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Mechanism of Poliovirus Inactivation by Bromine Chloride†

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The mechanism of poliovirus inactivation by BrCl was determined by exposing poliovirus to various concentrations of BrCl and correlating the loss of virus infectivity with structural changes of the virus. Concentrations of 0.3 to 5 mg of BrCl per liter resulted in 95% to total inactivation of poliovirus. However, the inactivated virus retained structural integrity, as determined by buoyant density measurements of poliovirus labeled with radioactivity. However, at concentrations of 10 to 20 mg of BrCl per liter, total inactivation of poliovirus was associated with the degradation of the structural integrity of the virus. Since infectious ribonucleic acid at similar concentrations could be recovered from untreated poliovirus and poliovirus treated with 0.3 mg of BrCl per liter, it was concluded that BrCl as HOBr or bromamines inactivates poliovirus by reacting with the protein coat of the virus. Moreover, this inactivating reaction does not result in the degradation of the structure of the virion, nor does it affect the biological activity of the internal ribonucleic acid of the virus.

Although chlorine continues to be used as the disinfectant of choice in the United States, its toxic and carcinogenic by-products plus its relative ineffectiveness in inactivating human enteric viruses in wastewater has led an Environmental Protection Agency Task Force to recommend that a safer and more effective disinfectant than chlorine be used (4). BrCl was cited as the newest and least studied of the promising alternative disinfectants to chlorine. In response to this challenge, we evaluated the effectiveness of BrCl against that of chlorine under laboratory conditions with small volumes and rapid, efficient mixing (6), as well as under practical field conditions with large volumes of sewage water flowing through a model contact chamber and with a continuous and automatic system of dosing with BrCl (7). Based on the results of these studies, it was determined that BrCl is superior to chlorine in inactivating poliovirus in clean as well as contaminated water.

It should be noted that the effectiveness of disinfectants in inactivating viruses is generally measured by the inability of the treated virus population to replicate in a tissue culture system. However, this assay procedure does not differentiate between the inactivation of viruses resulting from damage to the protein coat or to the nucleic acid. Significantly, some viruses, including members of the human enteric viruses, are unique in that they require only intact nucleic acid to infect and replicate within a host cell. Thus, viruses inactivated as a result of selective damage to the protein coat still retain biologically active nucleic acid. These inactivated viruses are still potentially infectious, as conditions that allow the nucleic acid from these viruses to enter the cell can result in the normal replication of viruses. On the other hand, viruses inactivated as a result of damage to the nucleic acid are generally rendered irreversibly noninfectious. Thus, from a public health point of view, a disinfectant which inactivates viruses by damaging the nucleic acid would be superior to a disinfectant which inactivates viruses by damaging the viral protein coat without destroying the infectivity of the viral nucleic acid. Based on these considerations, we previously proposed (12) that the mechanism of virus inactivation be included as a criterion in the evaluation of one disinfectant over another. This investigation determines the mechanism by which BrCl inactivates poliovirus and compares the results with those from an earlier study on the mechanism of chlorine inactivation of poliovirus (12).

MATERIALS AND METHODS

Preparation of virus and cell culture. The attenuated strain of poliovirus type 1 (LSc 2ab) was used throughout this study. Radioactive labeling of the protein coat or nucleic acid of the virus was done with ^{3}H- or ^{14}C-amino acid mixtures or [^{3}H]uridine, as described by Breindl (2). Growth of poliovirus in con-

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tinuous African green monkey kidney cells (BGM) as well as purification of the virus by differential centrifugation, treatment with trifluorotrichloroethane, and final isopycnic banding in CsCl have been previously described (6). This purified virus preparation was dialyzed against and stored in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at 4°C.

Assay for virus, RNA, and radioactivity. Virus infectivity was quantified by the standard plaque method on monolayers of BGM cells. Viral ribonucleic acid (RNA) was extracted from poliovirus preparations by the phenol method, and the extracted RNA was assayed for biological activity on BGM cells in the presence of 500 μg of diethylaminoethyl dextran per ml, as described by Pagano et al. (10). Radioactivity was determined by assaying 50 μl of sample diluted in 5 ml of Aquasol 2 (New England Nuclear Corp., Boston, Mass.) with a Packard liquid scintillation spectrometer (model 574).

