50}$. A sample from the virus control was processed and titrated at each time interval. Virucidal effects of the chlorinating agents were determined by the percent reduction in titer of virus in reaction mixtures compared to the control at each time of sampling. Each assay was repeated from two to four times on separate dates.

Agent I was found to be less efficient than HTH at inactivating poliovirus type 1 (Tables 5 and 6). A greater concentration of ionizable chlorine and a longer contact time were required for Agent I

TABLE 5. VIRUCIDAL EFFECT OF AGENT I AGAINST POLIOVIRUS TYPE 1 (CHAT STRAIN)

Conditions of assays			Percent reduction in titer by total chlorine from agent I[a,b]			
Contact Time	pH	Temp.	400 ppm	100 ppm	25 ppm	10 ppm
10 min	4.5	22 C	0	0	0	0
	7.0	4 C	0	0	0	0
	7.0	22 C	89.70	44.90	26.90	13.30
	9.5	22 C	93.70	15.80	15.80	32.20
	9.5	4 C	73.10	0	0	0
(WCW)	9.5	4 C	0	0	0	0
1 hr	4.5	22 C	56.20	0	0	0
	7.0	4 C	31.60	0	0	0
	7.0	22 C	97.90	77.20	57.80	34.30
	9.5	22 C	99.99	99.99	96.30	54.80
	9.5	4 C	99.99	94.40	49.20	28.00
(WCW)	9.5	4 C	99.30	43.90	15.80	15.80
2 hr	4.5	22 C	56.20	31.60	0	0
	7.0	4 C	31.60	0	0	0
	7.0	22 C	99.80	77.80	70.90	63.20
	9.5	22 C	99.99	99.99	99.99	95.40
	9.5	4 C	99.99	99.40	62.10	47.80
(WCW)	9.5	4 C	99.99	60.80	37.00	15.80
4 hr	4.5	22 C	56.20	31.60	0	0
	7.0	4 C	56.20	0	0	0
	7.0	22 C	99.99	88.40	68.00	67.20
	9.5	22 C	99.99	99.99	99.99	99.10
	9.5	4 C	99.99	99.99	96.10	74.00
(WCW)	9.5	4 C	99.99	99.70	60.80	25.00
24 hr	4.5	22 C	99.32	90.00	0	0
	7.0	4 C	99.90	91.80	17.80	0
	7.0	22 C	99.99	99.99	97.70	95.70
	9.5	22 C	99.99	99.99	99.99	99.96
	9.5	4 C	99.99	99.99	99.99	91.60
(WCW)	9.5	4 C	99.99	99.99	99.90	54.80

a. Numbers for percent reduction in titer are means of three or four separate assays, or the results of two separate assays in which the results were identical.
b. Number of 99.99 represents greater than or equal to 99.99 percent reduction in titer.

TABLE 6. VIRUCIDAL EFFECT OF HTH AGAINST POLIOVIRUS TYPE 1 (CHAT STRAIN)

Conditions of assays			Percent reduction in titer by total chlorine from HTH[a,b]			
Contact Time	pH	Temp.	50 ppm	25 ppm	10 ppm	5 ppm
10 min	4.5	22 C	99.99	99.99	99.90	ND[c]
	7.0	4 C	99.99	99.90	0	ND
	7.0	22 C	99.99	99.99	99.99	36.7
	9.5	22 C	99.99	99.99	99.99	ND
	9.5	4 C	99.99	99.99	0	ND
(WCW)	9.5	4 C	99.99	97.2	0	ND
30 min	4.5	22 C	99.99	99.99	99.90	ND
	7.0	4 C	99.99	99.99	0	ND
	7.0	22 C	99.99	99.99	99.99	ND
	9.5	22 C	99.99	99.99	99.99	ND
	9.5	4 C	99.99	99.99	17.80	ND
(WCW)	9.5	4 C	99.99	99.63	17.80	ND
1 hr	4.5	22 C	99.99	99.99	99.90	ND
	7.0	4 C	99.99	99.99	31.60	ND
	7.0	22 C	99.99	99.99	99.99	62.4
	9.5	22 C	99.99	99.99	99.99	ND
	9.5	4 C	99.99	99.99	56.20	ND
(WCW)	9.5	4 C	99.99	99.93	24.70	ND
2 hr	4.5	22 C	99.99	99.99	99.99	ND
	7.0	4 C	99.99	99.99	17.80	ND
	7.0	22 C	99.99	99.99	99.99	92.8
	9.5	22 C	99.99	99.99	99.99	ND
	9.5	4 C	99.99	99.99	76.10	ND
(WCW)	9.5	4 C	99.99	99.95	73.10	ND
4 hr	4.5	22 C	99.99	99.99	99.99	ND
	7.0	4 C	99.99	99.99	56.20	ND
	7.0	22 C	99.99	99.99	99.99	95.0
	9.5	22 C	99.99	99.99	99.99	ND
	9.5	4 C	99.99	99.99	90.00	ND
(WCW)	9.5	4 C	99.99	99.99	76.10	ND

a. Numbers for percent reduction in titer are means of three or four separate assays, or the results of two separate assays in which the results were identical.
b. Number of 99.99 represents greater than or equal to 99.99 percent reduction in titer.
c. No determination.

to inactivate poliovirus. A contact time of 10 minutes with HTH at 50 ppm free chlorine resulted in greater than a 99.99 percent reduction in titer at all assay conditions; whereas, 400 ppm total combined chlorine from Agent I effected only a 93.7 percent reduction in titer in 10 minutes at pH 9.5, 22°C, which were the optimal assay conditions for Agent I. A 99.99 percent reduction in titer was effected by Agent I at 100 ppm and 25 ppm total chlorine in 1 hour and 2 hours contact times, respectively, at pH 9.5, 22°C. At 10 ppm total chlorine Agent I reduced the titer by 99.96 percent in 24 hours of contact time at pH 9.5, 22°C. In contrast, 10 ppm free chlorine from HTH reduced the titer more than 99.99 percent in 10 minutes contact time at pH 7.0, 22°C and pH 9.5, 22°C. At 5 ppm free chlorine for HTH at pH 7.0, 22°C the titer was decreased by only 36.7 percent in 10 minutes and only 95.0 percent after 4 hours contact. The minimal effective concentration of HTH was only 10 ppm free chlorine, and this was dependent upon the assay conditions (Table 6).

The virucidal effects of both HTH and Agent I were decreased by the organic load of WCW, although the magnitude of the change was proportionally less for Agent I (Figures 7 and 8). Inactivation curves for Agent I against poliovirus type 1 consistently had a shoulder effect at the beginning of the curve (Figures 7, A-17, and A-18). The shoulder effect may have been due to aggregation of virions; however, it is possible that mechanism of interaction of Agent I with the protein surface of the poliovirus virion results in biphasic kinetics.

In summary, neither agent kills poliovirus type 1 in reasonable contact time at normal disinfection concentration of ionizable chlorine (2-5 ppm). HTH does do a better job against this organism than does Agent I, but Agent I will eradicate it given sufficiently long contact time.

PROTOZOA STUDIES

The IP-1 strain of Entamoeba invadens and the Portland strain of Giardia lamblia were grown in 15 mL borosilicate glass culture tubes containing TPI or TYI-S-33 medium (17). Stock cultures were maintained by transferring each week 1.5 mL of a 7-day culture into 10 mL of medium containing gentamycin and tetracycline at concentrations of 100 µg/mL. The transfer procedure involved chilling the cultures in an ice bath for 15 minutes to facilitate detachment from the surface of the culture tubes and mixing by inverting the tubes to establish a uniform suspension of organisms. The transfer procedures and processing for assays with the chlorinating agents were the same for both organisms.

For assays with Agent I and HTH, chilled organisms were washed three times in the appropriate buffer or WCW by suspending the contents of one culture tube in 50 mL of buffer and centrifuging at 750 x g for 15 minutes in a refrigerated centrifuge. The washed organisms were finally suspended in 10 mL of chilled buffer. The concentration of washed organisms was determined by adding 1.0 mL from the 10 mL

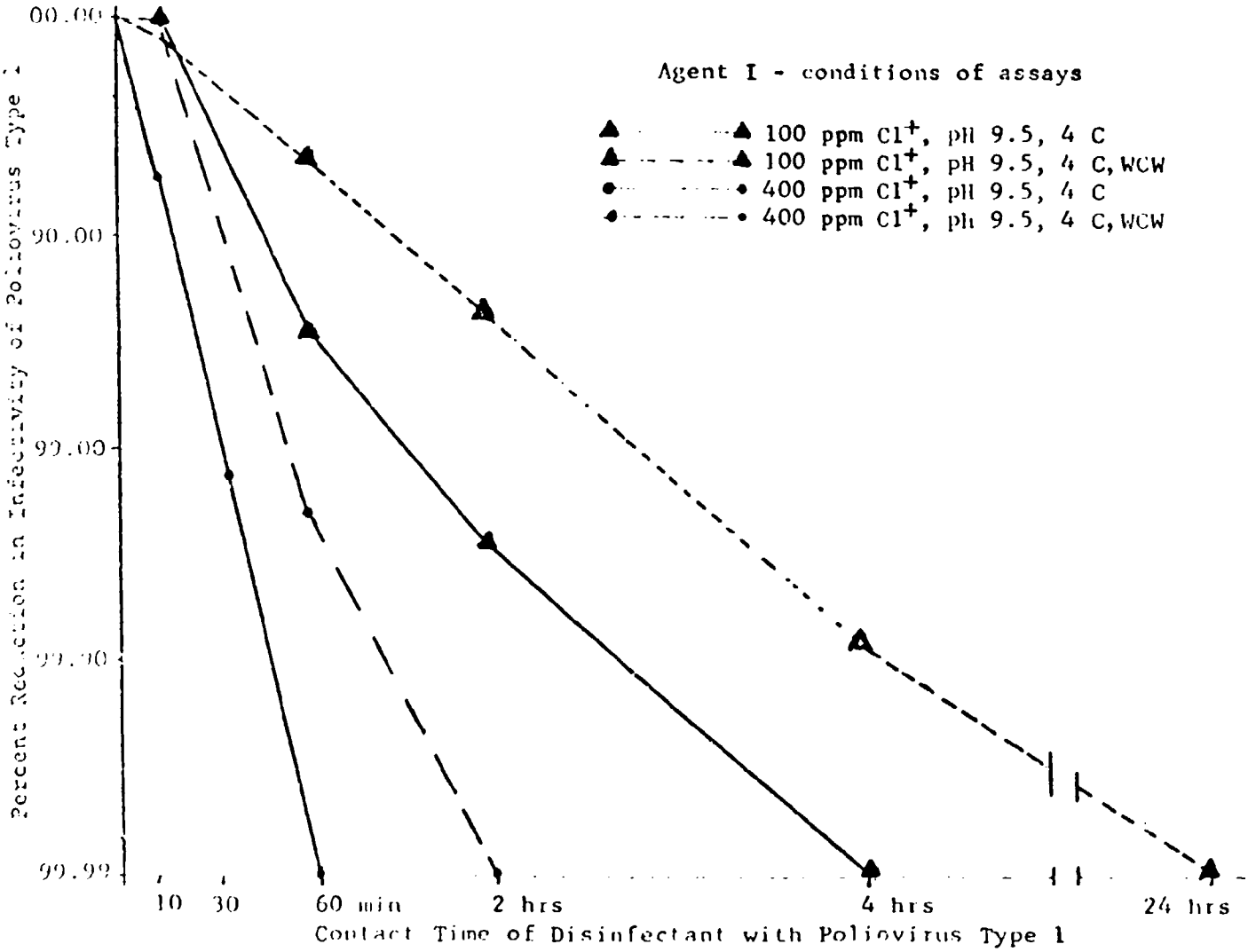


Figure 7. Inactivation of Poliovirus Type 1 (Chat Strain) by Agent I - Effect of Organic Load on Rate of Inactivation.

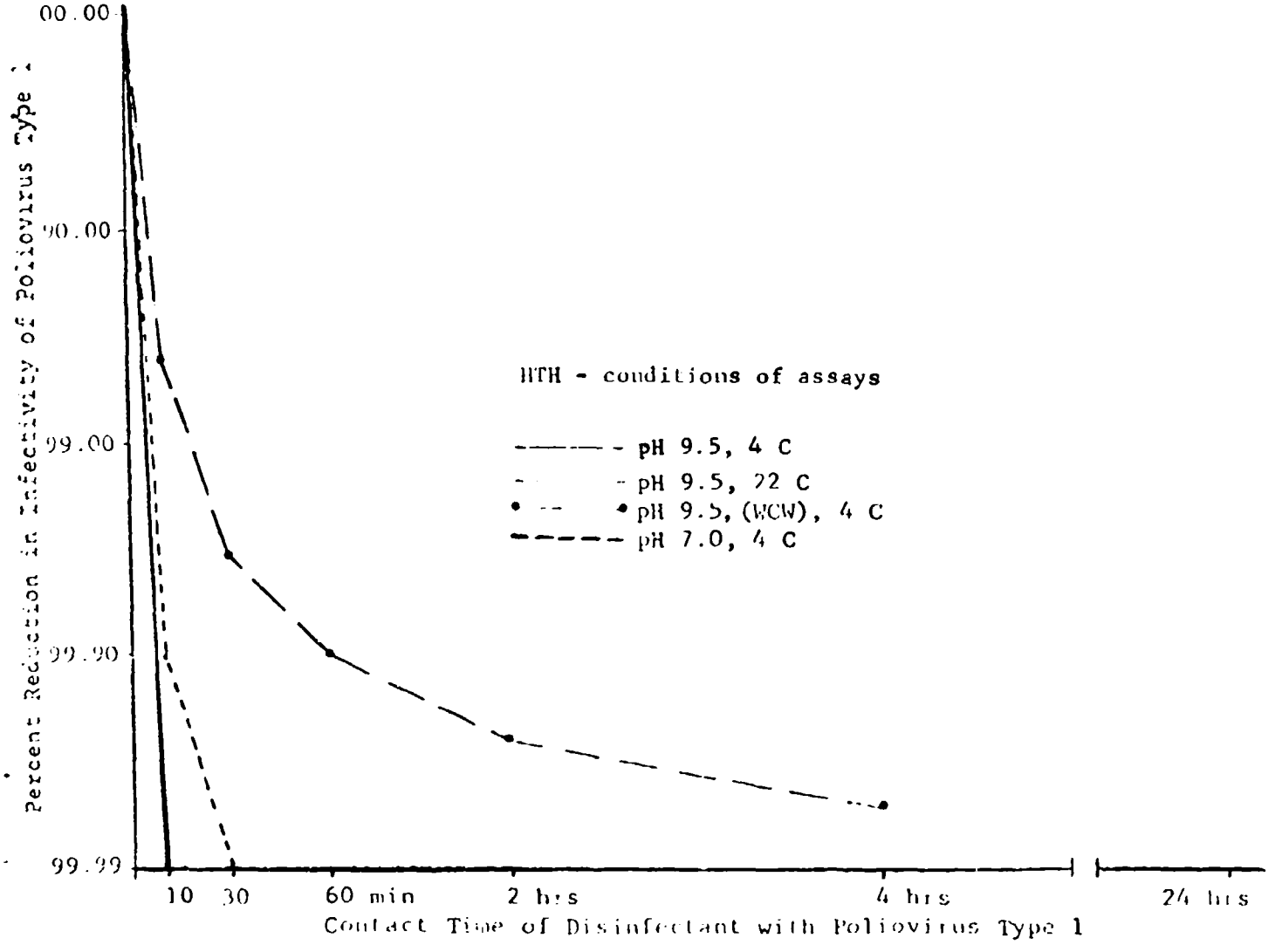


Figure 8. Inactivation of Poliovirus Type 1 (Chat Strain) by HTH at Concentration of 25 ppm Cl^+ - Effect of Organic Load on Rate of Inactivation.

suspension to 2.0 mL of saline with 2.0 percent formalin and counting with a model ZBI Coulter counter. The remaining 9.0 mL of organisms in buffer were diluted further to a concentration of 2 x 10^6/mL based on the cell count. Agent I and HTH were diluted in the appropriate buffer to concentrations of 20, 10, 4, 2 and 1 ppm ionizable chlorine and placed in a water bath at 4°C or 22°C to equilibrate for 15 minutes before mixing with the protozoa to assay for killing. It was found that pH 4.5 buffer rendered both E. invadens and G. lamblia nonviable, and therefore assays at pH 4.5 could not be done. After temperature equilibration (15 minutes for buffers and 60 minutes for WCW), equal volumes of disinfectant at each dilution and the suspension of protozoa were mixed and returned to the water bath. Controls consisted of mixing an equal volume of buffer with the suspension of organisms and maintaining in the water bath under the same conditions as the killing assay. At 2, 5, and 10 minutes after mixing, 1.0 mL samples were removed from each assay reaction tube and from the controls. The 1.0 mL samples were immediately added to 1.0 mL of sterile 0.02 N sodium thiosulfate to quench the chlorine and stop the action of the disinfectant. The individual samples in thiosulfate were centrifuged at 750 x g for 15 minutes in a refrigerated centrifuge. The supernatant was discarded, and the pellet of organisms was suspended in culture medium and transferred to borosilicate glass culture tubes and incubated at the appropriate temperature (25°C for E. invadens and 37°C for G. lamblia). The cultures were examined daily for evidence of viable cell growth. The results were based on microscopic assessment of growth of viable organisms in culture tubes. Growth of organisms processed from the buffer controls was the standard for comparison for evidence of killing by the disinfectants. A subjective scoring system was used to record the results, since reproducible methods of precise quantitation do not exist. The method for recording the results was as follows:

(+) - growth of organisms essentially the same as those of buffer controls.

(±) - greater than 90 percent reduction in growth of viable organisms compared to buffer controls based on subjective estimation.

(-) - no evidence of growth of viable organisms treated with Agent I or HTH compared to satisfactory growth of organisms from buffer controls.

