THE MECHANISMS OF POLIOVIRUS INACTIVATION BY CHLORINE DIOXIDE AND IODINE AND THE EFFECTS OF CATIONS ON HALOGEN INACTIVATION

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ABSTRACT

Chlorine dioxide and iodine inactivated poliovirus most efficiently at pH 6.0. Sedimentation analyses of viruses inactivated by chlorine dioxide or iodine at pH 10.0 showed that viral RNA separated from the capsids converting the virions from 156S structures to 80S particles. It was shown, however, that the separation of the RNA from the capsids was not the cause of virus inactivation by chlorine, chlorine dioxide or iodine. The RNA 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 isoelectric point 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 HeLa cells infected with chlorine dioxide-inactivated viruses showed a reduced incorporation of ^{14}C uridine into new viral RNA. Therefore, chlorine dioxide inactivated poliovirus by reacting with the virus RNA and impaired the ability of the genome to act as a template for RNA synthesis.

The presence of 0.1 M KCl or 0.1 M $MgCl_2$ enhanced the rate of inactivation of poliovirus by chlorine at both pH 6.0 and 10.0. Iodine inactivation of poliovirus was not affected by the presence of KCl or $MgCl_2$ at either pH 6.0 or 10.0. Inactivation of poliovirus by chlorine dioxide was reduced in the presence of KCl or $MgCl_2$ at pH 10.0 but no effect was seen at pH 6.0. Sedimentation analyses of viruses inactivated

in the presence of KCl or $MgCl_2$ indicated that the salts inhibited the separation of the RNA from the viral capsids under certain conditions. Moreover, the presence of $MgCl_2$ was found to stabilize chlorine or chlorine dioxide-inactivated viruses in an RNA-containing structure with a sedimentation coefficient of 120 to 130S. It was concluded, then, that the rates of inactivation of poliovirus by halogen compounds, as well as the gross structural changes associated with virus inactivation, are highly dependent on the pH of the reaction and on the ionic environment.

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CHAPTER I

INTRODUCTION

Chlorine was first utilized in the United States for the disinfection of drinking water in 1888 (61). Numerous reports have now accumulated on the kinetics of inactivation of bacteria and viruses by chlorine (18, 19, 32, 53). The reported data indicate that, on a general basis, viruses are more resistant to chlorine than bacteria, and that even within one virus group the degree of resistance to chlorine inactivation varies considerably. Nonetheless, the efficiency of chlorine for the treatment of water supplies is evident since the incidence of waterborne diseases has declined during this period. Possibly due to this efficiency very little interest has been given to the mechanisms of inactivation of microorganisms, especially of viruses, by chlorine. Most of the mechanistic studies have focused on the effects of chlorine on bacteria and on model organic compounds. The reactions of chlorine with organic matter appear to be of two main types: oxidative and substitutive, the latter resulting in the binding of chlorine atoms to the organic compound (12). It appears that in acid solution chlorination predominates while in alkaline media oxidation is more pronounced (4). It is also evident at that low pH values, hypochlorous acid, a dissociation product of chlorine in water, exists in greater proportions and is more virucidal than the hypochlorite ion which predominates at high pH values (32). Pereira et al. showed chlorine as hypochlorous acid to react with α-amino acids by oxidative decarboxylation to form nitriles and aldehydes (45). These workers also reported that exposure to hypochlorous acid resulted in the oxidation of cysteine

to cystine and cysteic acid, the chlorination of the tyrosine ring, and that the amide nitrogen bond of dipeptides was resistant to hypochlorous acid at room temperature. Hypochlorous acid can react with nucleotides resulting in the chlorination of pyrimidine bases and the degradation of the purine ring (13). The more practical studies of Venkobachar et al. (59) indicate that chlorine inhibits total dehydrogenase activity in the intact cells of Escherichia coli, and that the amount of inhibition correlated with the amount of bacterial inactivation. More recently, it was reported that low concentrations of chlorine cause changes in permeability and the complete inhibition of phosphate uptake by E. coli (60). It is now generally accepted that chlorine inactivates bacteria by the inactivation of sulfhydryl-containing enzymes (29, 47).

Mechanistic studies on the nature of the reactions of chlorine that lead to virus inactivation were not published until recently. Olivieri et al. (43) indicated that chlorine inactivated coliphage f2 by reacting primarily with the RNA of the virus. Later studies showed that ^{36}Cl was incorporated into f2 and that the label was mainly incorporated into the RNA (12). Moreover, the rate of incorporation of ^{36}Cl label paralleled the rate of inactivation of f2. Reports on the inactivation of poliovirus by chlorine are controversial. O'Brien and Newman (42) reported that inactivation of poliovirus by 1.0 mg/l chlorine was due to degradation of the RNA while the RNA was still associated with the viral particle, and that the isoelectric point of inactivated viruses was the same as that of control viruses. Tenno et al. (58) reported, however, that the infectivity of RNA extracted from chlorine-inactivated poliovirus was the same as that of control viral RNA. The chlorine concentration used in the latter study was 0.2 mg/l for 30 min as

compared to 4 min in the first study. It is possible, then that the different results are due to a difference in concentration.

Recently, the importance of evaluating agents that offer an alternative 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 material to produce chlorinated compounds, such as chloroform, which can be carcinogenic or hazardous (20). It has been reported that these compounds can be generated as a result of the chlorination of waters containing natural humic substances, or which are polluted from industrial sources or, more likely, in recycled water supplies (37). Another line of concern comes from the fact that chlorine-resistant enteroviruses have been isolated in drinking waters (52). Still another point that has been stressed is that an ideal alternative disinfectant should inactivate viral nucleic acids, since it has been pointed out that viable genomes can be released from inactivated viruses and, under certain conditions, can infect host cells (58).