Preparation and measurement of BrCl. The preparation of halogen-demand-free glassware, triple-distilled water, and nitrogen-free 0.05 M phosphate buffer (pH 6.0 to 7.5) has been previously described (6). A saturated aqueous solution of BrCl was initially prepared by bubbling BrCl gas from a pressurized tank of BrCl (Dow Chemical Co., Midland, Mich.) into distilled water. For working solutions, this stock solution was further diluted in phosphate buffer. Concentrations of stock BrCl were measured as HOBr by the amperometric method (1) and by multiplying the titration reading by 1.62 to convert the value to milligrams of BrCl per liter (3). The dose represents the initial calculated concentration of BrCl in the test solution based on the measured concentration in the stock solution of BrCl.

Experimental design. Poliovirus (0.2 ml) containing 10^8 to 10^9 plaque-forming units (PFU) was added to 3.8 ml of various concentrations of BrCl, and the mixture was allowed to react for various time periods at 24°C in a darkened room before aliquots were removed and immediately diluted into cold phosphate buffer containing 10 mg of sodium thiosulfate per liter to stop further reaction with BrCl. Samples were assayed for virus infectivity, RNA infectivity, or radioactivity as described above. For comparing the loss of virus infectivity with structural changes of the virion, 3.2 ml of the sample was added to 1.8 ml of 65% CsCl and centrifuged at 35,000 rpm for 24 h in a Beckman model L ultracentrifuge equipped with an SW50 rotor. The bottom of each tube was punctured, and 0.2-ml fractions were collected and assayed for infectivity, radioactivity, and refractive index, from which the buoyant density of each fraction was determined by the method of Ifft et al. (5).

RESULTS

Inactivation and structural analysis of virus. We previously determined that 0.15 to 0.30 mg of BrCl per liter was the minimum dose required to inactivate at least 99% of purified poliovirus suspended in phosphate buffer (15 min, 24°C, pH 7.0), whereas a dose of 1 to 5 mg of BrCl per liter resulted in >99.99% inactivation of the virus in the sample (6). For determining the effect of BrCl on the structure of poliovirus, 0.2 ml of [^{3}H]uridine-labeled poliovirus was added to 3.8 ml of BrCl solutions, resulting in doses of 0.3 and 1.0 mg of BrCl per liter. These samples were allowed to react for 15 min at 24°C before the reaction was stopped by the addition of sodium thiosulfate, and aliquots of these samples were assayed for residual virus infectivity. As expected, doses of 0.3 and 1.0 mg of BrCl per liter resulted in the inactivation of 1.7 and >4 logs of virus, respectively. The rest of the treated samples, as well as a sample of untreated, [^{3}H]-uridine-labeled poliovirus, were separately centrifuged to equilibrium in CsCl gradients, and the collected fractions were assayed for radioactivity, infectivity, and buoyant density. The results in Fig. 1 show that the shapes of the infectivity profiles of the three samples were similar, with peaks of infectivity observed at a density of 1.34 g/cm^3, the normal density of