The results of assays with E. invadens are presented in Table 7. Agent I was found to be more effective than HTH at rendering E. invadens nonviable. At pH 9.5, 22°C only 2 minutes time was required for 1 ppm total chlorine from Agent I to prevent viable growth of E. invadens. In 2 minutes contact time with HTH, 10 ppm free chlorine were required to prevent viable cell growth. Although the assays in WCW have not been completed for E. invadens, it is postulated that Agent I will perform considerably better than HTH because of the lesser effect of WCW on Agent I in virucidal and bactericidal assays. The

effect of Agent I on E. invadens did not change between 2 and 10 minutes contact time except at pH 7.0, 4°C which had a suppressing effect on Agent I, requiring a longer time for rendering organisms nonviable.

In addition to microscopic examination for lack of viable cell growth in cultures, a trypan blue exclusion test was used to examine E. invadens at various stages of treatment during the assays for Agent I and HTH.

TABLE 7. EFFECT OF AGENT I AND HTH ON VIABILITY OF ENTAMOEBA INVADENS[a]

Conditions of assays			Agent I - ppm					HTH - ppm Cl+				
Contact Time	pH	Temp.	10	5	2	1	0.5	10	5	2	1	0.5
2 min	7.0	4 C	-	±	+	+	+	±	+	+	+	+
	7.0	22 C	-	-	-	+	+	-	±	+	+	+
	9.5	22 C	-	-	-	-	+	-	±	+	+	+
	9.5	4 C	-	-	+	+	+	±	±	+	+	+
5 min	7.0	4 C	-	-	+	+	+	-	+	+	+	+
	7.0	22 C	-	-	-	±	+	-	±	+	+	+
	9.5	22 C	-	-	-	-	+	-	-	+	+	+
	9.5	4 C	-	-	±	+	+	-	±	+	+	+
10 min	7.0	4 C	-	-	±	+	+	-	±	+	+	+
	7.0	22 C	-	-	-	±	+	-	-	±	+	+
	9.5	22 C	-	-	-	-	+	-	-	+	+	+
	9.5	4 C	-	-	±	+	+	-	±	+	+	+

a. (-) = no evidence of viable cell growth; (+) = normal cell growth; (±) = estimated 90 percent reduction of viable cell growth.

Organisms of E. invadens that grow when placed in culture medium exclude trypan blue dye; this includes organisms taken directly from a stock culture and controls that have been processed with buffer only during assay procedures. Organisms that do not grow when placed in culture medium stain blue in the trypan blue exclusion test. This was a consistent finding of nonviable cells and trypan blue staining of E. invadens treated with Agent I and HTH at concentrations that rendered the organisms nonviable. Based on this observation with trypan blue dye, it appears that the disinfectants are in fact killing E. invadens rather than inducing a nonreplicating form of encystment.

Agent I was found to be even more efficient at rendering G. lamblia nonviable in that 1 ppm total chlorine prevented viable cell growth under all assay conditions except pH 7.0, 4°C which resulted in

a greater than 90 percent reduction in viable cell growth. Assays with G. lamblia revealed that both Agent I and HTH were more effective at rendering it nonviable compared to E invadens (Table 8). The results indicate that Agent I was more efficient than HTH against G. lamblia. Since all assays have not been completed, further studies must be done to better define the least concentration of Agent I that will cause G. lamblia to be nonviable. Studies have not been done as yet with trypan blue or other indicators of viable vs nonviable cell status with G. lamblia. These studies will be done to determine if the nonviable state of G. lamblia after treatment with chlorinating agents is indicative of cell death rather than induction of a nonreplicating form of encystment.

In summary, Agent I is better than HTH as a disinfectant against the two protozoa studied.

TABLE 8. EFFECT OF AGENT I AND HTH ON VIABILITY OF GIARDIA LAMBLIA[a]

Conditions of assays			Agent I - ppm Cl^+					HTH - ppm Cl^+				
Contact Time	pH	Temp.	10	5	2	1	0.5	10	5	2	1	0.5
2 min	7.0	4 C	-	-	-	+	ND[b]	-	-	-	+	ND
	7.0	22 C	-	-	-	-	ND	-	-	-	±	ND
	9.5	22 C	-	-	-	-	ND	-	-	-	±	ND
	9.5	4 C	-	-	-	-	ND	-	-	±	+	ND
(WCW)	9.5	4 C	ND	ND	ND	ND	ND	±	+	+	+	ND
5 min	7.0	4 C	-	-	-	-	ND	-	-	-	+	ND
	7.0	22 C	-	-	-	-	ND	-	-	-	±	ND
	9.5	22 C	-	-	-	-	ND	-	-	-	-	ND
	9.5	4 C	-	-	-	-	ND	-	-	-	±	ND
(WCW)	9.5	4 C	ND	ND	ND	ND	ND	±	+	+	+	ND
10 min	7.0	4 C	-	-	-	-	ND	-	-	-	+	ND
	7.0	22 C	-	-	-	-	ND	-	-	-	±	ND
	9.5	22 C	-	-	-	-	ND	-	-	-	-	ND
	9.5	4 C	-	-	-	-	ND	-	-	-	±	ND
(WCW)	9.5	4 C	ND	ND	ND	ND	ND	-	+	+	+	ND

a. (-) = no evidence of viable cell growth; (+) = normal cell growth; (±) = estimated 90 percent reduction of viable cell growth.
b. No determination.

FUNGI STUDIES

Agent I and HTH were compared for efficacy of inactivation of Candida albicans and Rhodotorula rubra both of which were obtained from

ATCC. The strain of Candida albicans (ATCC 44506) was originally isolated from a rectal swab collected from a human. The strain of Rhodotorula rubra (ATCC 16639) was originally isolated from sewage sludge. Both yeasts were grown on Saboraud's dextrose agar slants at 25°C. For disinfection assays, 48-hour cultures were harvested from slants with approximately 2 mL of buffer, pooled into a sterile tube with glass beads and vortexed to achieve single-cell suspensions. Absence of aggregation of yeast cells was determined by microscopic observation of a drop of each suspension on a glass slide. Each suspension was standardized by use of McFarland standards prepared from a nepholometer densitometer. The suspensions were diluted with the appropriate assay buffer, or WCW, to a concentration of 2×10^6 cells per mL and mixed with an equal volume of disinfectant for disinfection assays. The control tubes contained equal volumes of assay buffer and yeast suspension.

All disinfection assays were done with temperatures controlled at 4°C or 22°C and in buffers at pH 4.5, 7.0, or 9.5 or in WCW at pH 9.5. The disinfecting agents were diluted with the appropriate buffers to give concentrations in ppm of potentially ionizable chlorine of 96, 50, 20, 10, and 4 for Agent I, and 50, 20, 10, 5, and 2 for HTH. The assay tubes were equilibrated to a controlled temperature for 30 minutes before combining with an equal volume of yeast-cell suspension. The assay tubes were mixed by vortexing, and 1.0 mL samples were removed from each tube, including controls, at 1, 5, 10, 20, 30, and 60 minutes and again after 24 hour contact time after combining and mixing. The 1.0 mL samples were immediately added to 1.0 mL of 0.02 N sodium thiosulfate to quench the chlorine and stop the inactivation.

Serial log dilutions of the quenched samples were made in sterile saline, and duplicate plate counts were made on dilutions $10^{-1.0}$ through $10^{-4.0}$ of each test sample and each control. A micro-drop technique was used for the plate counts on Sabouraud's dextrose agar in 100 mm petri dishes. Duplicate colony counts were made from the dilution yielding the best countability. Counts were expressed as colonies per mL. The percent inactivation, or percentage kill, was calculated by the following formula:

$$\text{percent kill} = \frac{(\text{No. control cfu/mL}) - (\text{No. test sample cfu/mL})}{(\text{No. control cfu/mL})} \times 100$$

The results of from two to four replicate assays with both C. albicans and R. rubra indicated that HTH was considerably more efficient at killing these yeasts than Agent I (Tables 9 and 10). As found in assays with other microbes, Agent I was more efficient at pH 9.5, 22°C than at lower pH values or lower temperatures. In contrast, the killing efficacy of HTH decreased as the pH increased. Organic load from WCW had a greater effect on HTH than on Agent I; in fact, there was little difference in the efficacy of HTH and Agent I against R. rubra in WCW. Although higher concentrations of potentially

ionizable chlorine were required for Agent I to kill both yeasts, compared to HTH, as little as 2 ppm Cl^+ from Agent I killed more than 99.99 percent of cells from both C. albicans and R. rubra in 24 hours contact time, which was essentially the same as the results achieved with HTH.

A consistent irregularity observed in practically all assays with Agent I, and, to a lesser extent with HTH, was a greater percent kill after 5 to 10 minutes than at 20 to 30 minutes. Special assays were run at selected assay conditions using Tween 20 (polyoxyethylene sorbitan monolaureate) as a dispersing agent in buffers. Tween 20 did not eliminate the unusual characteristics of the rate of kill. Assays run with magnetic stirring bars operating throughout the entire sampling time tended to reduce the decrease in killing effectiveness after 20 to 30 minutes at higher concentrations of Agent I, but there was no improvement in the overall rate of kill by Agent I. Microscopic examinations were made on samples collected throughout the assays, and there was no evidence of aggregation of yeast cells. Thus, the reason for the peculiar behavior of Agent I with the two yeasts is not understood at this time.

TABLE 9. EFFECT OF CONDITIONS OF ASSAYS, TEMPERATURE, AND pH ON CONTACT TIME REQUIRED FOR AGENT I OR HTH TO INACTIVATE CANDIDA ALBICANS[a]

Conditions of Assays		Time (Min) for More than 99.99 % Kill by Agent I or HTH									
		Agent I - ppm Cl^+					HTH - ppm Cl^+				
pH	Temp	48	25	10	5	2	25	10	5	2.5	1
4.5	22 C	*	*	**	**	**	1	1	1	1	1
7.0	4 C	*	*	**	**	**	1	1	1	1	1
7.0	22 C	30	30	*	**	**	1	1	1	1	1
9.5	22 C	20	20	*	*	*	5	5	5	10	30
9.5	4 C	*	*	*	*	*	10	20	20	60	*
9.5 (WCW)	4 C	*	*	*	**	**	60	*	*	*	**

a. Numbers under columns are time of sampling when greater than 99.99 percent of yeast cells had been killed based on colony counts of dilutions.

*Less than 99.0 percent kill at 1 hour; greater than 99.99 percent kill at 24 hours.

**Less than 99.9 percent kill after 24 hours contact time.

TABLE 10. EFFECT OF CONDITIONS OF ASSAYS, TEMPERATURE, AND pH ON CONTACT TIME REQUIRED FOR AGENT I OR HTH TO INACTIVATE *RHODOTORULA RUBRA*[a]

Conditions of Assays		Time (Min) for More than 99.99 % Kill by Agent I or HTH									
		Agent I - ppm Cl^+					HTH - ppm Cl^+				
pH	Temp.	48	25	10	5	2	25	10	5	2.5	1
4.5	22 C	*	*	*	*	*	1	1	1	1	ND[b]
7.0	4 C	**	**	**	**	**	1	1	1	1	10
7.0	22 C	1	20	*	*	**	1	1	1	1	5
9.5	22 C	20	60	60	*	*	10	20	30	60	*
9.5	4 C	*	*	*	*	*	60	60	60	*	*
9.5 (WCW)	4 C	*	*	*	**	**	*	*	**	**	**

a. Numbers under columns are time of sampling when greater than 99.99 percent of yeast cells had been killed based on colony counts of dilutions.

*Less than 99.0 percent kill at 1 hour; greater than 99.99 percent kill at 24 hours.

**Less than 99.9 percent kill after 24 hours contact time.

b. No determination.

SECTION IV

TASK III (COMPARISON OF STABILITY OF AGENT I AND HTH)

The goal of Task III was to compare the stabilities of Agent I and HTH in water as a function of pH, temperature, and water quality.

One-liter flasks were filled with sterile, distilled, deionized, demand-free water, or in some cases WCW, buffered at a given pH, and the flasks were suspended in a constant temperature bath and allowed to stand under ambient lighting conditions. The flasks were stoppered with sterile porous cotton plugs which allowed chlorine gas to escape but prevented contamination by particulate matter. The initial concentration of the agents was always near 10 ppm ionizable chlorine. Aliquots (10 mL) were withdrawn from the flasks periodically (frequently during the first few hours and then once per week) over a time span of 6-8 weeks and titrated in triplicate for ppm total chlorine with a Wallace Tiernan amperometric titrimeter. Volume losses due to evaporation were checked before each titration, and, if appreciable, were compensated for by the addition of sterile demand-free water. The assay conditions were pH 4.5, 7.0, and 9.5 at 22°C; pH 9.5 at 4°C; WCW at pH 9.5, 4°C and at pH 9.5 at 27°C. A stability study was also performed for the two agents in the solid state in which they were placed in vials containing porous cotton plugs and allowed to stand at room temperature for a period of 4 months.

The stability studies for the solid state provide rather dramatic results. Over a 111-day period HTH lost 90.71 percent of its total chlorine content, while Agent I lost only 2.46 percent of its total chlorine content. This indicates that storage of HTH for long periods in containers which are not tightly sealed could lead to an agent which is not effective as a disinfectant in the portions normally employed in the field. The corresponding loss for Agent I was insignificant. It should be noted that HTH is more stable when stored in tightly sealed containers, particularly under refrigeration.

Results for the solution stability studies have been presented in Figures 9 and 10. Figure 9 indicates the performance of the two agents at 22°C in sterile, buffered, demand-free water. As can be seen, Agent I is essentially completely stable under these conditions at pH 4.5 and pH 7.0. In comparison HTH exhibited half-lives of 8.5 weeks and 6.4 weeks at pH 4.5 and 7.0, respectively. Figure 9 also shows that neither agent is extremely stable at pH 9.5, 22°C; the half-life of Agent I under these conditions is 6.25 weeks, while that of HTH is 4.9 weeks. The reason for loss of stability of Agent I at pH 9.5 is that the strong basic conditions promote hydrolysis of the compound to hypochlorite and its precursor amine. This equilibrium process lies far to the Agent I side at pH 4.5 and 7.0 (greater than 99 percent), but is obviously significantly shifted to the hypochlorite side at pH 9.5. The loss of total chlorine from HTH, however, remains greater. Both agents are quite stable at pH 9.5 in demand-free water if the temperature is reduced to 4°C.

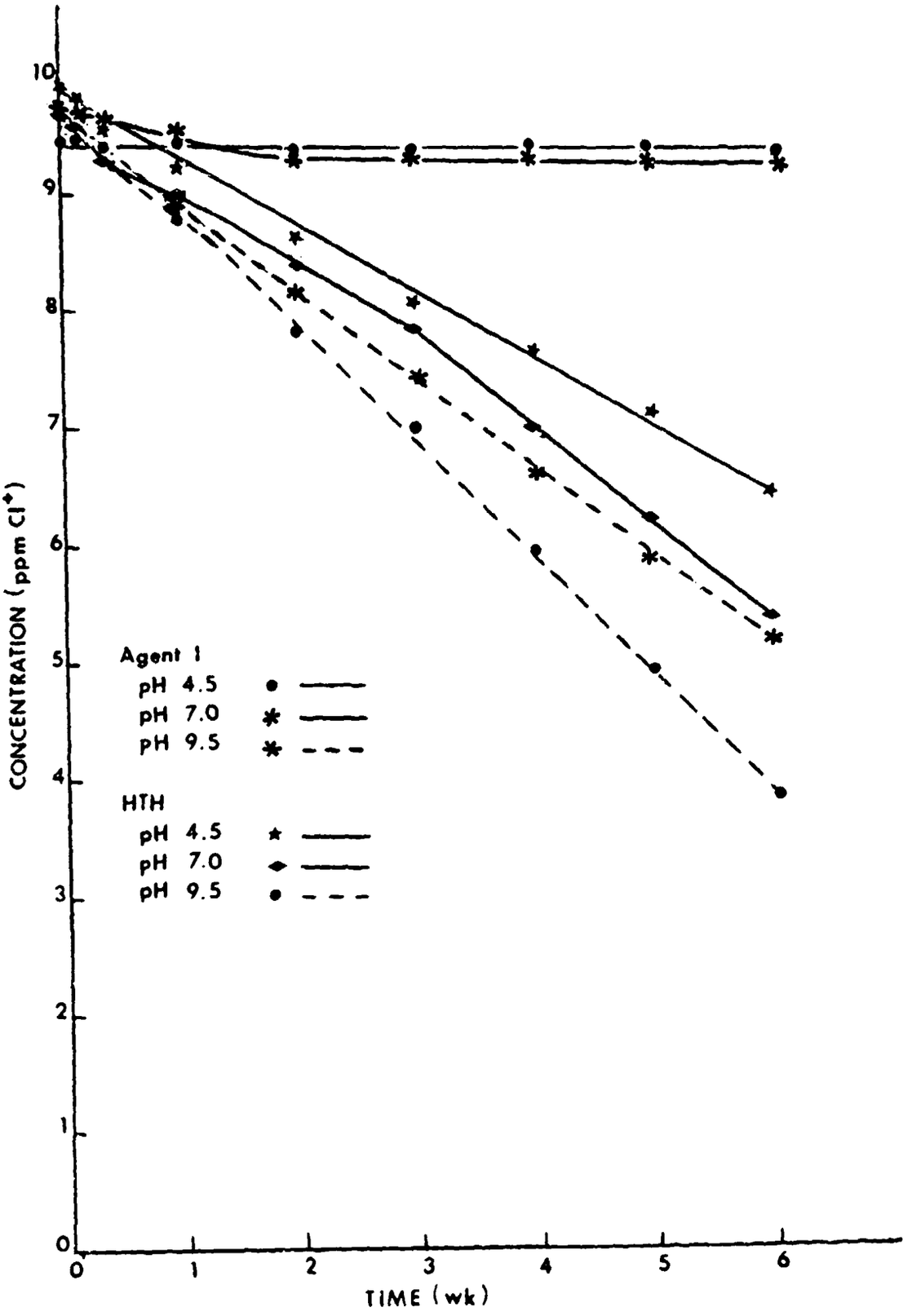


Figure 9. Stability of Agent 1 and HTH as a Function of pH at 22°C in Chlorine Demand-Free Water.

