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# Mechanisms of Inactivation of Poliovirus by Chlorine Dioxide and Iodine

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Chlorine dioxide and iodine inactivated poliovirus more efficiently at pH 10.0 than at pH 6.0. Sedimentation analyses of viruses inactivated by chlorine dioxide and iodine at pH 10.0 showed that viral RNA separated from the capsids, resulting in the conversion of virions from 156S structures to 80S particles. The RNAs released from both chlorine dioxide- and iodine-inactivated viruses cosedimented with intact 35S viral RNA. Both chlorine dioxide and iodine reacted with the capsid proteins of poliovirus and changed the pI from pH 7.0 to pH 5.8. However, the mechanisms of inactivation of poliovirus by chlorine dioxide and iodine were found to differ. Iodine inactivated viruses by impairing their ability to adsorb to HeLa cells, whereas chlorine dioxide-inactivated viruses were able to adsorb, penetrate, and initiate uncoating normally. Sedimentation analysis of extracts of HeLa cells infected with chlorine dioxide-inactivated viruses showed a reduced incorporation of [$^{14}$C]uridine into new viral RNA. We concluded, then, that chlorine dioxide inactivated poliovirus by reacting with the viral RNA and impairing the ability of the viral genome to act as a template for RNA synthesis.

The importance of evaluating agents that offer alternatives to chlorine in the disinfection of water and wastewater has become evident. One of the main factors that has prompted interest in alternative disinfectants is that chlorine can react with organic matter to produce potentially hazardous compounds (10). Furthermore, it has been reported that chlorine-resistant enteroviruses have been isolated from finished drinking waters (20). Although the mechanisms of inactivation of viruses by halogens are not well understood, it has been suggested that compounds that destroy the biological activity of enteroviruses by damaging RNA would be superior to chlorine as disinfectants (21). Two virucidal agents that have not been extensively studied in terms of mechanisms of inactivation of viruses are chlorine dioxide and iodine. Chlorine dioxide has been shown to be at least as efficient as chlorine as a disinfectant (19). Although iodine has not been studied extensively as a virucidal agent, its efficiency as a bactericidal and virucidal agent has been recognized (5, 7). In this report, the mechanisms of inactivation of poliovirus by chlorine dioxide and iodine are compared. The results indicate that chlorine dioxide

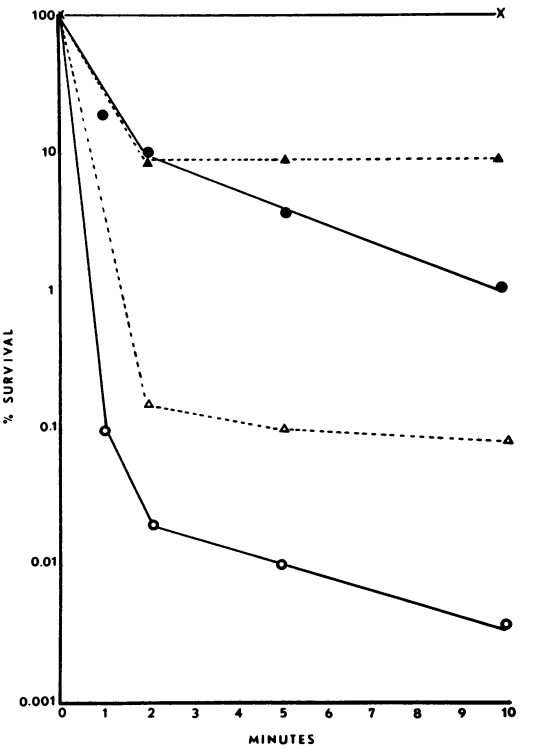


FIG. 1. Poliovirus inactivation by 1.0 mg of chlorine dioxide per liter or 2.5 mg of iodine per liter. Symbols: ●, chlorine dioxide at pH 6.0; ○, chlorine dioxide at pH 10.0; ▲, iodine at pH 6.0; △, iodine at pH 10.0; ×, untreated virus control at pH values 6.0 and 10.0.