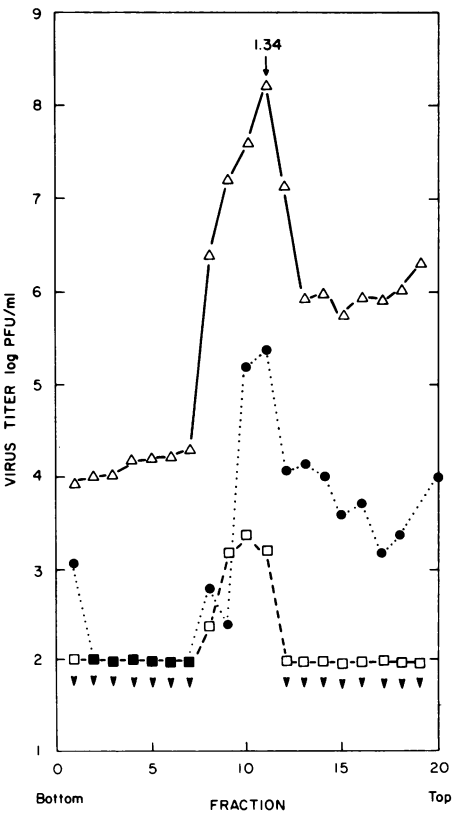

FIG. 1. *Distribution of poliovirus infectivity in CsCl gradient fractions before (△) and after treatment of poliovirus with 0.3 (●) and 1.0 (□) mg of BrCl per liter for 15 min at 24°C and pH 7.0. (▼) The final titer is something less than the level recorded.*

poliovirus. As expected, virus infectivity was highest in all fractions taken from the untreated sample, and correspondingly lower levels of virus infectivity were recovered from the samples treated with 0.3 and 1.0 mg of BrCl per liter, reflecting the effectiveness of virus inactivation by these concentrations of BrCl. However, the results in Fig. 2 show that the radioactivity profiles of these same three samples were very similar, with peaks of tritium activity at a density of 1.34 g/cm^3, indicating that BrCl-inactivated viruses still retained structural integrity.

For determining whether an effect on the protein coat of the virus could be detected, poliovirus protein coat was labeled with ^{14}C and treated with 0.3 and 5 mg of BrCl per liter for 15 min, and aliquots of the samples were analyzed for residual virus infectivity. As previously described, the rest of the samples were centrifuged to equilibrium in a CsCl gradient, and the fractions were assayed for infectivity and radioactivity. BrCl doses of 0.3 and 5 mg/liter were determined to inactivate 4.1 and >8 logs of virus, respectively. The results (Fig. 3) of the infectivity profiles of the CsCl fractions revealed that virus infectivity was higher in all fractions of the untreated sample, with the expected peak of infectivity at a density of 1.34 g/cm^3. A peak of much lower infectivity at this same density was also observed in the sample treated with 0.3 mg of BrCl per liter, reflecting the incomplete inactivation of the virus population by this concentration of BrCl. However, no infectivity was detected in any of the fractions from the sample treated with 5 mg of BrCl per liter, indicating that, at this high concentration of BrCl, total inactivation of the poliovirus population was achieved. Significantly, the radioactive profiles of the CsCl fractions from these same three samples were very similar, with a single peak of radioactivity at a density of 1.34 g/cm^3 (Fig. 4). These results indicated that, even at a dose of BrCl (5 mg/liter) which caused total inactivation of the poliovirus population, the overall structural integrity of the virus remained intact. However, when labeled virus preparations were

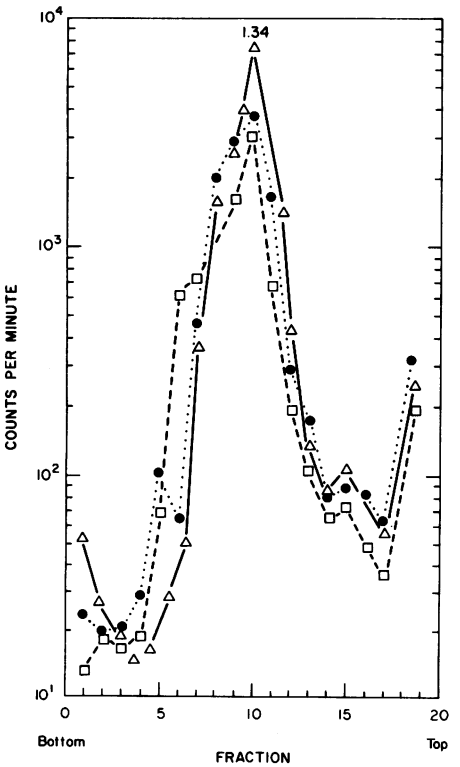

FIG. 2. *Distribution of ^3H activity in CsCl gradient fractions before (△) and after treatment of [^3H]uridine-labeled poliovirus with 0.3 (●) and 1.0 (□) mg of BrCl per liter for 15 min at 24°C and pH 7.0.*