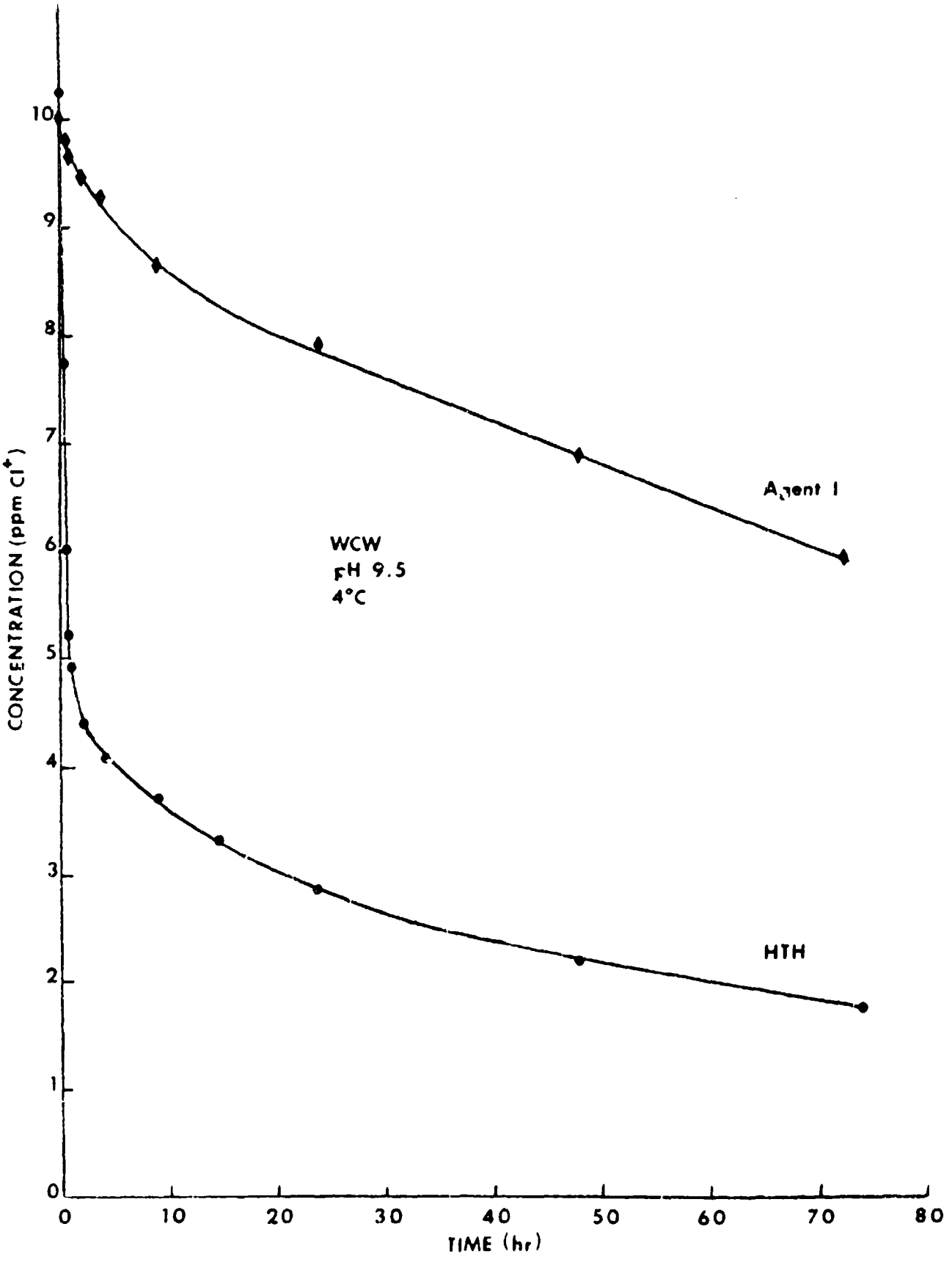


Figure 10. Stability of Agent I and HTH at pH 9.5, 4°C in WCW.

The results of the WCW experiments at pH 9.5, 4°C are shown in Figure 10. HTH is much more dramatically affected by organic load than is Agent I. The half-life of HTH (total chlorine) under these conditions is less than 1 hour, while that of Agent I is 96 hours. After 3 weeks in WCW, the total chlorine content of HTH had decayed from 10.25 ppm to 0.25 ppm; the corresponding loss for Agent I was from 10.0 ppm to 0.9 ppm. Therefore, neither agent holds up well for long time periods in the presence of heavy organic load, but Agent I is much more stable during the first week than is HTH. It is probable that the "free chlorine" from HTH disappears in seconds with the chlorinated organic residuals decomposing more slowly. Although pH 9.5 aids the decomposition of both agents at 22°C, this is not a factor at 4°C. The organic load in WCW is the more significant cause of loss of total chlorine content at low temperature. Experiments at 27°C in WCW at pH 9.5 have indicated that Agent I is significantly more stable (half-life of 2.5 hours as compared to 15 minutes) than HTH under these conditions. Also, Agent I is dramatically more stable than HTH in unbuffered water at room temperature (7).

SECTION V

TASK IV (COMPARISON OF THE ACTION ON MATERIALS BY AGENT I AND HTH)

The goal of Task IV was to compare the corrosive actions o Agent I and HTH on materials used by the military for water storage a d treatment.

A large sample of Agent I (12 g) was sent to Dr. Alan Riedinger of UOP, Inc., to test in their membrane test cell units. In their experiments UOP, Inc., flowed water containing Agent I at a concentration of 1 ppm or 10 ppm total chlorine through a membrane test cell unit containing eight TFC and SS-TFC membranes alternating in series. Twenty gallons of water containing 32 g/L sodium chloride, in addition to Agent I, was pumped through the unit at 1 gpm and 800 psi. The feed temperature was about 25°C, and the pH remained between 7 and 8 during the course of the experiment.

In the 1 ppm Cl^+ experiment the filters were exposed to the flowing Agent I/salt solution for 189 hours with no detectable change in either water flux or salt rejection; i.e., deterioration, during that time. The solution was then quenched with sodium bisulfite and observed again at 24 additional hours with still no detectable deterioration. Exposure of a 10 ppm total chlorine solution of Agent I caused no deterioration after 330 hours, but after quenching with bisulfite and rexamination after an additional 170 hours, some deterioration was noted, although small compared to similar experiments conducted with bleach (sodium hypochlorite) solutions. The active ingredient in HTH is the same as that in bleach, so it can be assumed that HTH and bleach would have had similar effects on the filter material. From this study UOP, Inc., concluded that Agent I causes "gradual" deterioration of the membrane filters but "significantly lower" deterioration than caused by 10 ppm free chlorine. They further speculated that their ROWPU units would function satisfactorily for "about 1 month" in the presence of 10 ppm chlorine supplied by Agent I.

Their conclusions seem unwarranted because all deterioration was noted after the addition of sodium bisulfite which, of course, quenches Agent I. It is likely that the sodium bisulfite or biofouling in the presence of no disinfectant caused the damage to the filters. Their data actually suggest that Agent I improves the performance of the filters when compared to their performance in the presence of no disinfectant.

For the experiments concerning the action of Agent I and HTH on military materials in these laboratories, six cells were designed and constructed to contain solutions in contact with two bladder materials. Those materials which included a UK Water Tank material (henceforth referred to as the black material) and a coated fabric for a 20,000 gal pillow tank (henceforth referred to as the tan material) were supplied by James C. Eaton of USAMBRDL. The cells consisted of 41 mm OD Pyrex® tubing which could be placed against a disk of the material backed by

an aluminum base plate clamped to the flared base of the Pyrex® tube, thus preventing leaks. For each of the two materials, three cells were employed containing either a control (distilled, deionized, demand-free water buffered at pH 7), or a solution of one of the agents at pH 7 at a concentration level of 100 ppm total ionizable chlorine. Each cell was placed in an upright position with the base plate/material down and Parafilm at the top to prevent contamination by particulate matter and was allowed to stand for 28 days at room temperature on a lab bench. At the end of the period, all samples were rinsed with distilled water, and three types of analyses were performed.

Small squares of material (1 cm^2) were cut out and subjected to analyses by electron microscopy. In Figure 11a-c three typical photographs of the black material at 500x magnification are shown. Two experts in the field here at Auburn say that these photographs indicate that both agents cause some damage to the material with HTH being more severe in its action. The HTH sample was not adjusted for chlorine concentration during the 28 day period. Since Agent I would remain at 100 ppm Cl^+ during that period, while HTH would decline by at least 30 ppm Cl^+ during the time (the cells were capped loosely with Parafilm® which contains organic demand), the study indicates that Agent I is clearly superior to HTH in terms of less corrosive action on the black material. For the tan material, little damage caused by either agent was evident. Figure 11d shows two crystals of Agent I adhered to the surface of the tan material (1000x magnification) for general interest.

Small 1.4 inch bone-like specimens of the materials exposed in the cells were subjected to tensile-strength analysis on an Instrom Universal Testing Machine. The results for the tan material were totally inconclusive because, being a laminated material, it broke in different layers in a random fashion upon exposure to stress. The results for the black material were somewhat better in that it is not laminated; however, we discovered during the tensile strength testing that the results depended upon the orientation of the sample "bones" when they were cut from the sample disk. In other words the black material has different strengths in different directions. Nevertheless, an expert in the Auburn Mechanical Engineering Department has concluded from the results in Table 11 that HTH probably caused more damage to the material than did Agent I. The rather large errors in Table 11 are due again to the fact that the samples could not be cut out in the same orientations for all runs. The trends noted in the table are certainly of questionable significance, but are in the direction expected, given the results of the electron microscopy studies.

Finally, 20 mL of each solution present in the cells for the 28 day periods were extracted with 3 mL of isooctane. One µL injections of the extracts were made into a Gow-Mac Gas Chromatograph with an FID detector (Silicone Chrom-P column held at 110°C). Under the detection limits of this instrument (100 ppm chloroform being the minimum amount detectable), no impurities were observed for solutions of either agent as compared to the controls. Of course, large polymeric compounds would have not been detected in this analysis.

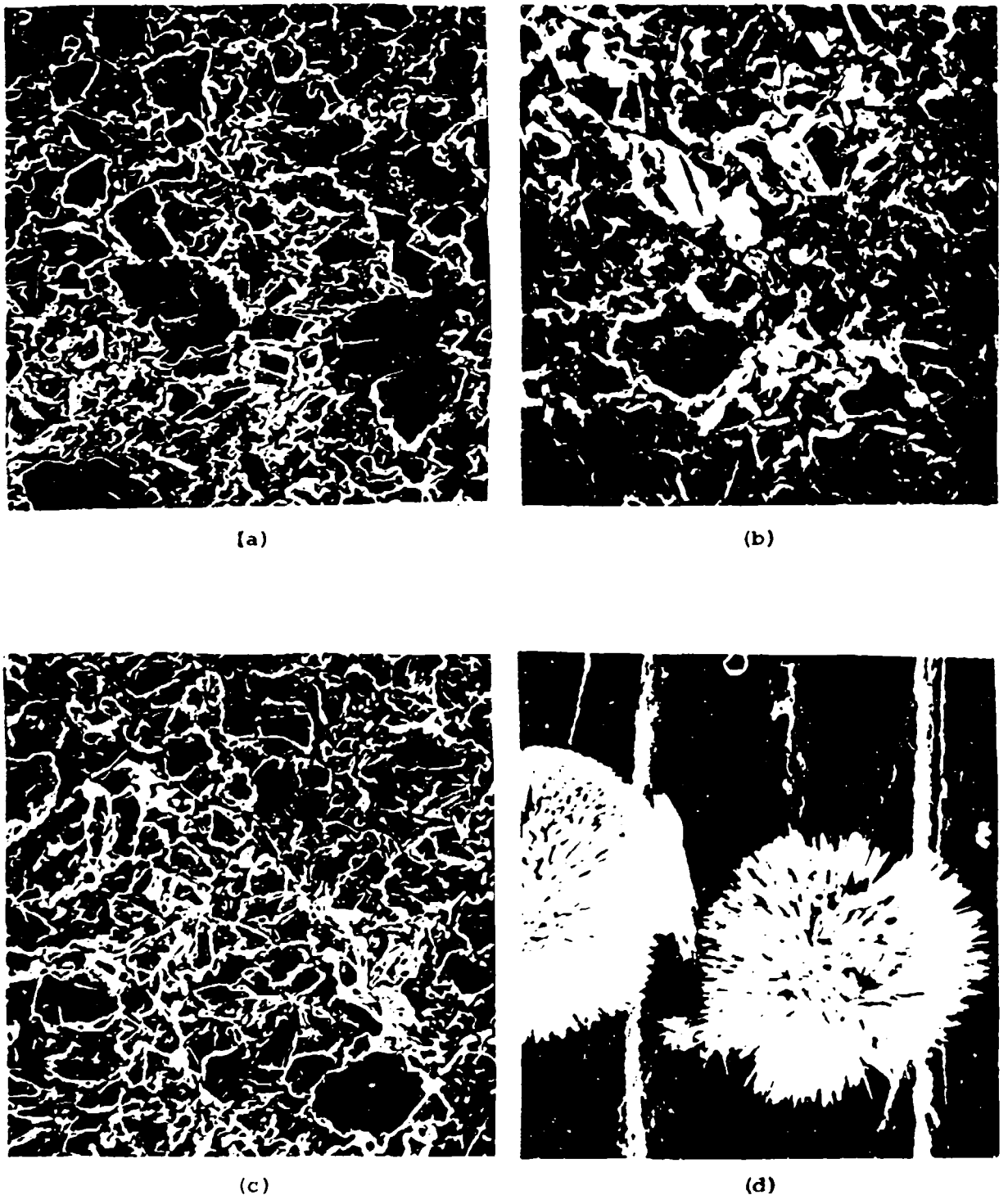


Figure 11. Electron Micrographs of Bladder Material Exposed to (a) pH 7.0 Buffer; (b) Agent I at 100 ppm Total Chlorine at pH 7.0; (c) HTH at 100 ppm Total Chlorine at pH 7.0; (d) Crystals of Agent I on Material. See text for additional information.

TABLE 11. TENSILE STRENGTH TESTING OF BLACK MATERIAL EXPOSED TO THE AGENTS FOR 28 DAYS AT 100 PPM Cl^+, pH 7.

Sample[a]	Young's Modulus psi	Yield Strength psi	Break Point sec
Control	16,694±367	5499±74	32.2±1.3
Agent I	15,181±3974	5115±1202	32.0±3.4
HTH	14,020±3643	4947±1171	31.4±1.3

a. Triplicate determinations.
b. Elongation was performed at 0.50 in/min.

SECTION VI

TASK V (COMPARISON OF AGENT I AND HTH IN THE PRODUCTION OF TRIHALOMETHANES).

The goal of Task V was to measure the production of toxic trihalomethanes in water solutions of Agent I and HTH which contained organic loads.

Solutions of Agent I and HTH (100 mL) buffered at pH 7.0 containing 10 ppm total ionizable chlorine were prepared. Two such solutions for each agent were doped with spectroscopic grade acetone at a concentration level of 3.8 percent by weight. Two others for each agent were doped with organic load in the form of WCW, except at pH 7.0. Two control solutions at pH 7.0 were prepared also. The water used for all of the solutions was distilled, deionized, and chlorine demand-free. One solution of each agent for each class (control, acetone, WCW) was exposed to sunlight for 10 hours at 25°C. A second solution of each agent for each class was kept in darkness for 7 hours. At the end of the reaction period, all solutions were quenched by 0.056 N sodium thiosulfate, and samples were prepared for analysis by gas chromatography.

The GC analyses were performed by the Alabama Department of Environmental Management Laboratory in Montgomery, Alabama, which is equipped with instrumentation specifically designed for trihalomethane analyses. Briefly, 20 mL of sample were extracted with 4 mL of pure isooctane. Five µL of each extract were injected by a Tracor 770 Autosampler into a Tracor 550 gas chromatograph equipped with an electron capture detector and a glass column packed with 3 percent SP 1000 on 100/200 mesh Supelcoport® held at 70°C. The instrument was calibrated for trihalomethanes using standard procedures developed by EPA (18).

The results of the GC analyses are shown in Table 12. As can be seen, a small amount of chloroform was detected for the Agent I samples in the presence of sunlight; the amount was greater for WCW than for acetone-doped water. The amount of chloroform was much greater for all samples containing HTH. Chloroform was the only trihalomethane detected in any of the solutions.

TABLE 12. CONCENTRATION OF CHLOROFORM DETECTED FOR SOLUTIONS CONTAINING ORGANIC LOAD AND THE TWO AGENTS BEING STUDIED.

Sample Conditions	ppb Chloroform	ppb Chloroform (corrected for control)
Control + Acetone + Light	5	
Agent I + Acetone + Light	9	4
HTH + Acetone + Light	56	51
Control + WCW + Light	4	
Agent I + WCW + Light	14	10
HTH + WCW + Light	58	54
Control + Acetone + Dark	11	
Agent I + Acetone + Dark	11	0
HTH + Acetone + Dark	29	18

It is thought that the 10 ppb figure derived for the Agent I/WCW sample is erroneously high because there was little difference for the HTH/acetone and HTH/WCW samples. Data in Table 12 show that sunlight expedites the reaction of both agents with organic load to form chloroform, because no chloroform was detected for Agent I/acetone in darkness, and considerably less for HTH/acetone in darkness. The reason for the higher chloroform content in the dark acetone control as compared to the sunlight acetone control was because the dark runs were made at different times with different stock solutions of acetone. Acetone always contains some chloroform as an impurity. It can be concluded that free chlorine from HTH is considerably more reactive with organics to produce chloroform than is combined chlorine in Agent I, but that both agents are probably safe to use for water stored such that sunlight is excluded. For water stored for long time periods in darkness or sunlight, however, HTH may be hazardous if appreciable organic load is present.