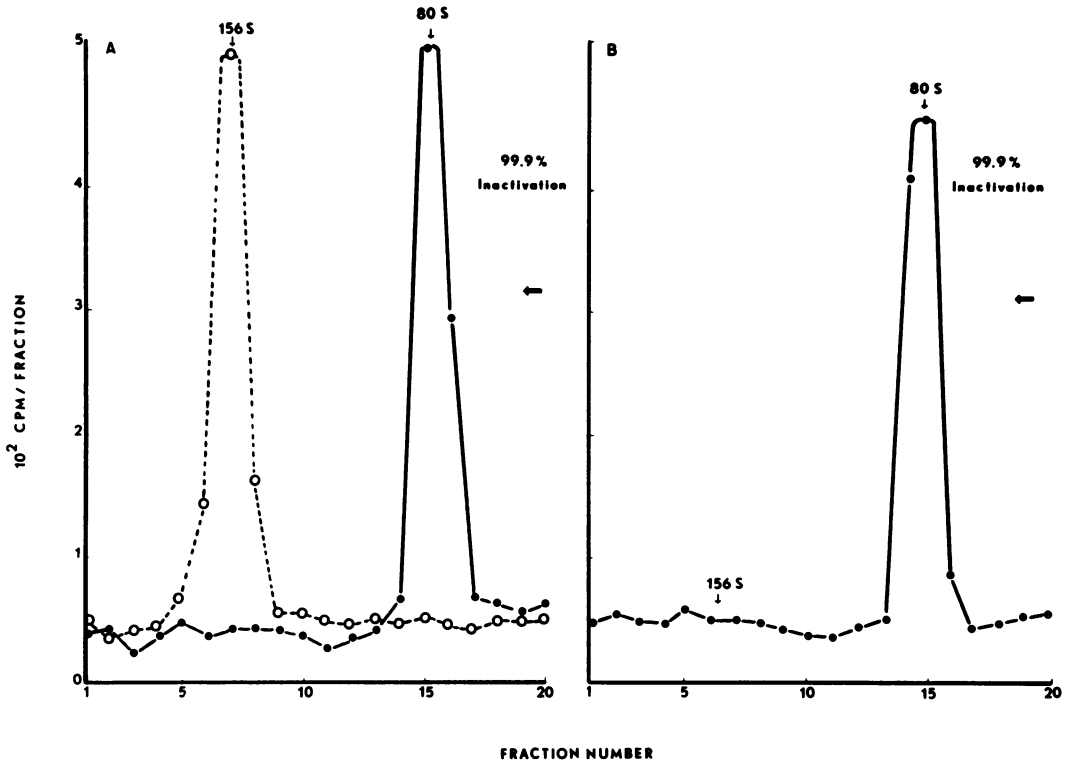


FIG. 2. Effects of chlorine dioxide and iodine on the sedimentation coefficients of capsid-labeled poliovirus at pH 10.0. Viruses were exposed to 1.0 mg of chlorine dioxide per liter for 1.0 min (A) or 2.5 mg of iodine per liter for 2.0 min (B) and then centrifuged at 30,000 rpm in 15 to 30% glycerol gradients for 3 h in an SW41 rotor. Control virus profile is shown by the dotted line. Direction of sedimentation is indicated by the horizontal arrows.

damages the RNA, whereas iodine reduces the ability of the virions to adsorb to host cells.

## MATERIALS AND METHODS

**Virus preparation and cell lines.** Poliovirus type 1 (Mahoney) was used in this study. We used HeLa cell monolayers to propagate and assay the viruses. The methods for plaque assay, radioactive labeling, and purification of virus suspensions have been described previously (17).

**Preparation and analysis of halogen solutions.** Chlorine dioxide was generated and its concentrations were determined by the methods described by Roller et al. (18). Chlorine dioxide concentrations used in the experiments are indicated in the text. A 2.5% iodine solution in 50% ethanol-water was used as the source of iodine. Iodine concentrations were determined by the diethyl-*p*-phenylene diamine method (12), which was modified for small volumes by the addition of 0.04 g of total halogen reagent to appropriately diluted iodine solutions in final volumes of 5.0 ml of glass-distilled water. Iodine concentrations used in inactivation experiments are indicated in the text.

**Virus inactivation experiments.** Halogen demand-free 0.05 M phosphate buffer (pH 6.0) and halogen demand-free 0.05 M borate buffer (pH 10.0) were used as suspending media for inactivation experiments. The final reaction volumes ranged from 1.0 to 3.0 ml, and all inactivation experiments were done at 25°C. The desired concentrations of halogen solutions were added to buffered virus suspensions containing $10^7$ to $10^8$ PFU/ml. At the end of the exposure times, residual halogen was inactivated by adding 0.1 to 0.3 ml of a 0.1% solution of sodium thiosulfate.

**Sedimentation and isoelectric focusing of viruses.** The procedures used for determination of the sedimentation characteristics of viruses and viral components and the isoelectric focusing techniques have been described previously (22).

