In this study of the effect of chlorine on enteric viruses, polioviruses and Coxsackie virus in water were inactivated by combined residual chlorine. The conditions under which this happens are discussed. Differences in resistance to chlorine were found among virus strains.

THE EFFECT OF CHLORINE IN WATER ON ENTERIC VIRUSES. II. THE EFFECT OF COMBINED CHLORINE ON POLIOMYELITIS AND COXSACKIE VIRUSES

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THIS REPORT extends the observations described previously[1] on the effect of residual chlorine on enteric viruses in water to include combined residual chlorine.

Some water supplies, and sewage especially, are chlorinated for disinfection by combined residual chlorine. This differs from free residual chlorine in that it has reacted with ammonia and nitrogenous organic compounds and is present as chloramines and chlororganic compounds. No systematic studies have been made of the effect of combined residual chlorine on enteric viruses. Unwittingly, earlier investigators contributed to this study by using virus preparations that were not pure and by failing to distinguish between free and combined residual chlorine. This presentation describes the conditions under which combined residual chlorine inactivates viruses in water.

Materials and Methods

In general, two kinds of observations were made: comparisons of the rates of inactivation of two strains of enteroviruses at different pH levels by one concentration of combined residual chlorine, and estimates of the times and concentrations of combined residual chlorine required for greater than 99.7 per cent inactivation of a resistant strain.

The precautions taken to reduce the chlorine demand to a minimum, the preparation of solutions and viruses, and the procedures were similar to those described previously,[1] with the exceptions noted below.

Stock solutions of combined chlorine were made by dosing a solution of ammonium chloride with chlorine water. After a one-hour combining period the solutions contained 10 ppm of residual chlorine by the orthotolidine test and 5 ppm of ammonia. Since these levels and the ratio of chlorine to ammonia remained constant in the dark at 5-10°C, the stock solutions were kept at these conditions. Dilutions, made with demand-free water at the time of experiment, contained chlorine and ammonia in a ratio of 2 to 1 and had no free residual chlorine. Residual chlorine was determined by the orthotolidine (OT) method.[2a] Colorimetric measurements

were made in a Klett-Summerson photoelectric colorimeter with Corning filter No. 42 (blue), previously calibrated with a series of temporary standards.[2b] Mono- and dichloramines were measured amperemetrically.[2c]

Virus strains were selected on the basis of their resistance to residual chlorine as demonstrated in the previous work.[1] Poliovirus Type 1 (MK 500) was more resistant than other strains tested, and Coxsackie virus Group B Type 5 (EA 80) the least resistant. Both had been isolated from sewage. A third strain, poliovirus Type 1 (Mahoney), a laboratory-cultivated strain obtained from Connaught Laboratories, Toronto, was used in a few experiments also.

Steps in the experimental procedure, carried out at 25°C, included: addition of virus to solutions containing residual chlorine in concentrations of from 0.5 to 10 ppm and ammonia in a ratio of 1:2 to give 50-per cent tissue-culture infectious doses per 0.1 ml in amounts from 300 to 3,000; removal of 5-ml aliquots initially and at intervals for determination of residual chlorine; removal of a 1-ml sample at intervals for infectivity estimates to 0.25 ml N/10 sodium thiosulfate to reduce the chlorine. The infectivity of suspensions before chlorination was estimated in a duplicate suspension in buffered water. During experimental periods the total residual chlorine decreased slightly (Figure 1).

Virus was measured by estimating dilution of virus giving a 50-per cent endpoint of infectivity ($\log \frac{1}{TCID_{50}}$) by the moving-average interpolation method of Thompson[3] and by counting the number of infectious particles as plaque-forming units (PFU). Plaque counts were made in bottle cultures of HeLa cells[4] or of monkey-kidney epithelial cells[5] which had been inoculated with 0.5 ml of suspension.

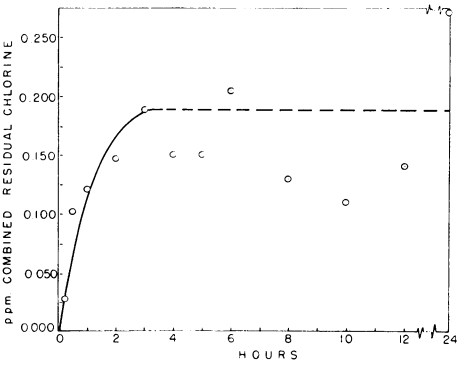

Figure 1—Average Loss of Combined Residual Chlorine During Experimental Period

Viruses were inactivated 99.7 per cent or more when one $TCID_{50}$ (50-per cent tissue-culture infectious dose) remained in 0.1 ml of suspension which had an initial infectivity titer of 300 $TCID_{50}$ or more, or when one PFU remained in 0.5 ml of suspension which had an initial plaque count of 300 PFU or more.