SECTION VII

CONCLUSIONS

Agent I is much more stable than HTH in both the solid state and in solution. This fact is evident from its retention of total chlorine over long time periods in demand-free, buffered solutions at normal pH values, its inert behavior toward materials relative to HTH, and its minimal reactivity with organic compounds to produce trihalomethanes in comparison to HTH. Neither agent is particularly stable in highly basic media (pH 9.5) at 22°C. On the other hand, HTH tends to act as a better disinfectant against most of the organisms in this study, notable exceptions being the protozoan species considered here. Thus, no general statement can be made concerning the efficacies of the two agents against all microorganisms. Some such organisms will be eradicated more rapidly by free-chlorine agents such as HTH; others will be killed more efficiently by the combined-chlorine Agent I, depending upon the mechanism for disinfection. It should be stated that, given sufficient contact time, Agent I will kill every organism against which it has been tested. Agent I performs much more efficiently than HTH in the presence of organic load. Both agents are better disinfectants at 22°C than at 4°C.

The reason for the exceptional stability of Agent I must relate to the presence of the two inductively-donating methyl groups in the 4 position on the ring. The anion resulting on nitrogen following dissociation of the N-Cl bond is destabilized, thus rendering the N-Cl bond exceptionally stable toward dissociation. Hence the loss of chlorine from Agent I solutions or the solid state is very slow at normal pH values. This molecular structural feature should be incorporated into the design of new N-chloramine disinfectants.

SECTION VIII

RECOMMENDATIONS

The results of this research would indicate that Agent I could be a better disinfectant than HTH for field water treatment in areas where the water contains appreciable organic contamination. It should also be considered because of its stability for remote areas for which storage and transportation of HTH are problems. Quite likely mixtures of the two agents (Agent I in excess) could be employed profitably with HTH being the rapid disinfectant and Agent I being the long-term disinfectant. Research addressing this question is a part of our renewal contract.

The original primary goal of this research was to demonstrate that organic N-chloramines, an example being Agent I, could possess advantages for disinfection of military water supplies when compared to the current disinfectant HTH. That fact has been demonstrated. It is recommended that further research be performed to develop new N-halamine compounds similar in structure to Agent I which may be even better disinfectants than Agent I. Such research is also a part of our renewal contract.

Finally, HTH is definitely a better disinfectant than Agent I at low temperatures (4°C) unless appreciable organic load is present. It is recommended that HTH be retained as a field disinfectant in cold climates. On the other hand, Agent I probably will be much better than HTH in tropical climates because of its stability. Research addressing this point is a part of our renewal contract.

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APPENDIX A

EXPERIMENTAL DATA

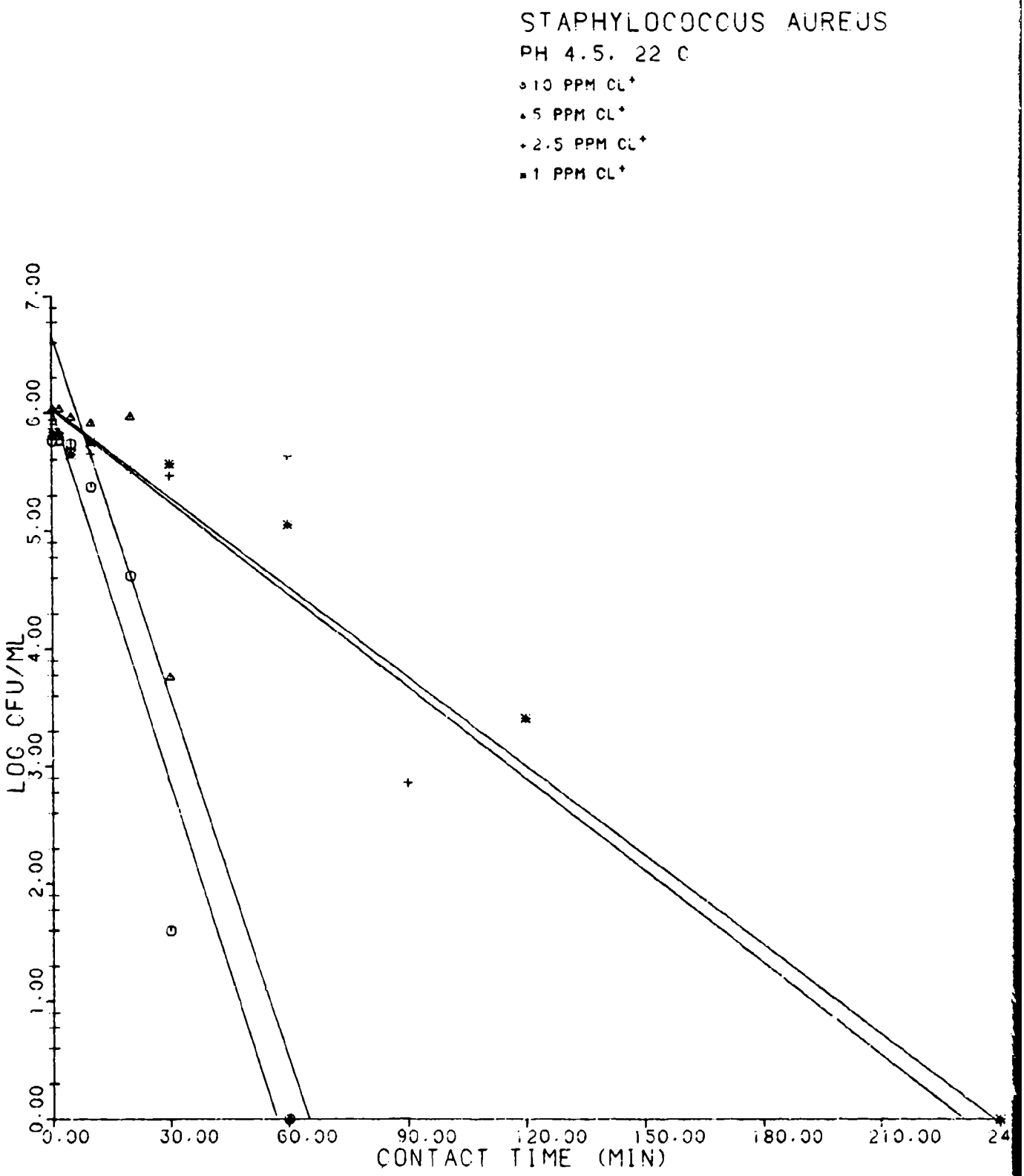

Figure A-1. Disinfection of Staphylococcus aureus by Agent I at pH 4.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

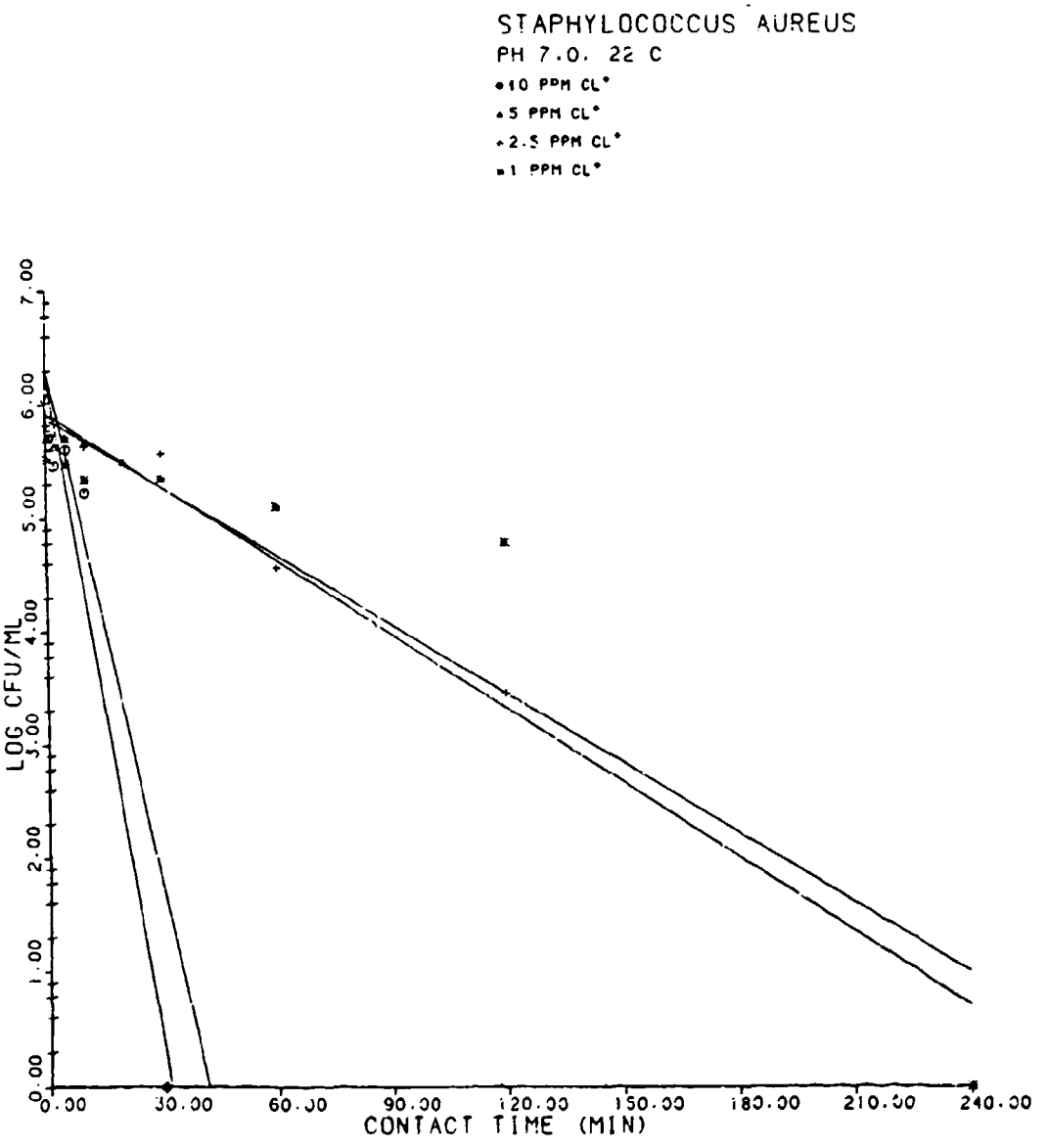

Figure A-2. Disinfection of Staphylococcus aureus by Agent I at ph 7.0, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

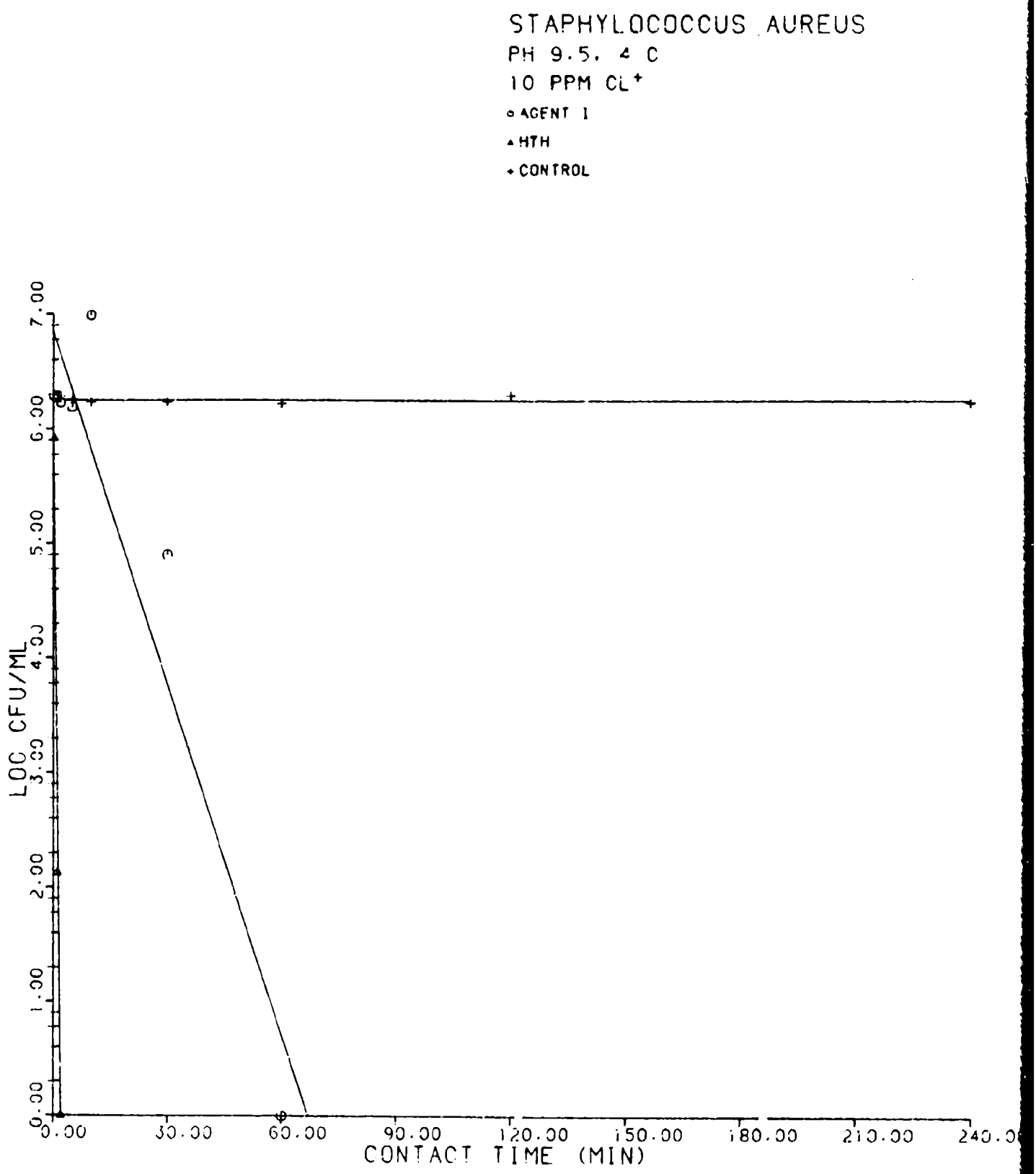

Figure A-3. Disinfection of Staphylococcus aureus by Agent I and HTH at pH 9.5, 4°C at 10 ppm Potentially Ionizable Chlorine.

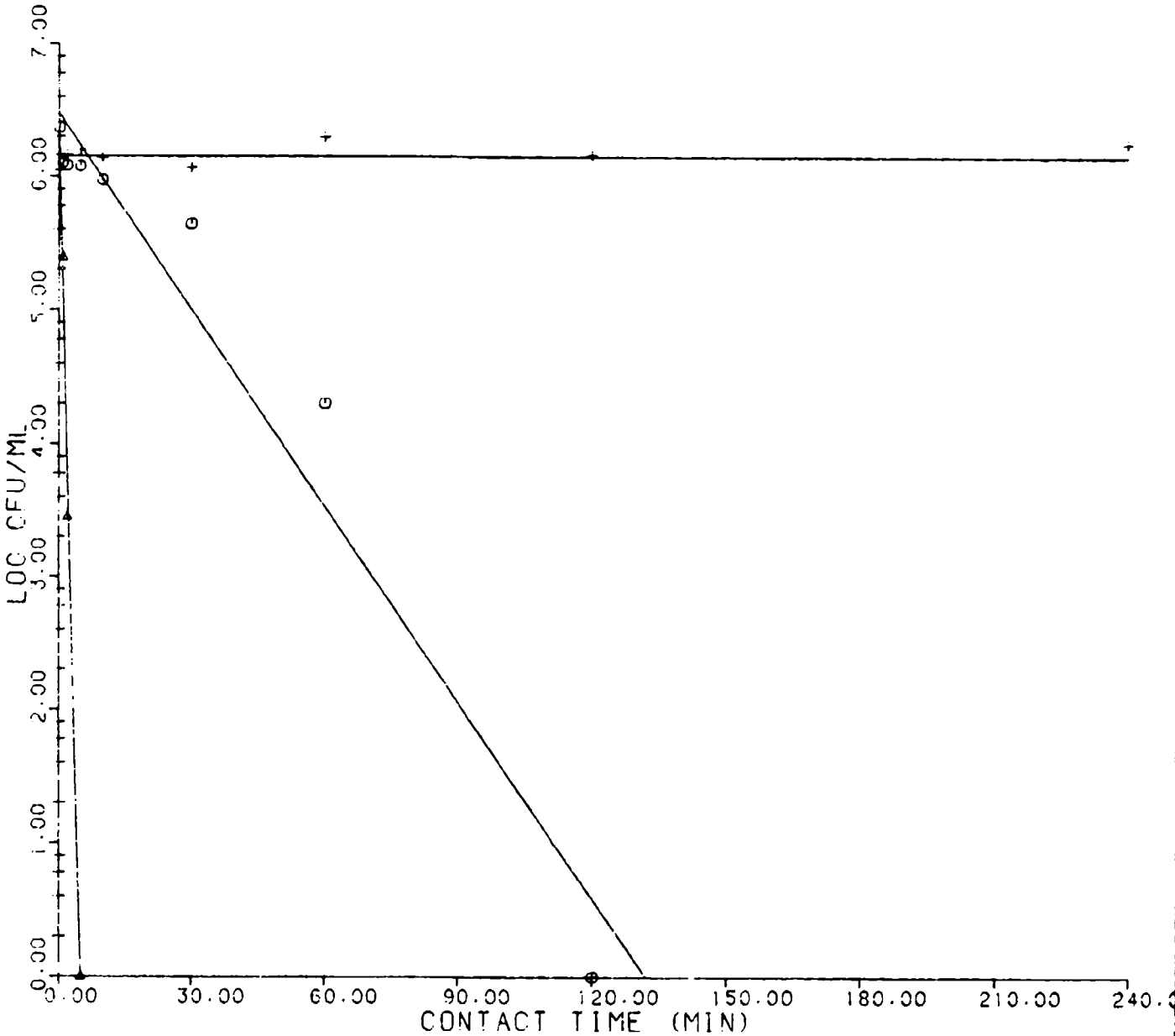

Figure A-4. Disinfection of Staphylococcus aureus by Agent I and HTH at pH 9.5, 4°C at 5 ppm Potentially Ionizable Chlorine.