**Binding of $^{125}$I to poliovirus.** Elemental $^{125}$I (150 mCi/mmol) was purchased from New England Nuclear Corp. and diluted to 90,000 cpm/ml (450,000 dpm) in unlabeled iodine stock solutions containing 25 mg of iodine per liter. Iodine binding and inactivation experiments were performed by adding 0.1 ml of stock halogen solution to 0.9 ml of buffer containing $10^7$ PFU of poliovirus, followed by sedimentation analysis as described above.

**Virus adsorption and penetration-uncoating experiments.** The adsorption of halogen-inactivated viruses to HeLa cells was determined by infecting each HeLa cell monolayer (ca. 14 $cm^2$) with 0.2 ml of control or

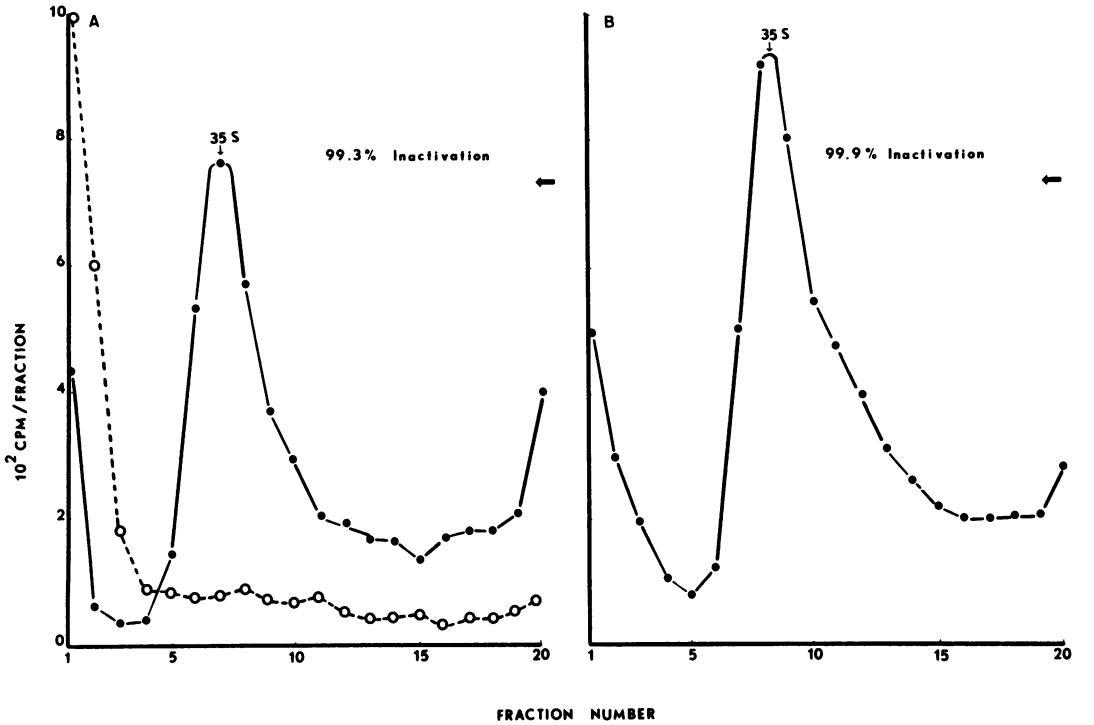


FIG. 3. Effects of chlorine dioxide and iodine at pH 10.0 on the sedimentation characteristics of poliovirus RNA. Viruses with labeled RNAs were exposed to 1.0 mg of chlorine dioxide per liter for 1.0 min (A) or 2.5 mg of iodine per liter for 2.0 min (B), and then the virus suspensions were centrifuged at 65,000 rpm in 5 to 30% glycerol gradients for 90 min in an SW65 rotor. Control virus profile is shown by the dotted line. Direction of sedimentation is indicated by the horizontal arrows.

halogen-inactivated, capsid-labeled virus suspension. After a 30-min adsorption period at 37°C, the monolayers were rinsed five times with phosphate-buffered saline. The cells were removed with trypsin-EDTA (GIBCO Laboratories), and the cell suspensions were cooled to 4°C by adding an equal volume of phosphate-buffered saline. The cells were sedimented by centrifugation at 8,000 rpm for 20 min, the pellets were suspended in 0.4 ml of 0.01% sodium dodecyl sulfate, and the cells were disrupted by sonication at 30 W for 10 s. Each disrupted cell suspension was added to 10.0 ml of scintillation cocktail, and the amount of radioactivity in each sample was counted in a liquid scintillation spectrometer. Adsorption of viruses to cells was determined by measuring cell-associated radioactivity. The virus penetration-uncoating experiments were performed similarly, except that the virions were allowed to interact with the cells for up to 60 min at 37°C. After the cells were removed and disrupted as described above, the samples were layered onto 15 to 30% glycerol gradients and centrifuged for 3 h at 30,000 rpm in an SW41 rotor at 4°C. The gradients were fractionated, and the fractions were analyzed by liquid scintillation spectrometry.