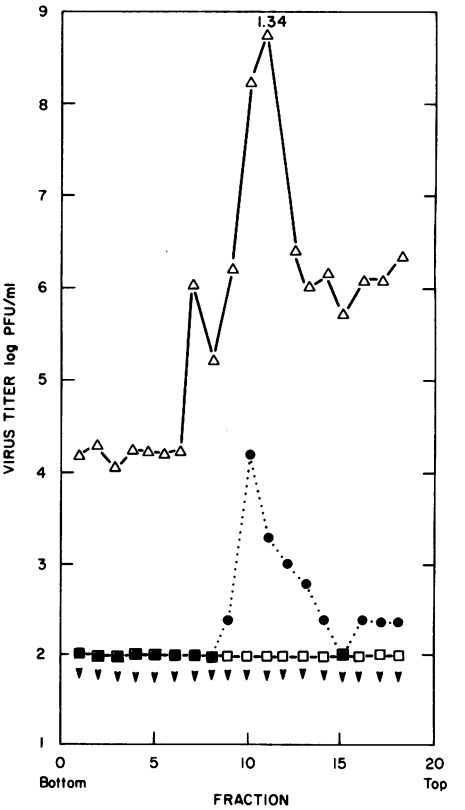

FIG. 3. *Distribution of poliovirus infectivity in CsCl gradient fractions before (△) and after treatment of poliovirus with 0.3 (●) and 5.0 (□) mg of BrCl per liter for 15 min at 24°C and pH 7.0. (▼) The final titer is something less than the level recorded.*

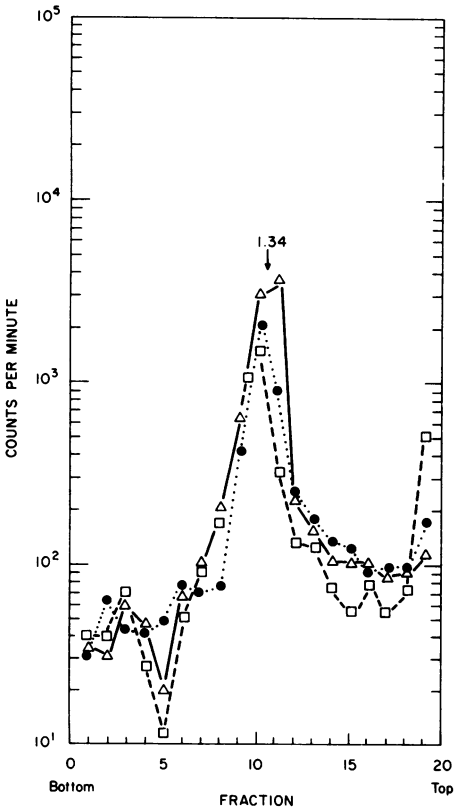

FIG. 4. *Distribution of ^{14}C activity in CsCl gradient fractions before (△) and after treatment of ^{14}C-protein-labeled poliovirus with 0.3 (●) and 5.0 (□) mg of BrCl per liter for 15 min at 24°C and pH 7.0.*

treated with 10, 15, and 20 mg of BrCl per liter and the samples were fractionated on a CsCl gradient, no peak of radioactivity was found in the fraction with a density of 1.34 g/cm^3. Moreover, for all three doses of BrCl, the major peak of radioactivity was found in fractions with a density of 1.50 to 1.56 g/cm^3. The data for virus treated with 0, 5, and 10 mg of BrCl per liter are plotted in Fig. 5. As was observed previously, the buoyant density profile of the virus sample treated with 5 mg of BrCl per liter was very similar to that of the untreated virus sample. These results indicated that degradation of the virus to denser ribonucleoprotein particles can be expected after an extensive reaction with BrCl.