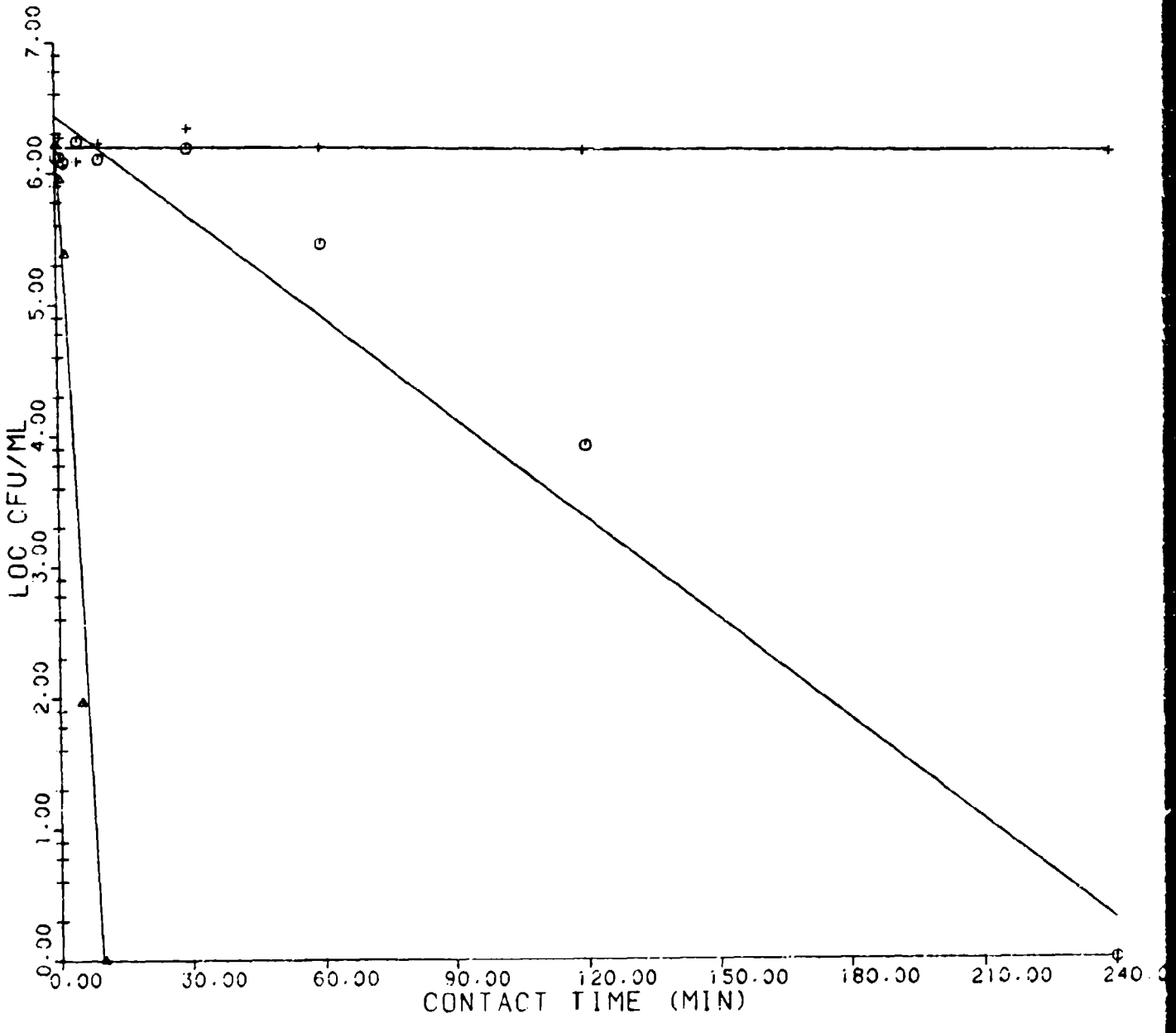

Figure A-5. Disinfection of Staphylococcus aureus by Agent I and HTH at pH 9.5, 4°C at 2.5 ppm Potentially Ionizable Chlorine.

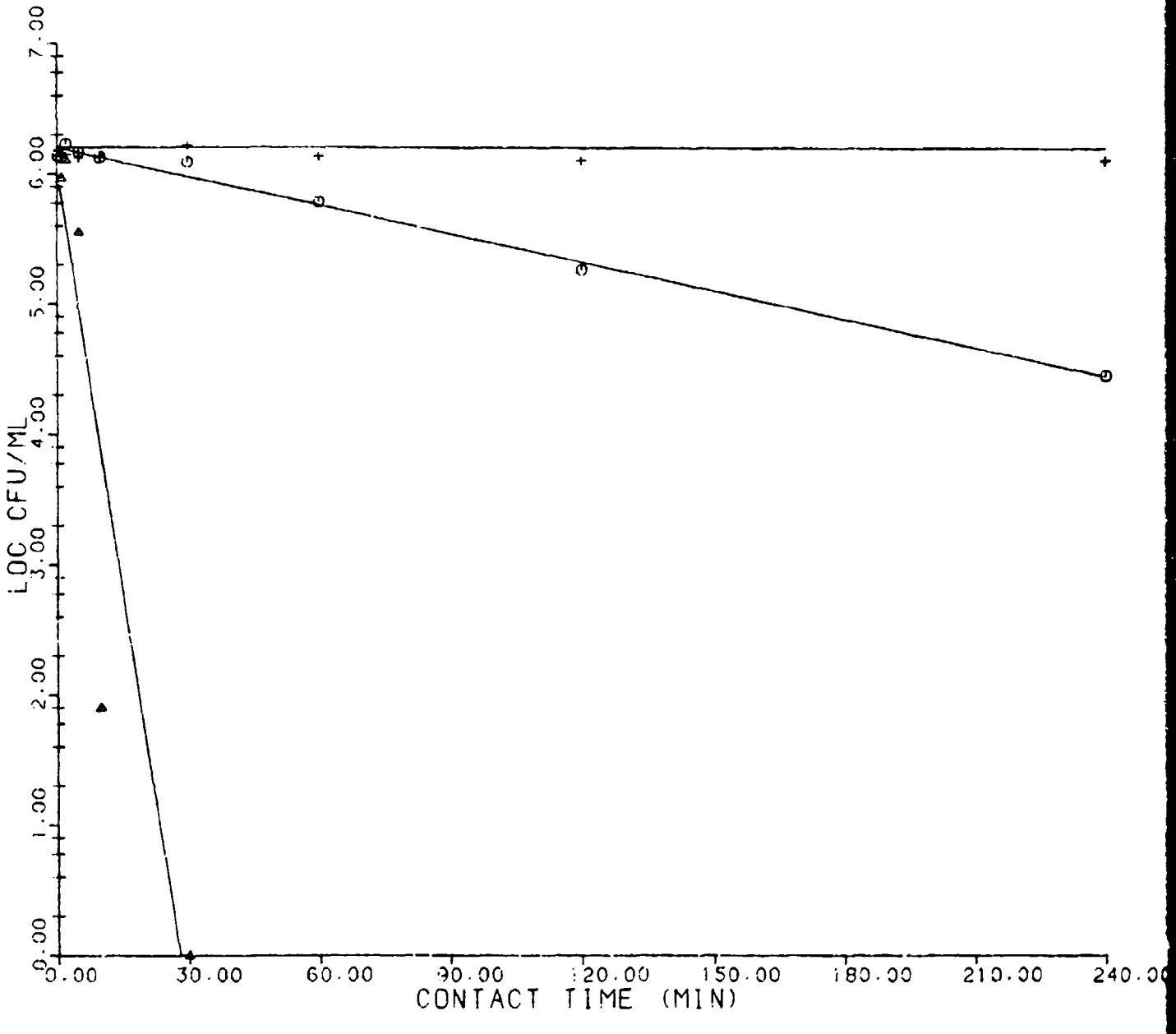

Figure A-6. Disinfection of Staphylococcus aureus by Agent I and HTH at pH 9.5, 4°C at 1 ppm Potentially Ionizable Chlorine.

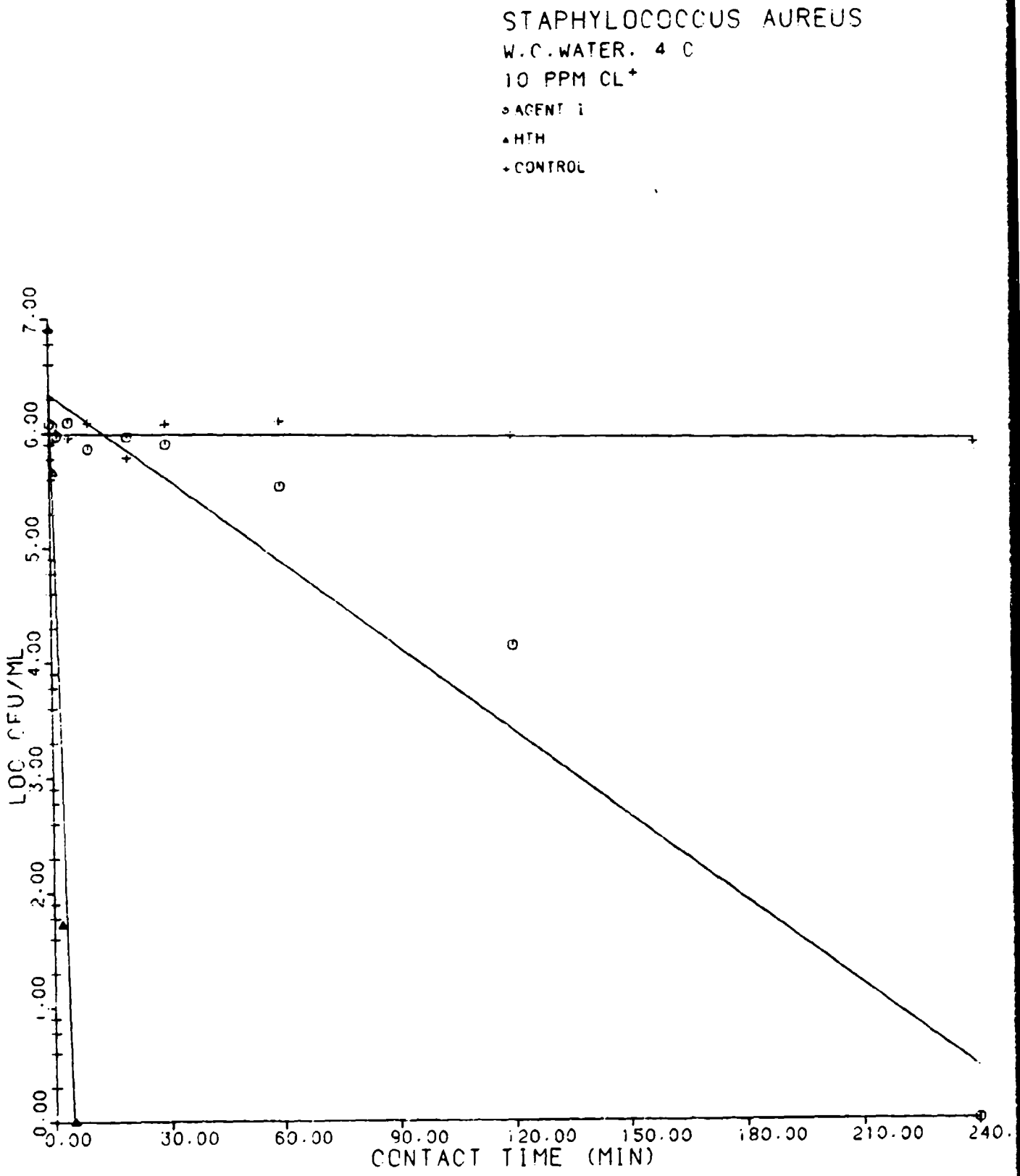

Figure A-7. Disinfection of Staphylococcus aureus by Agent I and HTH in WCW at 10 ppm Potentially Ionizable Chlorine.

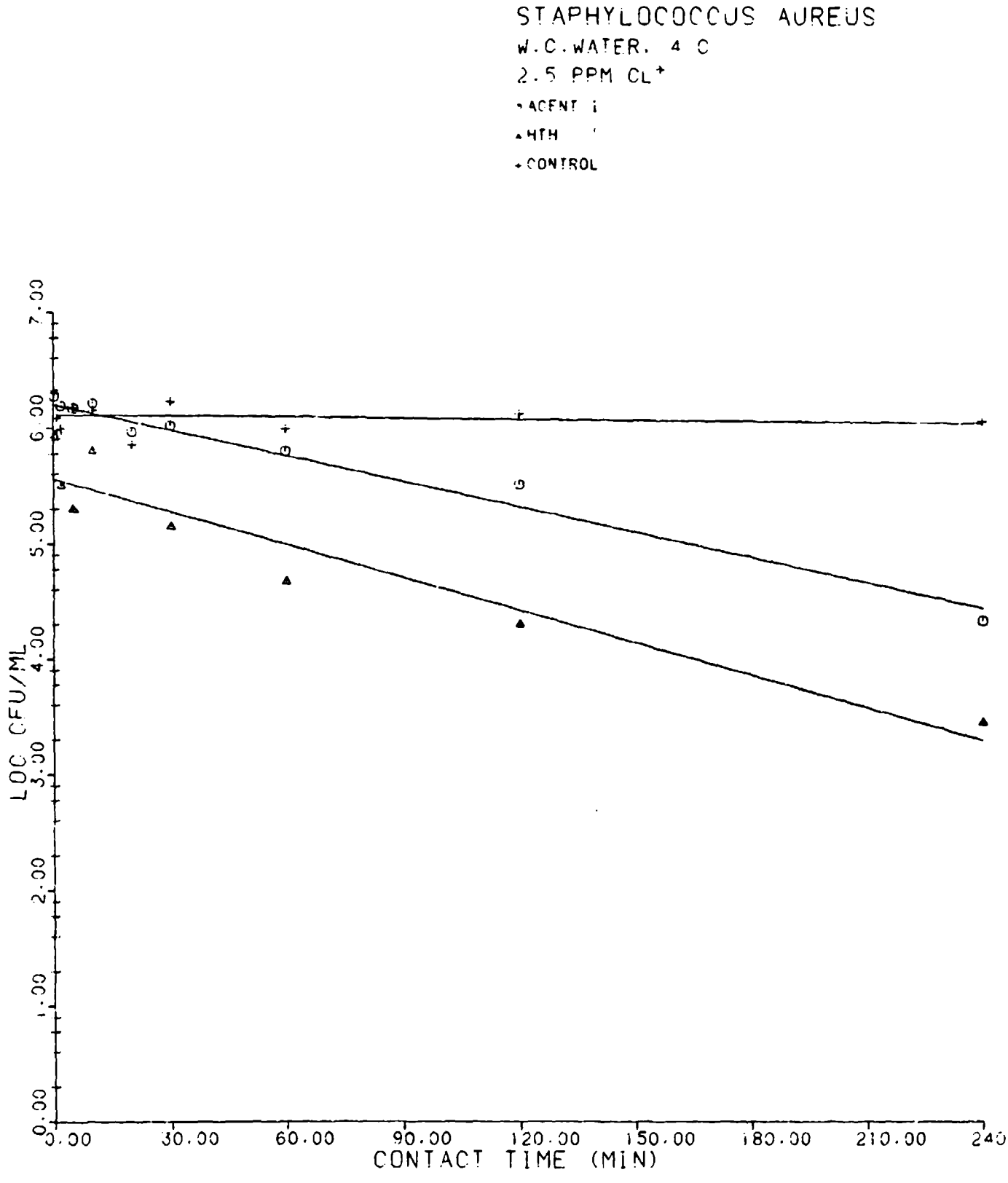

Figure A-8. Disinfection of Staphylococcus aureus by Agent I and HTH in WCW at 2.5 ppm Potentially Ionizable Chlorine.

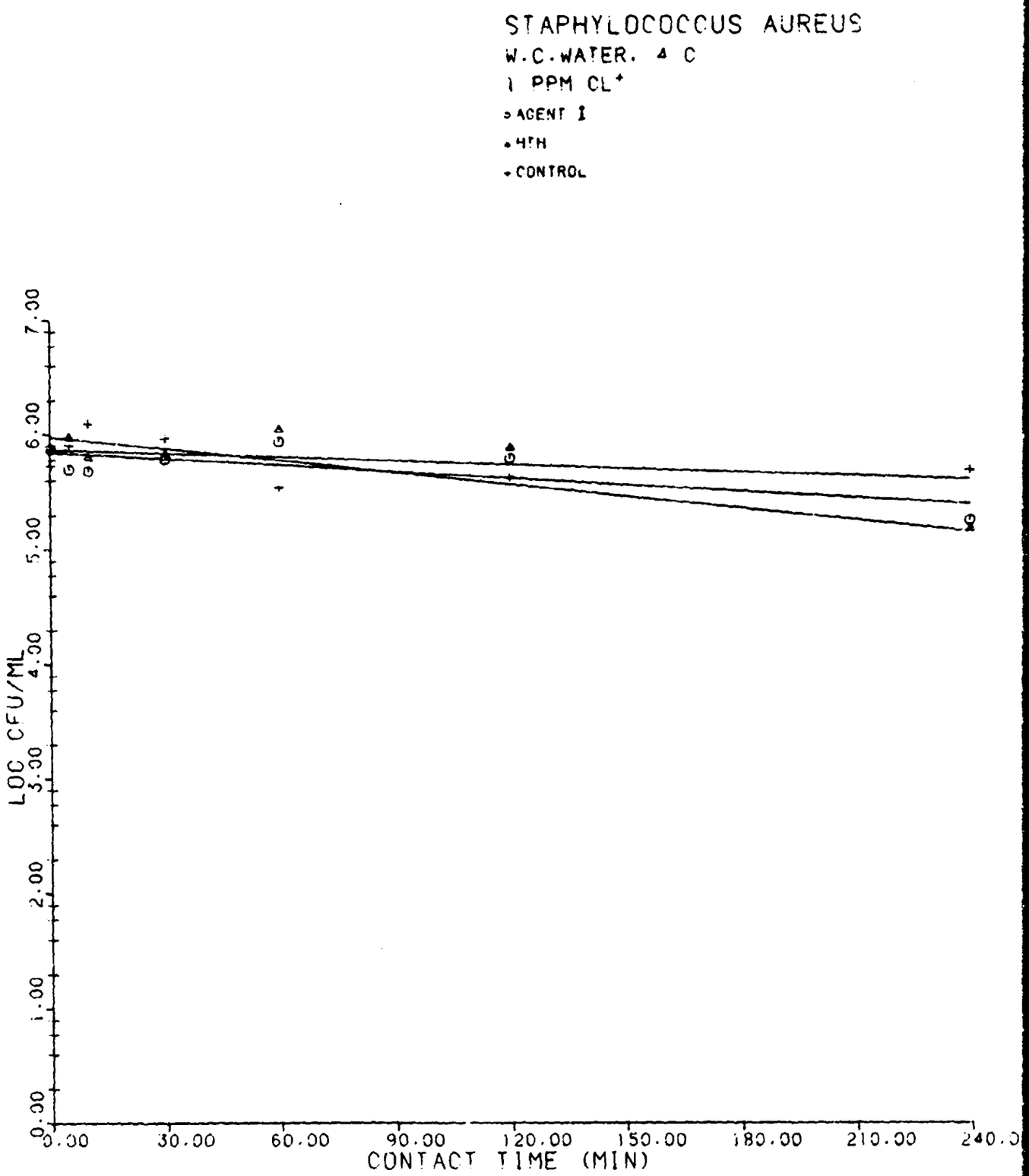

Figure A-9. Disinfection of Staphylococcus aureus by Agent I and HTH in WCW at 1 ppm Potentially Ionizable Chlorine.