**Effects of chlorine dioxide on poliovirus RNA synthesis.** Each HeLa cell monolayer (14 $cm^2$) was infected with 0.5 ml of a control or chlorine dioxide-inactivated virus sample. After 30 min, 5.0 ml of minimal essential medium containing 5% newborn calf serum was added, and the cultures were incubated at 37°C for 90 min. The medium was then replaced with 5.0 ml of minimal essential medium containing 5% newborn calf serum, 5.0 μg of actinomycin D per ml, and 0.5 μC of [$^{14}$C]uridine (53-mCi/mmol specific activity) per ml. The cultures were incubated for 3 h at 37°C to allow for the incorporation of the [$^{14}$C]uridine into new viral RNA molecules. The [$^{14}$C]uridine-containing medium was decanted, the monolayers were washed five times with phosphate-buffered saline, and the cells were removed with trypsin and centrifuged as described above. After suspension of the pellet in 0.3 ml of buffer (0.005 M Tris, 0.0005 M EDTA, 0.05 M NaCl, pH 7.5), sodium dodecyl sulfate was added to a final concentration of 0.01%. Since the cells were lysed by this treatment, the sonication step was omitted to avoid possible breakage of the newly synthesized viral RNA molecules. The lysed cells were layered onto preformed 5 to 30% glycerol gradients which were centrifuged at 40,000 rpm for 5 h at 4°C in an SW41 rotor. The gradients were fractionated, and the total counts per minute in all fractions were determined by liquid scintillation spectrometry.

## RESULTS

**Effect of pH on inactivation.** It has been reported that chlorine dioxide is more effective as a

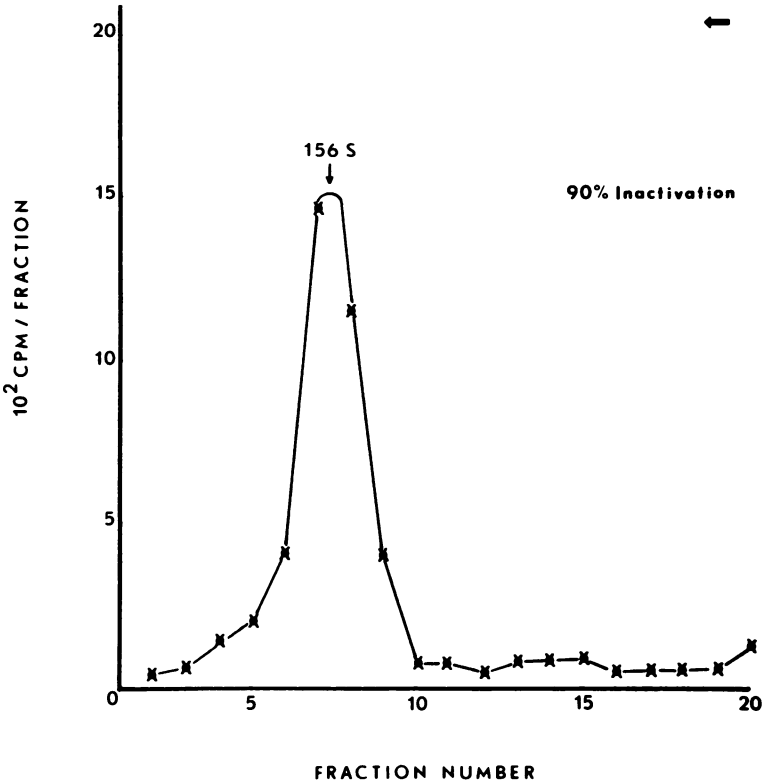


FIG. 4. Effects of chlorine dioxide and iodine on the sedimentation coefficients of poliovirus with labeled capsids at pH 6.0. Viruses were exposed for 2.0 min to 1.0 mg of chlorine dioxide per liter (●) or 2.5 mg of iodine per liter (×) and then centrifuged at 30,000 rpm in 15 to 30% glycerol gradients for 3 h in an SW41 rotor. Direction of sedimentation is indicated by the horizontal arrow.

virucidal agent at alkaline pH values (19). Our data agreed with these findings: we found that chlorine dioxide at a concentration of 1.0 mg/liter inactivated poliovirus more efficiently at pH 10.0 than at pH 6.0 (Fig. 1). Like chlorine dioxide, iodine also was more virucidal at pH 10.0 than at pH 6.0. The results indicate, however, that chlorine dioxide was a more efficient virucidal agent than iodine.