Results

The disinfecting action of combined residual chlorine for bacteria has been shown to be governed by several factors, among them the hydrogen-ion concentration.[6] Disinfection is accomplished faster and with less chlorine when the hydrogen-ion concentration is high. The dose of chlorine recommended for the disinfection of sewage, consequently, varies with the pH level.

In these experiments the hydrogen-ion concentration had a similar regulatory action on the inactivation of viruses by combined residual chlorine. The contact periods effecting inactivation (99.7 per cent or greater) lengthened as the hydrogen-ion concentration was decreased (Table 1). The rates of inactivation of viruses in contact with a combined residual chlorine concentration of 1 ppm differed according to the pH level. At

Table 1—Contact Time Required for Greater Than 99.7 Per cent Inactivation of Viruses at Different pH Levels by Combined Residual Chlorine at 1 ppm, 25°C

pH	Hours		
	Poliovirus 1 (MK 500)	Coxsackie virus B_5	Poliovirus 1 (Mahoney)
6	3	2	
7	3	3	3
8	>6	4	
9	6–8	5	
10	6–8	5	

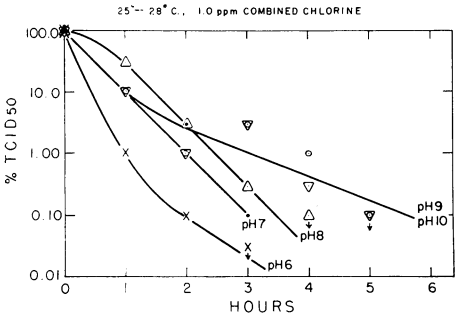

Figure 2—The Inactivation of Coxsackie Virus Group B Type 5 at Different pH Values (1 ppm Combined Residual Chlorine at 25°C)

a pH value of 6, Coxsackie virus was inactivated in three hours or less; at higher pH values, four or five hours were necessary (Figure 2). Poliovirus was also inactivated in three hours at a pH level of 6 but required more than six hours' contact time at pH values of 8 and above (Figure 3).

Butterfield's comparison[7] of strain resistance to chlorine among bacteria established the validity of the coliform index as a measure of pollution by other enteric bacteria as well. Similar information on virus resistance to chlorine is accumulating. Coxsackie virus required more intensive chlorination than did adenovirus.[8,9] Differences in resistance to free residual chlorine were encountered among strains of polioviruses and between polio and Coxsackie viruses.[1] In this experience, differences in resistance to combined residual chlorine were discerned in a comparison of the rates of inactivation (Figures 4a and 4b). At pH values of 8 and above, longer contact was required to inactivate poliovirus than Coxsackie virus. A third virus strain, poliovirus Type 1 (Mahoney), was inactivated at the same rate as the other viruses, at a pH level of 7.

The nature of the rate of inactivation was affected more by the pH level than by the strain of virus. The rates appeared constant for both poliovirus and Coxsackie virus at a pH level of 8. At higher pH levels the rates did not show a linear relationship with time (Figures 2 and 3). At lower pH values, rates approached linearity during portions of the inactivation periods.

When a comparison was made of the survival of these two viruses in contact with different concentrations of combined residual chlorine, a difference in resistance to chlorine again became apparent. The plaque-forming units which survived contact of three hours with concentrations of combined residual chlorine of up to 2.5 ppm at a pH value

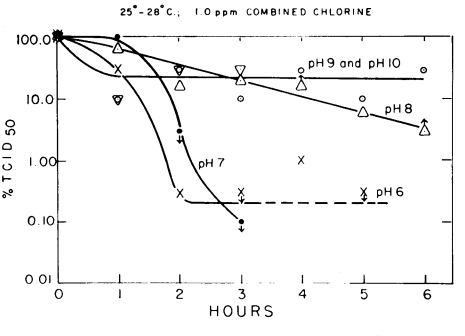

Figure 3—The Inactivation of Poliovirus Type 1 (MK 500) at Different pH Values (1 ppm Combined Residual Chlorine at 25°C)