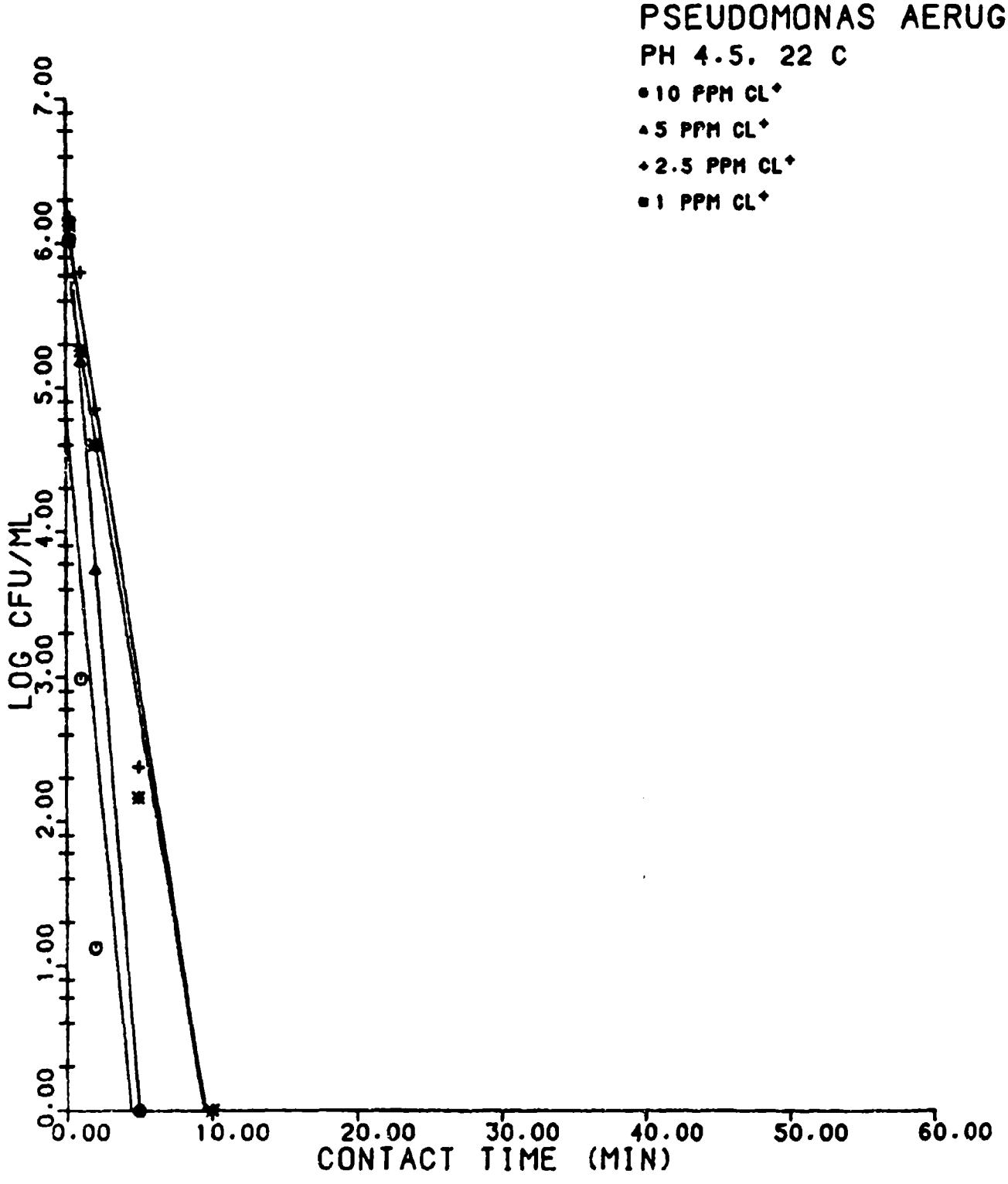

Figure A-10. Disinfection of Pseudomonas aeruginosa by Agent I at pH 4.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

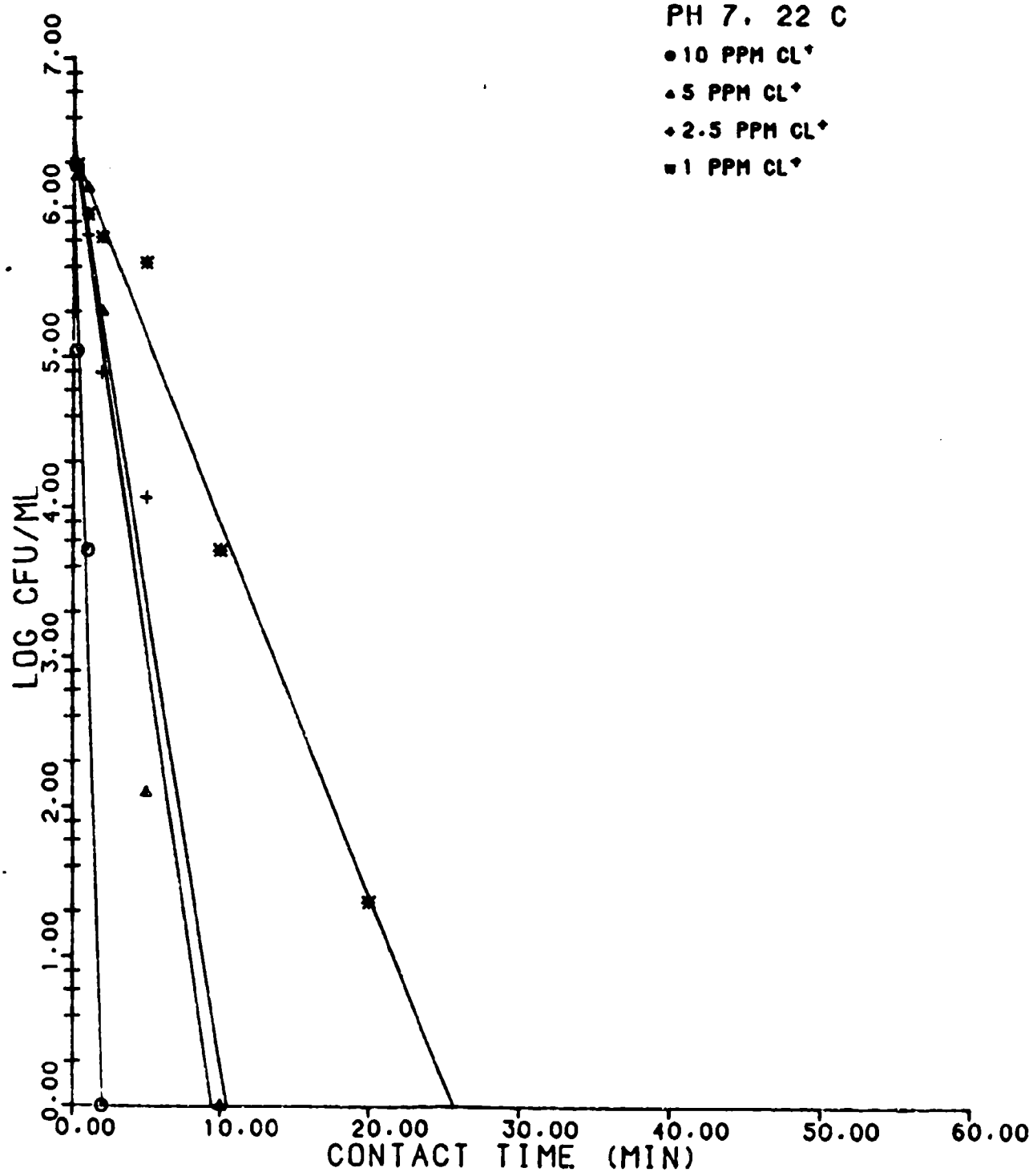

Figure A-11. Disinfection of Pseudomonas aeruginosa by Agent I at pH 7, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

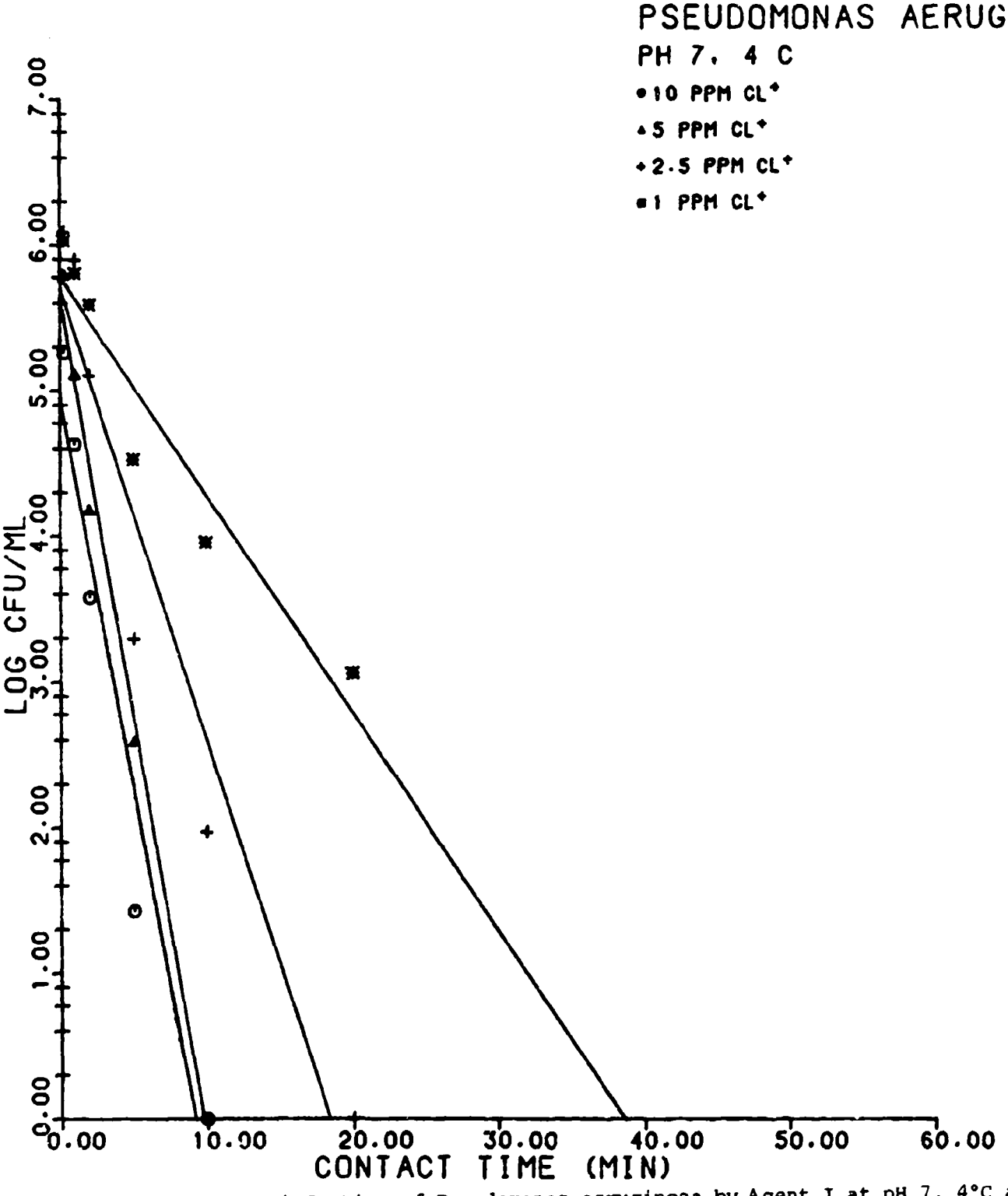

Figure A-12. Disinfection of Pseudomonas aeruginosa by Agent I at pH 7, 4°C as a Function of Concentration of Potentially Ionizable Chlorine.

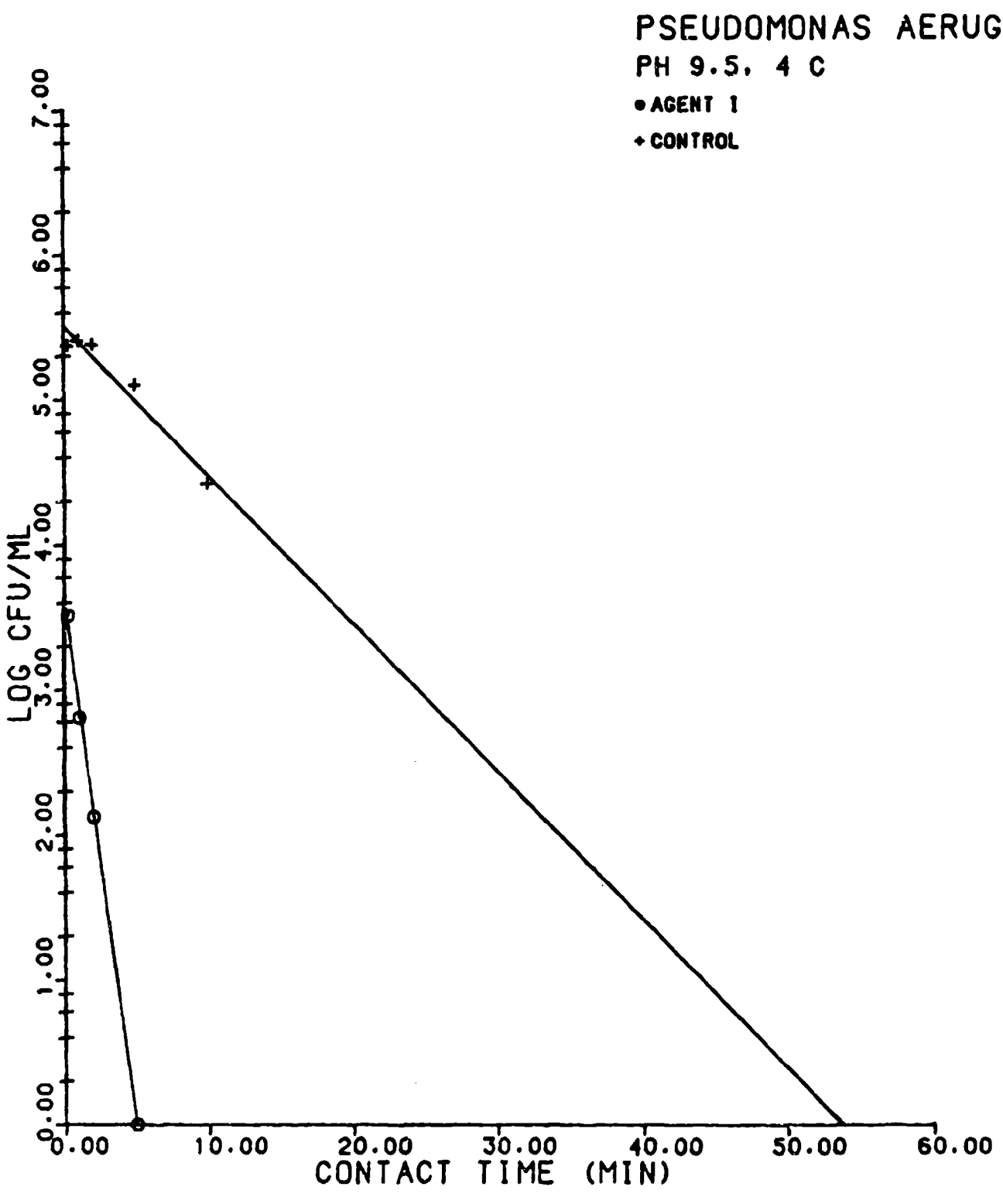

Figure A-13. Disinfection of Pseudomonas aeruginosa by Agent I at pH 9.5, 4°C at 2.5 ppm Potentially Ionizable Chlorine.