**Effects of chlorine dioxide and iodine on virus composition.** We observed effects on virus structure similar to those previously reported for chlorine (17): inactivation of poliovirus by 1.0 mg of chlorine dioxide per liter or 2.5 mg of iodine per liter at pH 10.0 resulted in the separation of the RNA from the capsids and the consequent conversion of the virions from 156S structures to 80S particles (Fig. 2). The RNAs released from chlorine dioxide- or iodine-inactivated viruses sedimented at the same position as 35S viral RNA (Fig. 3). At pH 6.0, exposure of poliovirus to 1.0 mg of chlorine dioxide per liter or 2.5 mg of iodine per liter for 2.0 min did not result in the loss of RNA from the capsids, and the viruses sedimented as 156S structures, although 90% of the virus population was inactivated (Fig. 4). It was reported previously for chlorine that the separation of viral components did not correlate with inactivation (1). These results indicated that the loss of RNA from the capsids of poliovirus is not the cause of inactivation. Consequently, experiments on the nature of virus inactivation by chlorine dioxide and iodine were done under conditions which minimized virus structural damage. For testing iodine, the experiments were done with solutions containing <1.0 mg of halogen per liter at pH 10.0. For testing chlorine dioxide, the experiments were done with solutions of 1.0 mg of halogen per liter at pH 6.0, at which the inactivation rate was slower (Fig. 1), since concentrations of <1.0 mg of chlorine dioxide per liter were difficult to determine reliably. In addition, the inactivated virus preparations were centrifuged in 15 to 30% glycerol gradients, and the fractions containing only 156S particles were used. A sensitive method for determining capsid alterations is isoelectric focusing. Accordingly,