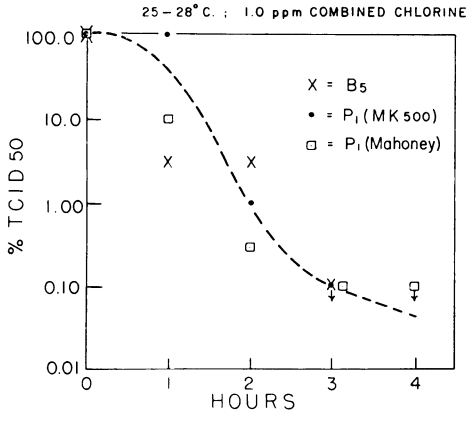

Figure 4a—The Inactivation of Two Virus Strains at pH 7 (1 ppm Combined Residual Chlorine at 25°C)

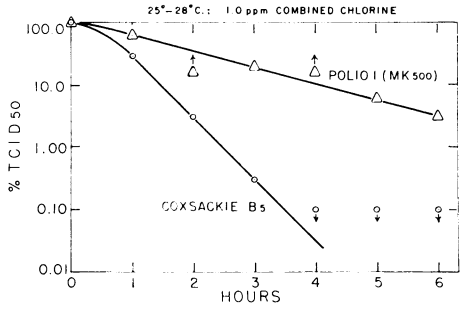

Figure 4b—The Inactivation of Two Virus Strains at pH 8 (1 ppm Combined Residual Chlorine at 25°C)

of 7 in suspensions with an initial count of from 400 to 8,000 PFU/ml were counted. The per cent recovery of plaque-forming units is illustrated in Figure 5. About 8 per cent of the poliovirus suspension survived contact with 2 ppm, while less than 0.2 per cent of Coxsackie virus preparations survived. More intensive chlorination with 2.5 ppm of combined residual chlorine reduced the number of surviving particles of poliovirus to 0.9 per cent.

An attempt was made to determine whether the poliovirus surviving chlorination represented an inherently resistant fraction. Virus in plaques which formed following contact with 2.5 ppm of residual chlorine for three hours at a pH value of 7 was subcultured. The survival of the progeny line during contact with different concentrations of residual chlorine was no greater than that of the parent strain, suggesting that the apparent resistance of the poliovirus suspension may not be of genetic origin.

These experiments made it clear that the present methods of disinfecting sewage with chlorine may not be adequate to destroy viruses, especially resistant strains. Indeed, viruses have been isolated from final plant effluents that had been chlorinated.[10] An attempt was

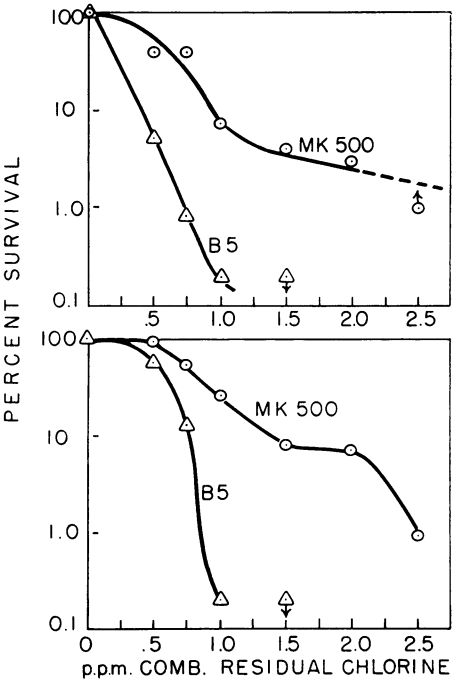

Figure 5—The Per cent Survival of Two Virus Strains After Contact with Different Concentrations of Combined Residual Chlorine for Three Hours at pH 7: (a) Infectivity Measured as 50 Per cent Tissue-Culture Dose; (b) Infectivity Measured as Plaque-Forming Unit.

Table 2—Time Required for Inactivation of Greater Than 99.7 Per cent of Poliovirus Type 1 MK 500 by Combined Residual Chlorine

	Hours ppm Combined Chlorine											
pH	0.5	0.75	1.0	1.5	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
6	6		3		2	3	2	>1½	1½	1			>½
7	>7	3	3	3	3	3	1½	1½	1	1	1	½	>½
8	12		7		4	4	4	>2	>2	2	1½	1	1
9	8		>6		6	4	4	>2	2	2	1½	1½	1

made, therefore, to determine the minimum contact periods with combined residual chlorine concentrations which would be required to inactivate the resistant poliovirus strain encountered, Type 1 (MK 500) (see Table 2). The concentration recommended for disinfection in many states, 0.5 ppm residual chlorine, inactivated virus only after contact periods longer than six hours. Another common practice, a 15-minute contact period, required concentrations greater than 9 ppm.