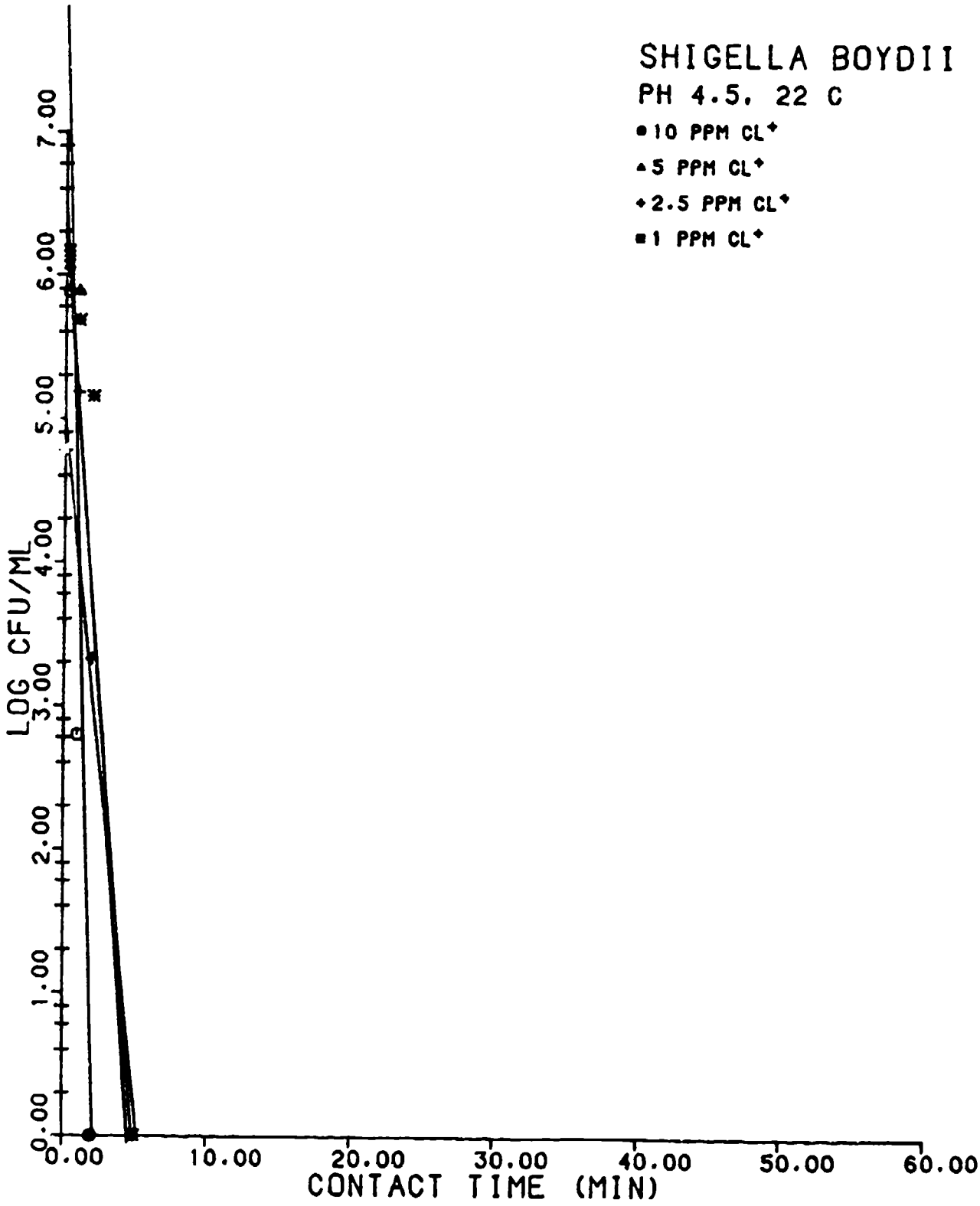

Figure A-14. Disinfection of Shigella boydii by Agent I at pH 4.5, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

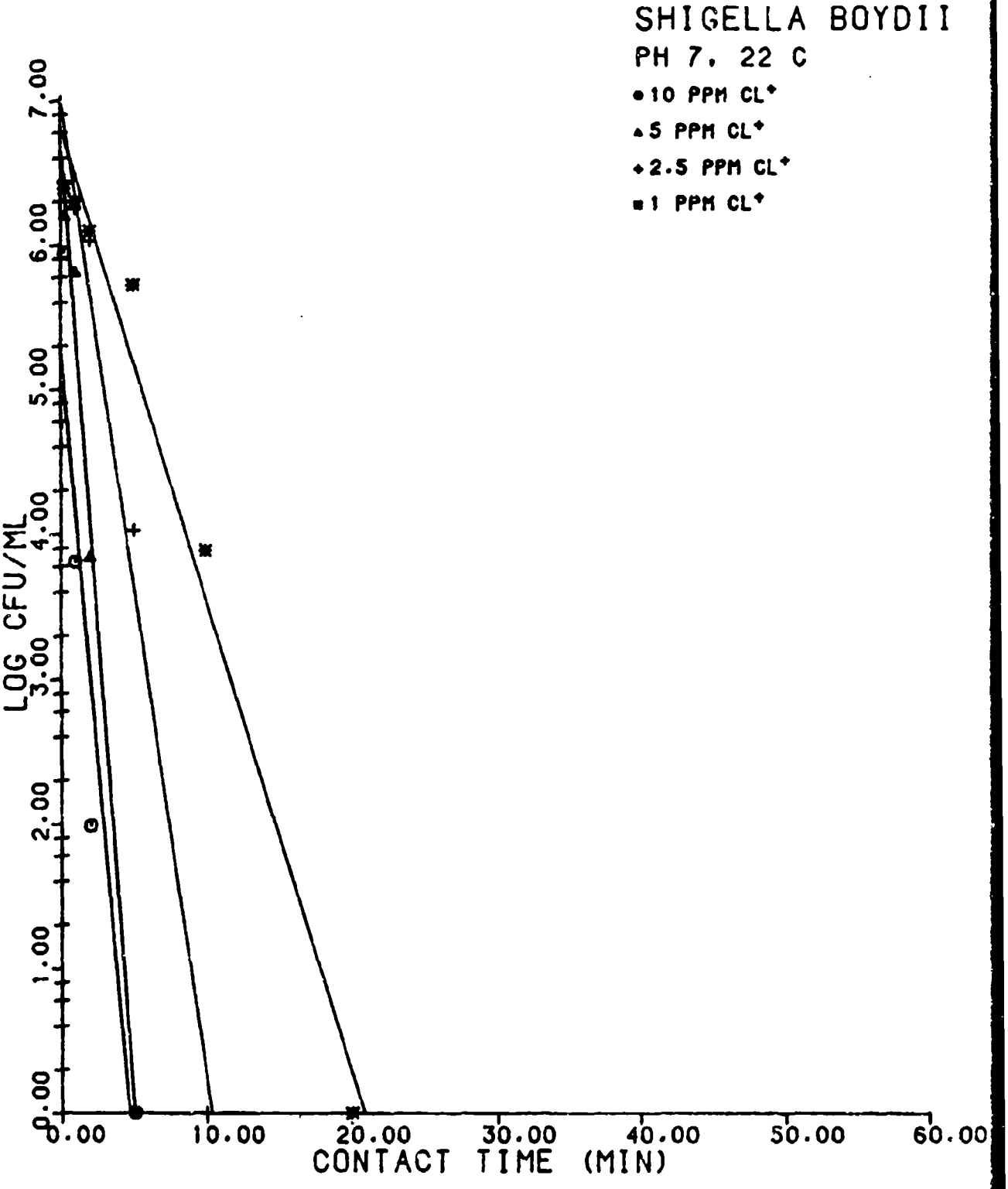

Figure A-15. Disinfection of Shigella boydii by Agent I at pH 7, 22°C as a Function of Concentration of Potentially Ionizable Chlorine.

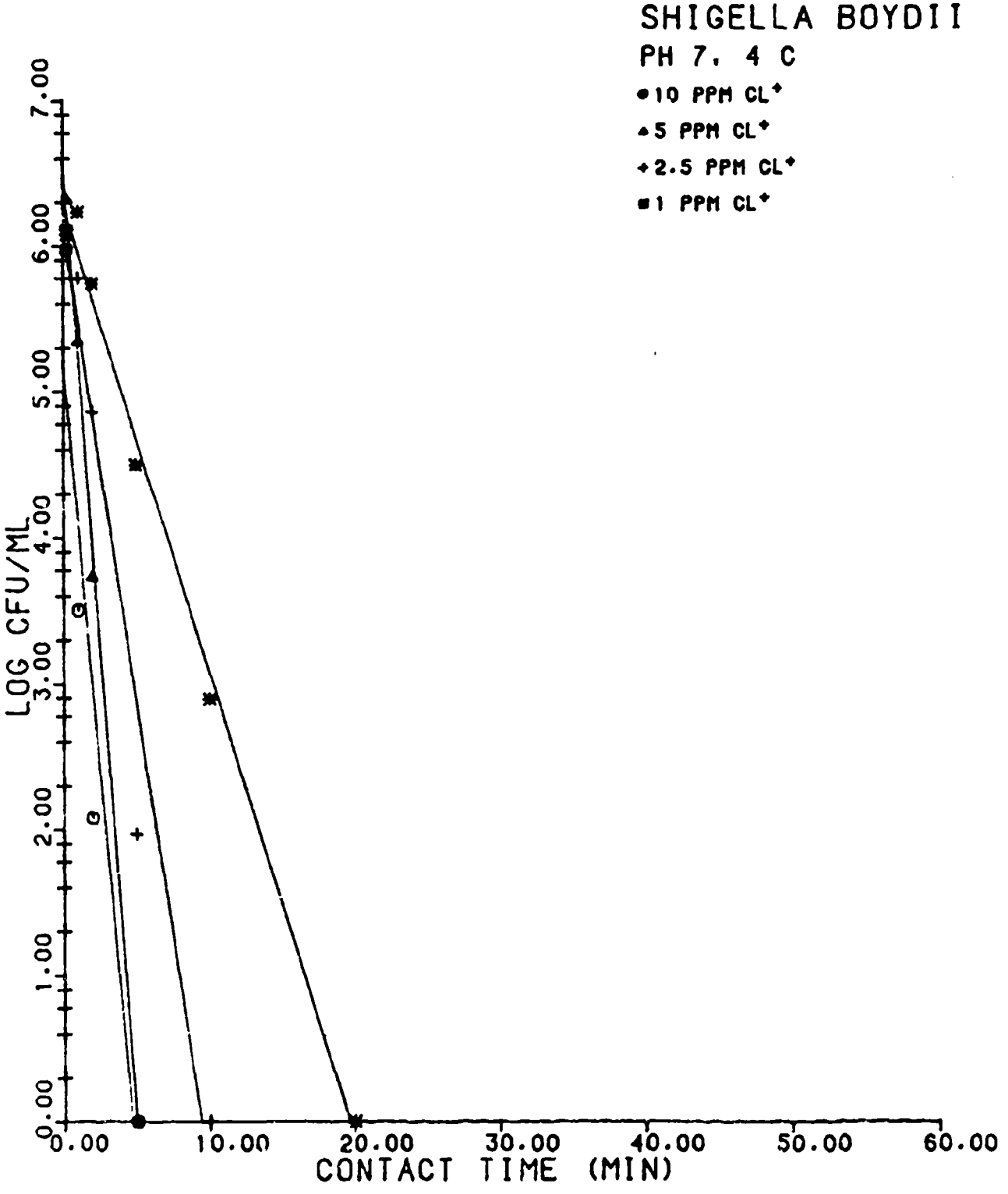

Figure A-16. Disinfection of Shigella boydii by Agent I at pH 7, 4°C as a Function of Concentration of Potentially Ionizable Chlorine.

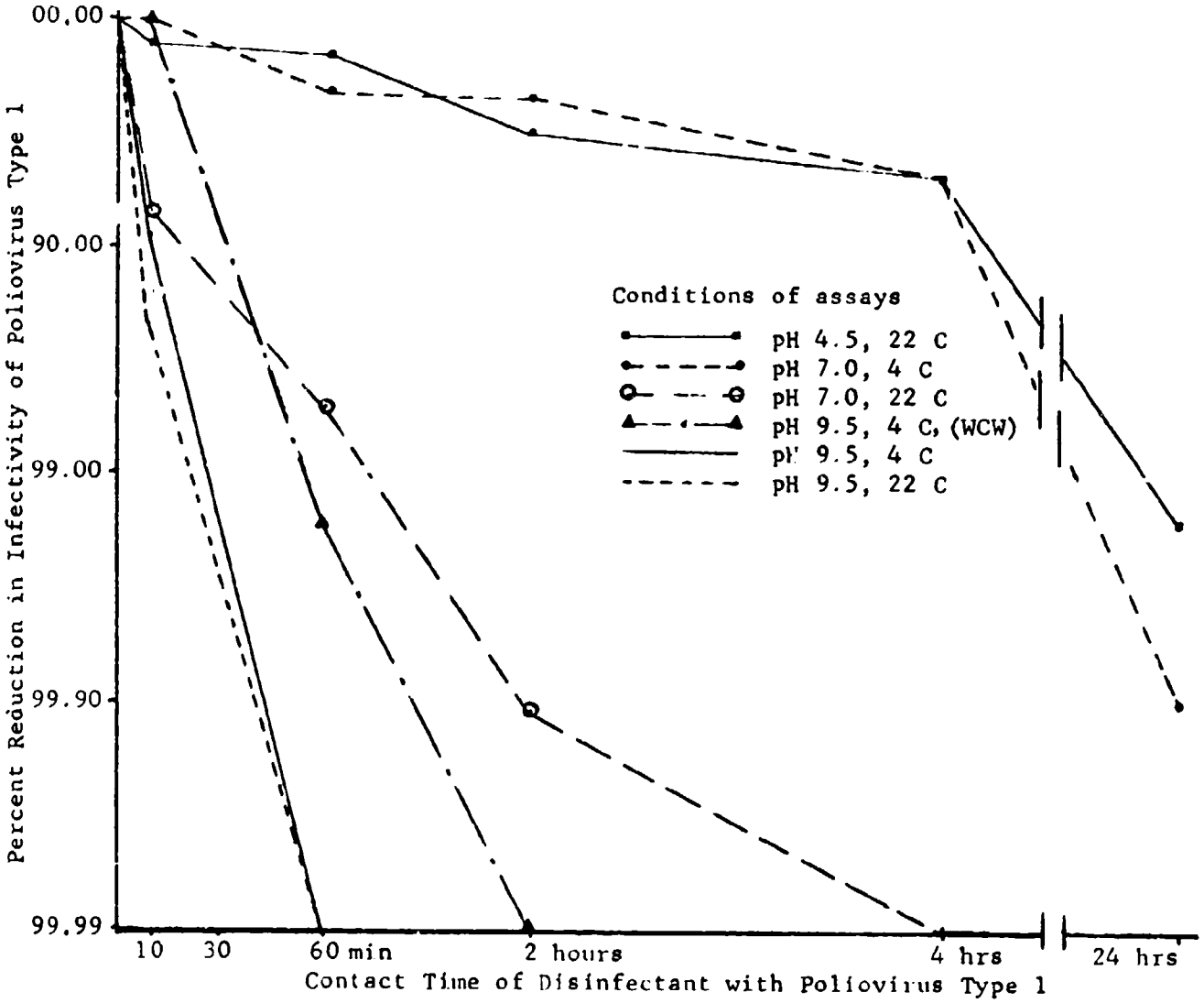

Figure A-17. Effect of Assay Conditions on Rate of Inactivation of Poliovirus Type 1 (Chat strain) by Agent I at Concentration of 400 ppm Potentially Ionizable Chlorine.

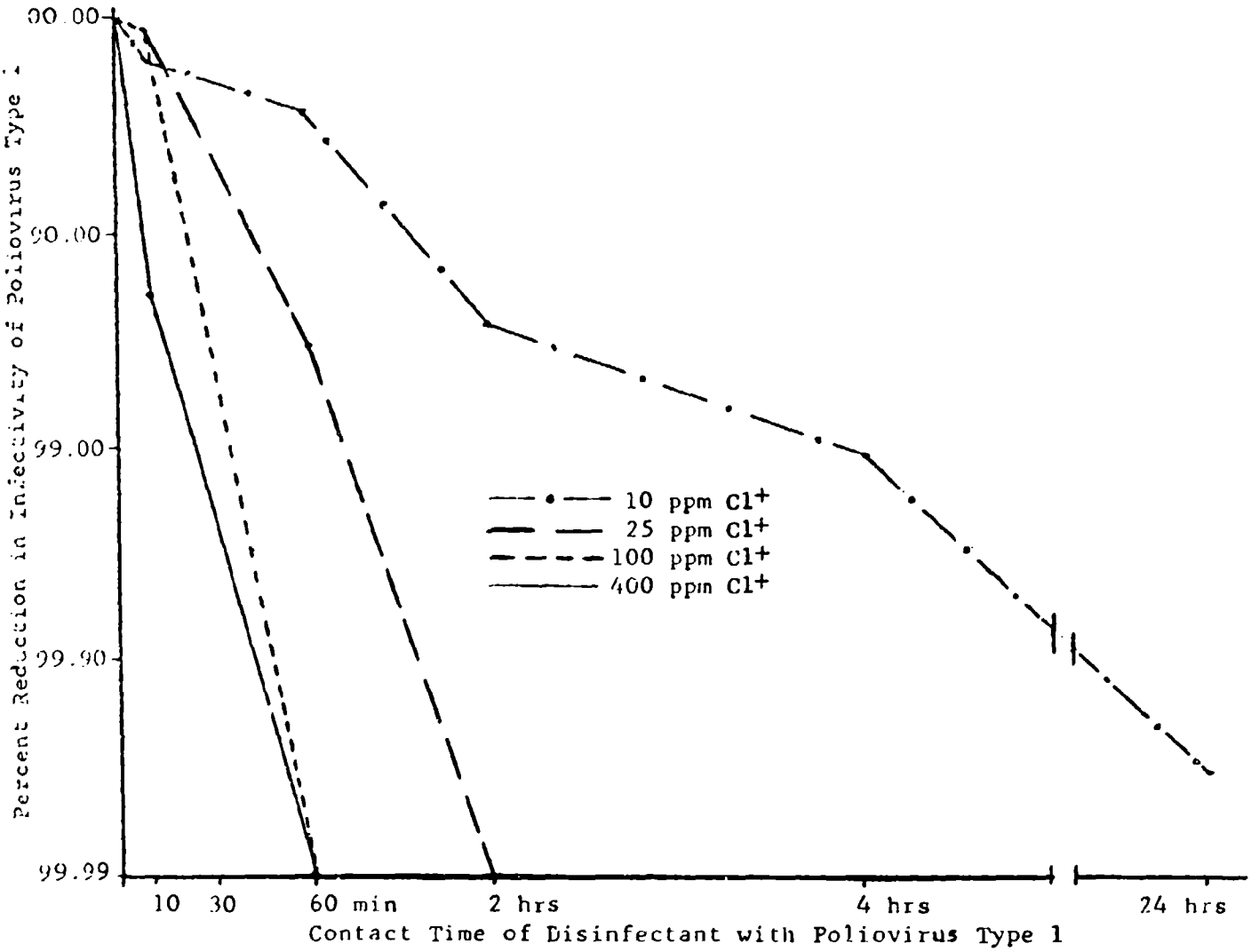

Figure A-18. Rate of Inactivation of Poliovirus Type 1 (Chat Strain) by Different Concentrations of Agent I at pH 9.5, 22 C.