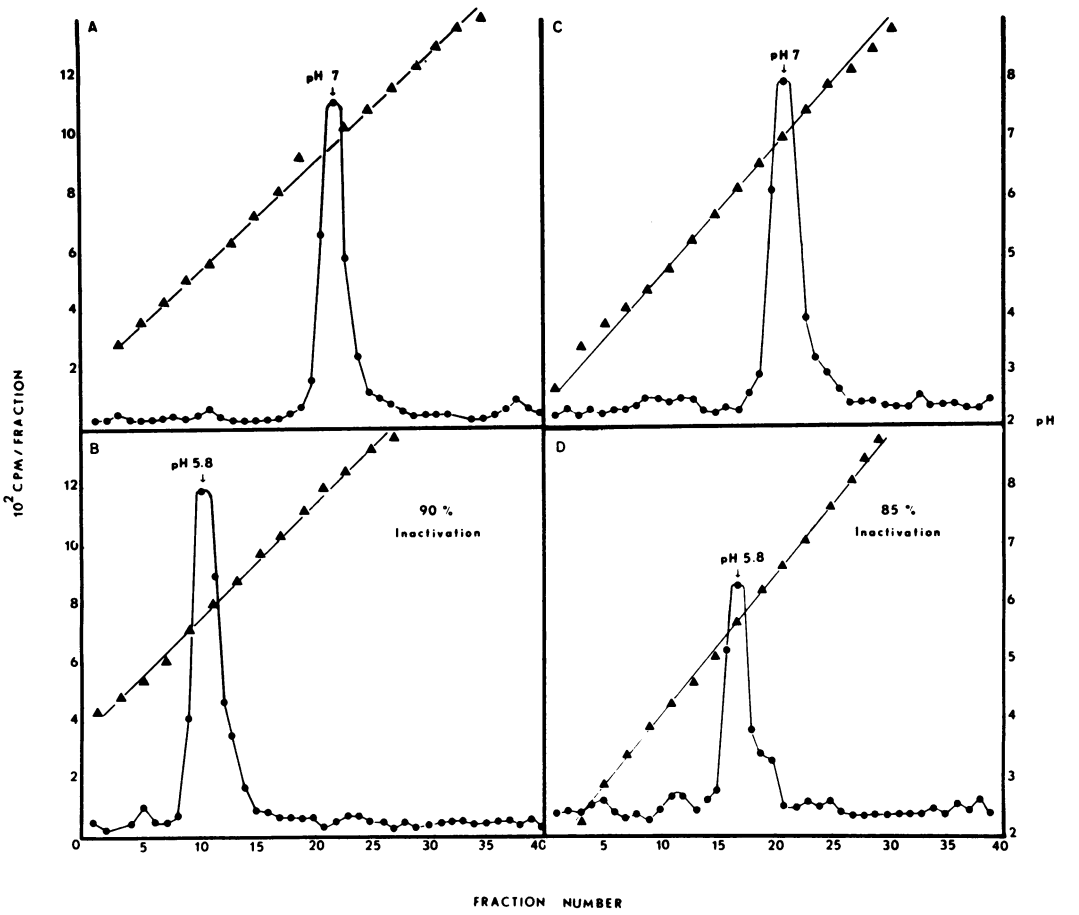


FIG. 5. Effects of chlorine dioxide and iodine on poliovirus pI values. Viruses with labeled capsids were exposed to 1.0 mg of chlorine dioxide per liter at pH 6.0 for 2 min (B) or 0.8 mg of iodine per liter at pH 10.0 for 15 min (D). The virus suspensions were then centrifuged in 15 to 30% glycerol gradients for 3 h at 30,000 rpm in an SW41 rotor. The gradient fractions containing 156S virus particles were pooled and focused in sucrose gradients. (A and C) pI values for control viruses. Direction of migration during isoelectric focusing was from right to left. The pH gradient is shown (▲).

the pI values of inactivated virions were compared with those of infective viruses. The pI values for both the chlorine dioxide- and iodine-inactivated virions were irreversibly shifted from pH 7.0 to pH 5.8 (Fig. 5). According to Mandel (15), heat-treated poliovirus with a stabilized pI value of pH 4.5 can not adsorb to host cells. Therefore, the adsorption to HeLa cells of viruses with altered pI values was compared with the adsorption of control virions. Chlorine dioxide-inactivated virions adsorbed to HeLa cells as efficiently as did control viruses (Table 1). In contrast to these results, the data clearly show that iodine-inactivated viruses had impaired adsorption capabilities and that the reduced adsorption correlated with the amount of inactivation. Thus, chlorine dioxide and iodine inactivated poliovirus by different mechanisms, although the effects on the pI values were similar.

In view of the effects of iodine on the adsorption of polioviruses to host cells, it was of interest to determine if iodine became associated with the virus particles. Accordingly, $^{125}I$ was added to iodine solutions used to inactivate unlabeled polioviruses. After the viruses were exposed to the halogen, the samples were analyzed by rate zonal centrifugation in 15 to 30% glycerol gradients as described above. Results of these experiments are shown in Fig. 6, which shows that no $^{125}I$ became associated with the viruses.

Since chlorine dioxide-inactivated viruses adsorbed to HeLa cells as efficiently as did control

TABLE 1. Effects of chlorine dioxide and iodine on poliovirus adsorption to HeLa cells[a]

| Virus treatment | Halogen concn (mg/liter) | Adsorption of inactivated virus[b] | % Reduction in adsorption of inactivated virus[c] | % Inactivation[d] |
|---|---|---|---|---|
| Control | 0 | 100 | 0 | 0 |
| Chlorine dioxide | 1.0 | 100 | 0 | 90 |
| Iodine | 0.4 | 60 | 40 | 50 |
| Iodine | 0.8 | 13 | 87 | 97 |

[a] HeLa cells were infected with viruses with labeled capsids. Adsorption was determined by measuring viral radioactivity associated with the host cells.

[b] Expressed as values relative to control virus adsorption as 100%.

[c] Expressed as values relative to reduction in control virus adsorption as 0%.

[d] Percent reduction in PFU after exposure to indicated concentration of halogen.

viruses, experiments to determine the ability of chlorine dioxide-inactivated viruses to penetrate into HeLa cells and initiate the uncoating process were done. The gradient profiles of lysed cells infected with control or chlorine dioxide-treated virus are presented in Fig. 7, and it is evident that there were no differences between control and inactivated viruses. In these experiments, the early modified and further modified articles described by DeSena and Torian (8) were observed in gradient profiles of cells infected with control or chlorine dioxide-inactivated virus.

Since no differences were detected in the adsorption, penetration, or uncoating between chlorine dioxide-treated virus and control virus, the possibility that chlorine dioxide reacted with viral RNA was considered. Although RNA released from chlorine dioxide-inactivated virus was found to cosediment with intact viral RNA (Fig. 2), the possibility still remained that the molecule had suffered damage not detectable by sedimentation analysis. Accordingly, experiments were done to determine the ability of the RNA from inactivated virions to direct the incorporation of [$^{14}$C]uridine into new viral RNA. Control or chlorine dioxide-inactivated virions were allowed to adsorb to HeLa cells, penetrate, uncoat, and initiate RNA replication. Actinomycin D was added at the same time as [$^{14}$C]uridine to stop DNA-directed RNA synthesis, thus assuring that the radioactive label was incorporated into viral RNA. The infected cells were lysed, and the newly synthesized viral RNA containing [$^{14}$C]uridine was then analyzed by rate zonal centrifugation and liquid scintillation spectrometry. Less [$^{14}$C]uridine were incorporated into new RNA molecules by chlorine dioxide-treated samples than by controls (Table 2). Furthermore, the percent reduction (determined by the total number of counts per minute incorporated into viral RNA) correlated reasonably well with the percent inactivation value.