The failure of residual chlorine in concentrations of 6 ppm and higher to reduce the survival of virus proportionately suggests that a fraction of the suspension was resistant, an idea reinforced by the data on rates of inactivation and effective concentrations. Extending the period of contact was a more effective way of enhancing the disinfecting action of combined chlorine for this strain of virus than was increasing the concentration above 6 ppm.

Discussion

The measure of inactivation, 99.7 per cent or greater, was chosen because it represented the limit of detectability of virus in most of the suspensions, which usually contained about 300 tissue-culture infectious doses. A larger dose would have permitted greater opportunity to observe the rate of inactivation, yet would have been less appropriate to the basic purpose of the study, which was to determine the effect of combined residual chlorine on viruses in amounts that might be encountered in sewage or water supplies.

The slower rate of inactivation of viruses as the pH value was increased may be attributed to the kind of combined chlorine formed at various pH levels. Amperemetric titration of combined residual chlorine solutions showed that at lower pH values of 6 and 7, dichloramines were formed together with monochloramines (Table 3). As the pH values were increased, monochloramine formation was favored. The disinfecting property of combined residual chlorine for bacteria and protozoans is

Table 3—Formation of Mono- and Dichloramines at Various pH Levels. Chlorine to Ammonia Ratio 2:1

	ppm Residual Chlorine		
pH	Monochloramine	Dichloramine	Total
6	0.69	0.52	1.21
7	0.73	0.43	1.16
8	0.82	0.41	1.23
9	0.83	0.38	1.21
10	0.86	0.41	1.27

Figures are the average of several values.

attributed mainly to the dichloramines present.

The failure to make a distinction in the resistance to chlorine between the progency of survivors and the parent line has had counterparts in studies of heat resistance. Stanley found subcultures of survivors of heat treatment to be inherently more resistant than the original virus suspension.[11] Youngner's experience[12] with many strains of poliovirus indicated that the thermal inactivation rate of a parent line was the sum of the rates of all the particles in the line.

The nature of the relationship between virus concentration and time of inactivation, i.e., the rate of inactivation, is not simple. As noted in the previous observations on the effects of free residual chlorine on viruses,[1] conditions favoring inactivation—low pH value and sensitive strain—for example, favored a linear relationship of virus concentration with time. This state could be interpreted as meaning that the inactivation rate is determined by several factors and may be described by a family of curves that approach linearity as conditions for a maximum inactivation rate are met. The kinetics of bacterial disinfection have long enjoyed an anomalous description,[13] and the many recent studies of inactivation of poliovirus by formaldehyde, heat, and ultraviolet irradiation indicate that virus disinfection may be subject to the same controversies.

The table summarizing minimum concentrations and contact periods for the inactivation of poliovirus indicates that the current recommendations for sewage treatment may not disinfect it of viruses. Since disinfection is defined as destruction of all pathogens, the doses meeting present-day standards are obviously not adequate. More intensive chlorination and its financial backing may be necessary. The data suggest that a longer contact period with small amounts of chlorine may be more effective in destroying resistant strains of viruses than increasing the concentration. An alternative to intensified chlorination may be the addition of a second type of disinfection, such as heat, ionizing radiation, or ultraviolet irradiation, i.e., agents to which strains resistant to chlorine may be sensitive.[14]

Summary

Polioviruses and Coxsackie virus in water were inactivated by combined residual chlorine, the effective concentration depending upon hydrogen-ion concentration, contact period, and strain of virus. At 25°C and a pH value of 7, a concentration of at least 9 ppm was necessary for inactivation of poliovirus with a contact period of 30 minutes, and of 6 ppm with a one-hour contact time; 0.5 ppm required a contact period of more than seven hours.

Decreasing the hydrogen-ion concentration decreased the rate of inactivation. Differences in resistance to chlorine were found among strains of virus. The inactivation rate of a progeny line which survived chlorination was similar to that of the parent line.

The dose of combined chlorine recommended for the disinfection of sewage did not inactivate the viruses studied.

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Behavioral scientists will focus on mental health problems of the community and society at the 37th annual meeting of the American Orthopsychiatric Association, February 25-27, Sherman Hotel, Chicago, Ill. Included in the program is a joint session with the Mental Health Section of the American Public Health Association, chaired by Hyman M. Forstenzer, director, Community Mental Health Services, New York State Department of Mental Health.

The scientific sessions and workshops cover a wide range of topics with important emphasis on the problems of childhood and adolescence, including juvenile delinquency. Training of workers, narcotic addiction, adult therapy, and hospitalized patient are others.

The American Orthopsychiatric Association is a membership organization for which qualified psychiatrists, psychologists, and social workers are eligible. In addition a number of outstanding persons in anthropology, sociology, education, pediatrics, and law are invited to membership.

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