APPENDIX B

EVALUATION OF "AGENT I" IN TWO IN VITRO MUTAGENICITY ASSAYS

This Appendix contains material produced by Arthur D. Little, Inc. for Air Force Aerospace Medical Research Laboratory, Air Force Systems Command, Wright-Patterson AFB, Ohio 45433. The material contained herein is reprinted as written. Table numbers are changed to avoid confusing the reader.

LIST OF ABBREVIATIONS

CHO	Chinese hamster ovary
HGPRT	hypoxanthine-quanine phosphosphoribosyltransferase
S9	9000 xg supernatnant
EMS	ethylmethanesulfonate
FCS	fetal calf serum
FCM	macromolecular (dialyzed) fraction of fetal calf serum
EDTA	disodium ethylenediamine-tetraacetate
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
KCl	potassium chloride
IITRI	Illinois Institute of Technology Research Institute

OBJECTIVE

The objective of this study is to evaluate the mutagenic potential of "Agent I", a water disinfectant of interest to the United States Air Force. Two in vitro short-term assays, a microbial Salmonella/mammalian microsomal mutagenicity assay (Ames assay) and a mammalian specific locus gene mutation assay (CHO/HGPRT assay), were utilized for this purpose.

The Ames mutagenicity assay measures the ability of chemical agents to induce mutations in certain strains of bacteria. The suspect chemicals are tested with five specially constructed mutants of Salmonella typhimurium, selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by chemical mutagens. By adding homogenate of rat liver (S9) to the plates, thus incorporating an aspect of mammalian metabolism in vitro, the assay may also detect potential mutagens which require metabolic activation.

The CHO/HGPRT assay measures the ability of a test compound to induce forward mutations at the enzyme hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) locus of Chinese hamster ovary cells on the basis that presumptive mutants are defective in the enzyme HGPRT are unable to convert purine analogues such as 6-thioguanine to toxic metabolites. Hence, in a selection medium containing 6-thioguanine, the mutant cells will be able to grow, while the wild type cells are killed.

MATERIALS AND METHODS

TEST COMPOUND

The test compound, "Agent I", was provided by the United States Air Force and used as such without further analyses. The text compound was stored in a 4°C refrigerator designated for hazardous substances. According to information provided by the U.S. Air Force, 42 mg/liter of this material contains 10 mg/liter of total "chlorine" as Cl^-, or 20 mg/liter of Cl_2 if the Wallace-Tiernan titrator is used for the measurement. There is no "free chlorine" for this material. "Agent 1" was tested on a total weight basis and not as ppm of total chlorine. The stability of the test material during the treatment period of the mutation assays is not known.

The positive control compound ethylmethanesulfonate (EMS) was provided by the National Cancer Institute through IITRI, 2-aminoanthracene, 2-nitrofluorene were obtained from Aldrich Chemical Company and 9-amino-acridine and sodium azide were from Sigma Chemical Company. These control compounds are stored in a -20°C freezer or a 4°C refrigerator designated for hazardous substances.

SALMONELLA/MAMMALIAN-MICROSOMAL MUTAGENICITY ASSAY (AMES)

Salmonella Tester Strains

The Salmonella typhimurium stains used in this study were obtained from Dr. Bruce Ames, University of California, Berkeley, California, and are identified as TA-98, TA-1538, TA-100, TA-1535, and TA-1537. Their properties and specific details of the assay have been described by Ames and co-workers (Ames et al., 1973). Master cultures from which working cultures are prepared are maintained frozen in liquid nitrogen. Working cultures are maintained at -80°C. Confirmation of strain performance is conducted every six months.

Ames Assay Test Procedure

The Ames assay was conducted according to our standard operating procedure #CB/M-812a. "Agent I" was screened for toxicity in a standard plate incorporation assay with or without metabolic activation (Aroclor 1254-induced rat liver microsomal fraction, S9) with TA-100 at seven concentrations from 0.001 to 1000 µg/plate in triplicate plates. "Agent 1" was assayed for mutagenicity in the standard plate incorporation assay against strains TA-98, TA-100, TA-1535, TA-1537 and TA-1538 with and without metabolic activation at concentrations of 5, 1, 0.2, 0.04 and 0.008 µg/plate in duplicate plates. The organism (0.1 ml), the appropriate sample dilution (0.2 ml) and the S-9 mixture (0.5 ml, if required), were added to 2.0 ml of 0.6% molten top agar containing the histidine/biotin supplement. The molten top agar mixture (in duplicate) was mixed by vortexing and poured on minimal glucose agar plates. The solvent control was the highest volume of DMSO used in each experiment. Positive controls are listed below and included compounds which do and do not require metabolic activation.

Tester Strain	Positive Control Chemicals
All Strains	2-Aminoanthracene, 10 µg/plate
TA-98, TA-1538	2-Nitrofluorene, 10 µg/plate
TA-100, TA-1535	Sodium azide, 10 µg/plate
TA-1537	9-Aminoacridine, 50 µg/plate

After incubation for 48 hours at 37°C, mutant colonies were counted and results reported as (average) total number of revertants per plate ± the standard deviation.

Quantitation of Data

The mean number of revertants and the standard deviation were calculated for each condition and the results expressed as mean number of revertants per plate.

Acceptability of Assays and Criteria for Response

The criteria used to determine the validity and results of an assay include the following:

- Solvent control values must be within the normal range.
- Number of revertants induced by positive control chemicals must be within the historical normal range.
- There must be a healthy (background) "lawn" of cells indicating that the test chemicals have not been assayed at concentrations that are so cytotoxic that all induced mutants have been killed.
- Concentrations of materials tested should include toxic doses since mutagenicity and toxicity are related, but non-toxic doses must also be tested.
- A twofold increase in numbers of revertants over the spontaneous number of revertants with a positive dose-response relationship is considered a positive test.
- Dose-response curves should be reproducible.
- A positive dose-response relationship which does not double the number of spontaneous revertants is an indication of an inconclusive (±) test.

CHO/HGPRT MUTAGENESIS ASSAY

Cell Cultures

The cells used in the mutagenesis study were subclone of BH_4 strain k_1 of the Chinese hamster ovary (CHO) cell line. The stock cultures were originally obtained from Dr. Abraham Hsie's Laboratory (Oak Ridge National Laboratory, Oak Ridge, Tennessee) in April, ±982 and stored in liquid nitrogen. The CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin. They were grow in monolayer culture at 37°C in an atmosphere of 5% CO_2 and 95% humidity. For subculture, the cells were detached with 0.05% trypsin/0.02% EDTA solution. Under these culture conditions, the cells have a doubling time of approximately 12 hours and maintain a stable karyotype of 19-20 chromosomes.

Cytotoxicity Determination

Initial Screening - The cytotoxic effect of "Agent I" on CHO cells was determined by a direct cloning assay which measured a reduction in colony-forming ability of the cells following a 24-hour treatment with the

test compound. Cells were plated at 300 cells per 100 mm tissue culture dish 24 hours prior to treatment with 0.1 - 1000 µg/ml of "Agent I". After 24 hours of treatment, the medium was removed, the cells were rinsed two times with PBS, and fresh medium added. The cells were incubated for 7 days to allow colonies to develop, then rinsed with PBS, fixed in 100% methanol and stained with 5% Giemsa. Colony counts were made and cloning efficiencies and surviving fractions determined. The selection of concentrations for the CHO/HGPRT mutagenesis assay was based on the results of this clonal assay.

Parallel Cytotoxicity Assay - In parallel with the mutation assay, CHO cells were plated in duplicate at 200 cells/100 mm plate to determine the cytotoxic effect of the test compound at the concentrations selected for the mutagenicity assay. The cells were then fixed with methanol, stained with Giemsa and colonies counted. The % cloning efficiency for each test condition was calculated based on the number of cells plated. The surviving fraction was determined by comparing the cloning efficiency of the treated to the control.

CHO/HGPRT Test Procedure

Mutation induction at the HGPRT locus was measured using our standard operating procedure #CB/M-806 for this assay. Briefly, cells were plated at 5×10^5 cells per 100 mm dish in Ham's F12 medium containing 5% dialyzed fetal calf serum (FCM). After 24 hours, duplicate cultures were treated with "Agent 1", the primary stock of which was prepared in DMSO. The solvent control was the highest concentration of DMSO used in the experiment and the positive control ethylmethanesulfonate (EMS) was used at 248 µg/ml. The cells were treated for 16 hours after which the medium was removed, the cells were rinsed twice with PBS, and fresh Ham's F12 medium containing 5% FCM was added. After 24 hours of incubation, the cells from each dish were trypsinized, counted, and replated in duplicate at 1×10^6 cells per 100 mm dish in 10 ml of Ham's F12 medium containing 5% FCM. To allow for phenotypic expression, 1×10^6 cells were subcultured from each of the dishes every 2-3 days for a total expression time of 9 days. The cells were then plated for selection of 6-thioguanine resistant mutants. Cells from duplicate plates were trypsinized, pooled, and plated at a concentration of 2×10^5 cells per 100 mm dish (5 dishes per set) in 10 ml of Ham's F12 medium without hypoxanthine and with 10 µM 6-thioguanine. From the same stock cells, duplicate 60 mm dishes were plated with 200 cells in 5 ml of Ham's F12 medium with 5% FCM for determination of cloning efficiency.

After 7 days of incubation, the 60 mm dishes for cloning efficiency determinations were rinsed with PBS, fixed in methanol, and stained with 5% Giemsa. THe same was done with the 100 mm dishes for mutant colonies, after 9 days of incubation. Colony counts were made and cloning efficiencies and surviving fractions determined by pooling the results of duplicate sets.

Quantitation and Statistical Analysis of Data

The mutation frequency was calculated by dividing the total number of mutant colonies by the total number of cells plated (2×10^6) corrected for the cloning efficiency. Mutation frequency was then expressed as the number of mutants per 10^6 clonable cells. To determine the statistical significance of the mean number of mutant colonies per plate of the treated condition versus the control, the following two-tailed t-test was carried out.

$$s = \sqrt{\frac{(n_c-1)s_c^2 + (n_t-1)s_t^2}{n_c + n_t - 1}}$$

$$t = \frac{|\bar{x}_c - \bar{x}_t|}{s\sqrt{\frac{1}{n_c} + \frac{1}{n_t}}}$$

n_c = sample size of control population;
n_t = sample size of test population;
s_c = standard deviation of control population;
s_t - standard deviation of test population;
$\bar{x}_c$ = mean of control population;
$\bar{x}_t$ = mean of test population.

A value of $p<0.05$ was considered statistically significant.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- The cloning efficiency of CHO cells is 60% or better.
- The spontaneous frequency is between 0-20 mutants per 10^6 clonable cells.
- The positive control, EMS at 248 µg/ml induced at least 300 mutants per 10^6 clonable cells.
- An agent is considered positive in the assay if it induces a statistically significant ($p<0.05$) mutation frequency above the control, (or solvent control, whichever has a higher frequency) and responds in a dose-dependent manner.

RESULTS

Ames Assay

Results of the cytotoxicity assay of "Agent I" to TA-100 are shown in Table 1. Concentrations of 1000 and 100 µg/plate caused total destruction of the background lawn on one plate and sparse backgrounds on two plates. The highest concentration used in the mutagenicity assay was 5 µg/plate.

The results of the mutagenicity assay (Table 2) show that the highest concentration tested (5 µg/plate) was slightly toxic to all strains except TA-1538 and no mutagenic response was evident at any of the concentrations tested. All positive control chemicals elicited a positive response and DMSO control backgrounds were acceptable.

CHO/HGPRT Assay

Data on the cytotoxicity of "Agent I" to CHO cells are presented in Table 3. Over a five-log concentration range of 0.1-1000 µg/ml tested, "Agent I" was cytotoxic at concentrations of 10 µg/ml and above, resulting in no clonal growth of CHO cells at those concentrations. The highest concentration tested in the mammalian cell mutagenicity assay was 5 µg/ml.

Table 4 shows the results of the cytotoxicity and mutagenicity assays conducted with "Agent I". Under the conditions of the assay, "Agent I" was non-toxic at 5 µg/ml. Even though the CHO cells were exposed to "Agent 1" for 24 hours in the initial assay compared to 16 hours in the mutagenicity assay, these results nevertheless suggest "Agent I" exhibited an abrupt cytotoxic profile on the CHO Cells.

The data in Table 4 show that "Agent I" is not a mammalian cell mutagen under the conditions of the assay, since the total numbers of thioguanine-resistant mutant colonies scored in the test conditions were not statistically different than those of the untreated and solvent controls. The mutation frequency of the untreated control was slightly higher than generally observed. However, the assay was determined to be acceptable based on the mutation frequency obtained in the solvent control and the positive control.

CONCLUSION

Under the conditions of the assays, "Agent I" induced no mutagenic response in either the Salmonella/mammalian microsomal mutagenicity assay (Ames assay) or the CHO/HGPRT gene mutation assay. The test compound was relatively cytotoxic to both microbial and mammalian cells.

TABLE B-1

TOXICITY TEST - "AGENT I"
AMES ASSAY

	Revertants Per Plate[a] TA-100 -S9
Negative Control[b]	120±13
2-Aminoanthracene[b] (10 µg/plate)	159±16
Sodium Azide[c] (10 µg/plate)	TNTC
"Agent I"[b]	
1000 µg/plate	d
100 µg/plate	d
10 µg/plate	e
1 µg/plate	121±7
0.1 µg/plate	95±5
0.01 µg/plate	100±17
0.001 µg/plate	125±14

[a]Mean of triplicate plates ± standard deviation

[b]Dissolved/diluted in dimethylsulfoxide (DMSO)

[c]Dissolved/diluted in sterile distilled water

[d]Toxic effect seen, total destruction of the lawn

[e]Toxic effect seen, one plate with total destruction of background lawn and two with sparse background lawn.

TABLE B-2

AMES ASSAY OF "AGENT I"

	Revertants Per Plate[a]									
	TA-98		TA-1537		TA-1538		TA-100		TA-1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative Control	21±1	29±2	4±1	7±2	9±1	16±1	134±2	117±10	22±2	11±1
2-Aminoanthracene[b] (10 µg/plate)	28±1	TNTC	12±1	143±23[d]	6±1	346±98[d]	179±19	TNTC	21±0	54±6[d]
2-Nitrofluorene[b] (10 µg/plate)	TNTC	TNTC			TNTC	TNTC				
9-Aminoacridine[b] (50 µg/plate)			136±18	64±6						
Sodium Azide[c] (10 µg/plate)							TNTC	TNTC	TNTC	TNTC
"Agent I"[b]										
5 µg/plate	13±0[e]	25±0	1±1[e]	5±3	3±1	22±3	102±4[e]	105±2	20±2[e]	11±1
1 µg/plate	20±5	26±4	5±1	11±0	9±1	20±5	101±1	107±9	16±3	8±1
0.2 µg/plate	16±3	28±3	5±1	8±0	6±1	20±3	97±4	125±6	26±3	13±3
0.04 µg/plate	20±3	25±2	7±2	5±2	8±4	13±4	108±5	119±12	17±1	9±1
0.008 µg/plate	20±1	27±4	6±1	9±2	6±2	23±2	95±14	120±15	22±0	12±1

[a]Mean of two replicate plates ± standard deviation

[b]Dissolved/diluted in dimethylsulfoxide (DMSO)

[c]Dissolved/diluted in sterile distilled water

[d]Slight toxic effect seen in selected areas of the plate

[e]Grainy background indicating slight toxicity

TABLE B-3

CYTOTOXICITY OF "AGENT I" ON CHO CELLS
CLONAL DETERMINATION

Concentration (µg/ml)	Colonies/Plate ±S.D.[a]	Cloning Efficiency (%)	Treated/Control
0	286±25.8	95.3	-
0.1	285±19.8	95.0	0.99
1.0	256±3.4	85.3	0.90
10.0	0.8±0.5	0.3	0.003
100.0	0	-	-
1000.0	0	-	-
Solvent Control (2% DMSO)	259±20.9	86.3	0.91

[a]Plating density: 300 cells; mean of 4 plates ± standard deviation.

TABLE B-4

CHO/HGPRT MUTAGENICITY ASSAY ON "AGENT I"

Chemical	µg/ml	Cytotoxicity		Mutagenicity		
		% Cloning Efficiency	Treated/ Control	Total Colonies	% Cloning Efficiency	Mutation Frequency*
Control	0	105	1.00	88	109	40.4
Solvent Control (0.1% DMSO)	0	92	0.87	30	84	17.9
"Agent I"	0.5	92	0.87	34	73	23.3
	1.0	95	0.90	46	80	28.8
	2.5	99	0.94	57	51	62.1
	5.0	97	0.92	48	97	24.7
Ethylmethanesulfonate	248	52	0.49	1407	58	1212.9

*Mutation frequency is expressed as the number of mutants per 10^6 clonable cells.