## DISCUSSION

The results presented in this report show that separation of the RNA from the capsids of poliovirus occurred under conditions that enabled more efficient inactivation by chlorine dioxide and iodine. The same phenomenon has also been reported for poliovirus inactivation by chlorine and bromine chloride (14, 17). Therefore, under appropriate conditions, the separation of viral components appears to be a generalized phenomenon of enterovirus inactivation by halogens. However, in no case does separation of viral components appear to be the cause of virus inactivation.

Iodine and chlorine dioxide were found to inactivate poliovirus more efficiently at pH 10.0 than at pH 6.0. The nature of the most active species of these compounds in disinfection has not been studied in detail. However, chlorine

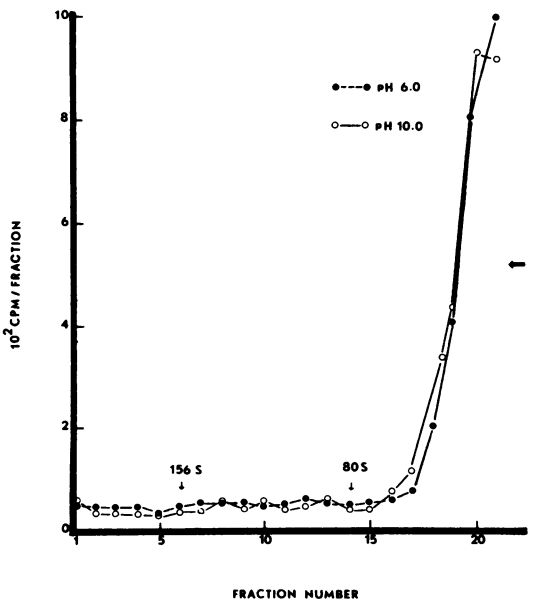


FIG. 6. Binding of $^{125}$I by iodine-inactivated polioviruses. Viruses were suspended in a solution of 2.5 mg of iodine per liter containing $^{125}$I. Virus suspensions were centrifuged in 15 to 30% glycerol gradients at 30,000 rpm for 3 h at 4°C in an SW41 rotor. Positions of 156S and 80S particles are shown. Direction of sedimentation is indicated by the horizontal arrow.

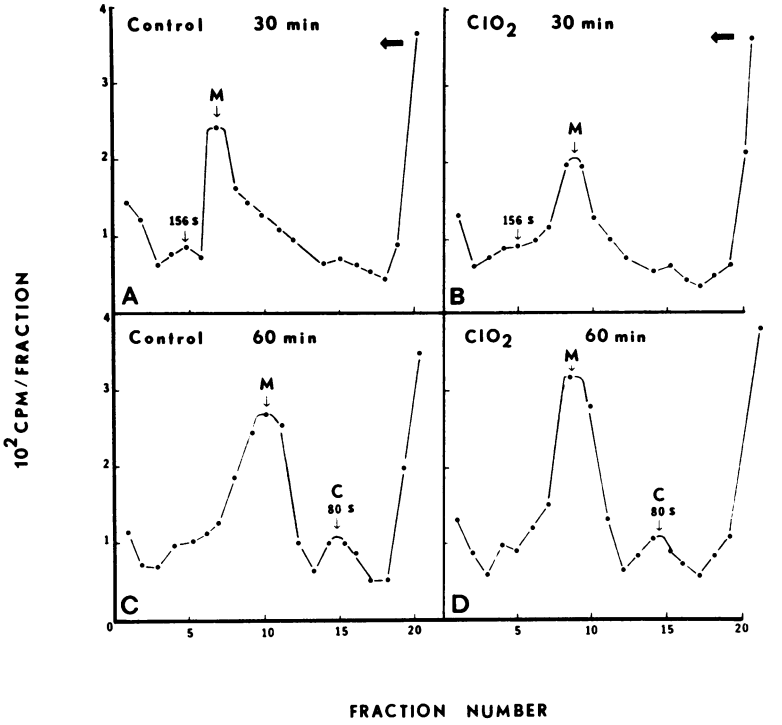


FIG. 7. Effects of chlorine dioxide on uncoating of poliovirus with labeled capsids in HeLa cells. Virus suspensions were inactivated 90% in solutions of 1.0 mg of chlorine dioxide per liter at pH 6.0 for 2.0 min. HeLa cells were infected with control or inactivated virus, and at the indicated times, the cells were lysed, and the cell extracts were centrifuged in 15 to 30% glycerol gradients for 3 h at 30,000 rpm in an SW41 rotor. The positions of the early modified (M) and further modified (C) particles are shown. Direction of sedimentation is indicated by the horizontal arrows.