Bromamine inactivation of virus. One of the factors with regard to the determination of the mechanism of halogen inactivation of viruses is the difficulty in determining which species of halogen is responsible for the inactivation of viruses. It has been clearly established that both HOCl and HOBr are the most effective disinfecting species, as compared with the various species of combined forms of halogens. It cannot be assumed that the mechanisms of virus inactivation by free and combined forms of BrCl are similar. For assessing the effect of bromamine on the infectivity and structure of poliovirus, the same experiment with ^{14}C-protein-labeled poliovirus was carried out, except that the virus was treated with 5 mg of BrCl per liter prereacted for 4 min with 0.01 and 0.1 M glycine to ensure the total conversion of HOBr to bromamines. Under these conditions, only 1.5 and 1.9 logs of poliovirus were inactivated by 5 mg of BrCl per liter mixed with 0.01 and 0.1 M glycine, respectively, presumably because of the lower virucidal activity of bromamines. Analysis of the CsCl gradient fractions for infectivity and radioactivity revealed results which were similar to those shown in Fig. 3 and 4. Thus, as with HOBr, the mechanism of virus inactivation by bromamines does not appear to cause a detectable change in the structural integrity of the virion.

Inactivation and infectivity of viral RNA. Since the data indicated that the inactivation of poliovirus by BrCl occurred long before the structure of the virus was degraded, the possibility existed that the mechanism of virus inactivation involves damage to the biological activity of viral RNA, without affecting the overall

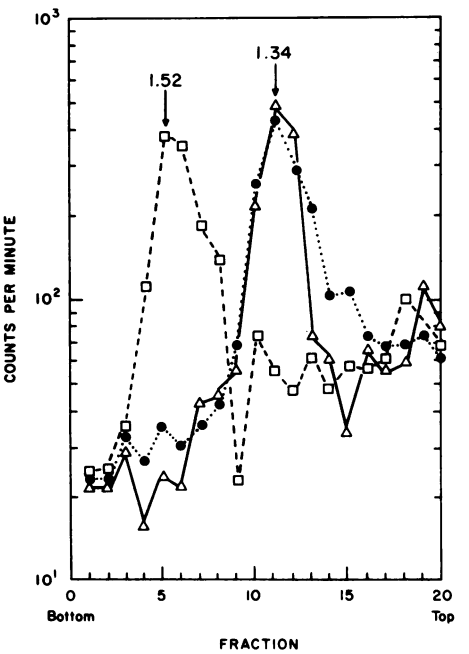

FIG. 5. *Distribution of ^3H activity in CsCl gradient fractions before (△) and after treatment of ^3H-protein labeled poliovirus with 5.0 (●) and 10.0 (□) mg of BrCl per liter for 15 min at 24°C and pH 7.0.*

structure of the virion. For assessing this possibility, RNA was extracted from virus samples before and after they were treated with 0.3 mg of BrCl per liter, and the infectivity of the extracted RNA was determined and compared. The results of three separate experiments are shown in Table 1. In each experiment, the infectivity titer of the untreated virus preparation, which ranged from 2.7×10^7 to 3.5×10^7 PFU/ml, was reduced to less than 10 PFU/ml after the virus was treated with 0.3 mg of BrCl per liter. However, the infectivity titer of RNA extracted from untreated virus, which ranged from 1.1×10^2 to 4.7×10^2 PFU/ml, was only slightly higher than the infectivity titer of 4.9×10^1 to 1.1×10^2 PFU/ml for the RNA extracted from the poliovirus samples treated with 0.3 mg of BrCl per liter. Evidence that the RNA extract samples revealed a true measurement of RNA and not of residual virus in the sample was obtained by the observation that treatment of these samples with 1 μg of ribonuclease per ml for 20 min at 24°C resulted in the total loss of infectivity in these samples. Thus, the drastic loss of virus infectivity, with little loss in the infectivity of RNA extracted from the inactivated virus, indicates that the mechanism of poliovirus inactivation by BrCl does not involve damage to the RNA of the virus.