dioxide has been reported to remain undissociated in aqueous solutions at pH values from 4.0 to 8.4 (2). In an alkaline solution it disproportionates to chlorite ($ClO_2-$) and chlorate ($ClO_3-$) via the following reaction: $2ClO_2 + 2OH- \rightleftharpoons H_2O + ClO_2- + ClO_3-$ (8). Since the end product formed as a result of the oxidation of organic matter by undissociated chlorine dioxide is the $ClO_2-$ ion (9, 16), it is possible that $ClO_3-$ is the most active species in inactivation of poliovirus at alkaline pH levels. However, according to Chen (6), the amount of $ClO_2-$ or $ClO_3-$ formed as the result of the disproportionation of $ClO_2$ at pH 10 is less than 5%. It is not clear at the present time whether this amount of chlorite or chlorate is sufficient to account for the increase in the rate of inactivation of poliovirus observed at pH 10.0. An alternative explanation would be that the high pH increases the sensitivity of the virus to $ClO_2$ attack. In iodine solutions at pH 10.0, more than 88% of the iodine is in the form of hypoiodous acid (HIO), whereas at pH 6.0, 90% of the iodine is in the form of elemental iodine (3). Thus, it appears that the most active species of iodine in virus inactivation is the HIO molecule.

It is not surprising, then, that chlorine dioxide and iodine inactivate poliovirus by different mechanisms. Our results indicate that when HIO was the inactivating agent, the ability of the viruses to adsorb to host cells was impaired. Since the percent inactivation values correlated well with the percent reduction in adsorptive capabilities, we suggest that HIO reacts with the protein coat of the virus and not with the RNA. This proposal is in agreement with the findings of Hsu, who reported that poliovirus and phage f2 RNAs are fully resistant to iodine (13). The reactions of HIO with the protein coat of poliovirus appear to be oxidative rather than substitutive since we found no evidence of $^{125}I$ binding to polioviruses. Iodine has been shown to act on other proteins and viruses by oxidation of sulfhydryl groups, which results in little or no binding of the halogen (11).

Brigano et al. suggested that chlorine dioxide at pH 7.0 inactivates viruses by denaturing the protein coat (4). However, this hypothesis was

TABLE 2. Incorporation of [$^{14}$C]uridine into viral RNA in HeLa cells infected with control or chlorine dioxide-inactivated poliovirus in the presence of actinomycin D[a]

| Cultures treated with: | % [$^{14}$C]uridine incorporation[b] | % Reduction of [$^{14}$C]uridine incorporation[c] | % Virus inactivation[d] |
|---|---|---|---|
| Nothing | 0 | —[e] | — |
| Infective virus | 8 | — | — |
| Inactivated virus | 2.5 | 69 | 88 |

[a] Poliovirus suspensions were inactivated with chlorine dioxide before HeLa cells were infected. We added 5 μg of actinomycin D per ml and [$^{14}$C]uridine to infected cell cultures simultaneously.

[b] Determined from the ratio counts per minute of [$^{14}$C]uridine incorporated into RNA:total counts per minute of [$^{14}$C]uridine added.

[c] Expressed as percentage of [$^{14}$C]uridine incorporated by cells infected with inactivated viruses/percentage of [$^{14}$C]uridine incorporated by cells infected with infective viruses.

[d] Percent reduction in PFU by chlorine dioxide.

[e] —, 0% reduction.

based on thermodynamic analysis of inactivation curves rather than on structural and functional analysis of inactivated viruses. Our results for inactivation at pH 6.0 indicate that, although chlorine dioxide reacts with the protein coat and changes the pI, the critical target appears to be the viral RNA, which is less capable of acting as a template for viral RNA replication. The possibility that the mechanism for poliovirus inactivation at pH 10.0 differs from the mechanism for inactivation at pH 6.0 cannot be eliminated by the present results.

It has been suggested that the most effective virucidal compounds used for water disinfection should inactivate the viral genome (21). Our results, then, indicate that chlorine dioxide may be a good primary or secondary disinfectant since viral RNA appears to be the critical target at pH 6.0. Clearly, further studies on the nature of virus inactivation by halogen compounds are needed if more effective virucidal agents are to be developed.

## ACKNOWLEDGMENTS

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