DISCUSSION

In previous studies (6, 7), we determined that BrCl was superior to chlorine in inactivating poliovirus under both controlled laboratory conditions and field conditions of treating wastewater. Thus, BrCl was proposed as a possible alternative to chlorine for disinfecting all types of water. Since chlorine is the current disinfectant of choice, we initially evaluated the virucidal effect of chlorine and determined that the mechanism of poliovirus inactivation by chlorine was caused by an effect on the protein coat of the virus rather than by destruction of the biological activity of the viral RNA (12).

TABLE 1. *Recovery of RNA infectivity from untreated poliovirus and poliovirus treated with 0.3 mg of BrCl per liter (30 min, 24°C, pH 7.0)*

Sample	Recovered infectivity (PFU/ml)		
	Expt 1	Expt 2	Expt 3
Untreated virus	2.7×10^7	3.5×10^7	3.1×10^7
BrCl-treated virus	<10	<10	<10
RNA extracted from untreated virus	1.6×10^2	1.1×10^2	4.7×10^2
RNA extracted from BrCl-treated virus	4.9×10^1	1.1×10^2	7.7×10^1

However, in contrast to our studies, O'Brien and Newman (8) reported that chlorine inactivated poliovirus by penetrating the capsid structure of the virus to inactivate the internal RNA of the virus.

In this study, measurements of virus inactivation and structural changes of the virion were compared after exposing poliovirus to various concentrations of BrCl for 15 min at 24°C and pH 7.0. With minimal concentrations of BrCl (0.3 to 1.0 mg/liter), inactivation of 2 to 8 logs of poliovirus was consistently observed. On the other hand, doses of 5 to 20 mg of BrCl per liter resulted in the total inactivation of poliovirus. Despite the drastic inactivation of poliovirus by 0.3 to 5.0 mg of BrCl per liter, the buoyant density of the inactivated virus particle remained unaltered when assayed by isopycnic centrifugation with a CsCl gradient. Moreover, the RNA remained associated with the inactivated virus particle, and no degradation of the capsid protein was associated with the loss of infectivity of the virion. Similar results were obtained when poliovirus was treated with 5 mg of BrCl per liter in the presence of 0.01 to 0.1 M glycine to ensure the conversion of HOBr to bromamines. However, when poliovirus was treated with 10 to 20 mg of BrCl per liter, degradation of the capsid structure of the virus was detected. Finally, similar concentrations of infectious RNA could be extracted from untreated poliovirus as well as poliovirus treated with 0.3 mg of BrCl per liter. These results indicate that HOBr and bromamines inactivate poliovirus by reacting with the protein component of the virus and that the inactivating reaction does not result in any detectable change in the structure of the virion, nor does it affect the infectivity of the viral RNA. Although bromine rather than BrCl was used and different viruses were studied, similar results were obtained in previous studies. For example, Olivieri et al. (9) reported that 15 mg of bromine per liter inactivated f2 bacteriophage by reacting with the protein coat of the virus and that the viral RNA was not affected. Moreover, Sharp et al. (11) reported that the overall structure of reovirus was retained even after the virus was inactivated by 0.5 mg of BrOH per liter.

In conclusion, evidence was obtained to indicate that BrCl inactivates poliovirus by reacting with the protein coat of the virus and that the virus-inactivating event does not result in the inactivation of the internal viral RNA. The exact reaction by which BrCl renders the virus noninfectious remains unknown but is probably related to preventing the virus from properly attaching onto the cell or to preventing proper uncoating of the virus to allow the penetration

of the RNA into the cell. Based on our earlier study (12), this same conclusion can be reached with regard to the mechanism by which chlorine inactivates poliovirus. We therefore conclude that, since chlorine and BrCl appear to use the same or similar mechanism to inactivate poliovirus, there is no advantage of selecting one disinfectant over the other, based on the mechanism by which these disinfectants can be expected to inactivate viruses.

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