Two compounds that have not been studied in relation to the mechanisms of inactivation of viruses are chlorine dioxide and iodine. The use of chlorine dioxide in water disinfection has been limited in the United States (38), and in most cases it has been used as a postchlorination process to control phenolic tastes and odors (23, 40, 46, 61). The efficiency of chlorine dioxide as a bactericidal and virucidal agent, however, has been reported to equal and, in some cases, to exceed that of chlorine (7, 51, 55). Very few reports have been published on the reactions of chlorine dioxide with model organic compounds and on the mechanisms of inactivation of bacteria and viruses.

Using chromatographic techniques, Benarde et al. (6) indicated that chlorine dioxide did not react significantly with free amino acids including arginine, asparagine, histidine, leucine, proline, and valine. Chlorine dioxide oxidizes cystine, methionine, tyrosine, and tryptophan (20, 36). There is no information available on the reactions of chlorine dioxide with nucleic acids, but sugars are apparently oxidized at the hydroxyl group without destruction of the furanose of pyranose ring (20). Of particular interest to the public concern about water disinfection is the fact that chlorine dioxide does not produce chloroform or other trihalomethanes (7, 20).

With respect to the mechanisms of inactivation of bacteria by chlorine dioxide, Benarde et al. (6) reported that protein synthesis in E. coli was abruptly inhibited by chlorine dioxide and that no leakage of cell contents occurred after exposure to the compound. Roller et al. (47) showed that total dehydrogenase activity and protein synthesis were inhibited in Haemophilus influenzae by chlorine dioxide but that the reactions were not sufficient to account for cell death. They also reported that DNA transformability was not significantly altered and concluded that the cause of inactivation must have been other than the inhibition of protein synthesis or the denaturation of the DNA.

The mechanisms of inactivation of viruses by chlorine dioxide have not been investigated, but Brigano et al. (9), based on thermodynamic analyses, indicated that the kinetic parameters of inactivation were consistent with those of protein denaturation and suggested that poliovirus could be inactivated through reactions with the protein coat.

Although iodine has not been used extensively in the treatment of water supplies, except under emergency conditions, its efficiency as a

bactericidal compound has been recognized (8, 11). The virucidal effectiveness of iodine has been shown with a variety of viruses including influenza A, Newcastle disease virus, rhino-viruses, coxsackieviruses, and poliovirus (2, 10, 26). Iodine has been reported to react with proteins by the oxidation of sulfhydryl groups to form disulfide bonds or sulfenyl iodides, and by the binding of iodine atoms to tyrosine and histidine (24). Studies on the effects of iodine on enveloped viruses have indicated that iodine has a high affinity for the carbon-carbon double bonds in unsaturated fatty acids, and that this reaction results in a reduction of the ability of the virus membrane to fuse with host cell membranes (2). Poliovirus RNA and coliphage f2 RNA have been shown to be fully resistant to iodine (26). These results were confirmed for f2 by Olivieri et al. (43), who found that RNA extracted from both untreated and iodine-inactivated viruses was resistant to iodination and concluded that iodine reacted with f2 through the iodination of tyrosine. There are no reports available on the reactions of model compounds with iodine that could be applicable to virus studies. However, it is of interest to mention that, although iodine forms trihalomethanes, the yield is significantly less as compared to the amount of trihalomethanes produced by chlorine (20).

The present study was undertaken to investigate the mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. For comparative purposes, chlorine was included in some of the experiments. Since the pH and the presence of cations have been shown to influence the rate of inactivation of enteroviruses by chlorine, these two variables were also studied. The results and discussion are divided into two sections. The first section is presented in Chapter III where the

effects of chlorine dioxide and iodine on poliovirus structure and function are reported and discussed. Chapter IV deals with the effects of KCl and $MgCl_2$ on poliovirus inactivation by chlorine, chlorine dioxide, and iodine. Finally, a summary and comments section is given in Chapter V.

CHAPTER II

MATERIALS AND METHODS

Virus preparation and cell line

Poliovirus type 1 (Mahoney) was used in this study. HeLa cell monolayers served as host cells for the propagation and in the assay of viruses by the plaque method as described before (41). Radioactively labeled virus suspensions were prepared by adding minimal essential medium (MEM) containing 5% serum, and either ^{3}H uridine (50 μC/ml of medium; 5 C/mmole) or ^{3}H leucine (25 μC/ml;165 C/mmole) and ^{3}H valine (25 μC/ml; 37C/mmole) to cell monolayers infected 2 h earlier with poliovirus. Following overnight incubation at 37°C, the infected monolayers were frozen and thawed three times and removed from the glass as previously described (41). Virus purification was accomplished by one cycle of differential centrifugation (8,000 rpm for 20 min; 40,000 rpm for 30 min in an SW-41 rotor) followed by ultracentrifugation at 30,000 rpm in preformed 15 to 30% glycerol gradients (15 to 30% glycerol in buffer containing 0.1 M NaCl, 0.01 M tris (hydroxymethyl) aminomethane, and 0.001 M EDTA disodium salt, pH 7.5) for 3 h at 4°C using an SW-41 rotor. Fractionation of the gradients was accomplished by collecting 0.6-ml fractions from the bottom of the tube and counting 10-μl aliquots by liquid scintillation spectrometry. The scintillation cocktail contained 16.0 g omnifluor, 2.0 l toluene, and 2.0 l 2-ethoxyethanol. Fractionation of the gradients followed by liquid scintillation counting of 10 μl aliquots of each fraction provided a reliable method to locate the fractions that contained viruses. In a 20-fraction gradient, the viruses usually banded at fractions 5 through 8. These fractions were

pooled and the mixture centrifuged at 40,000 rpm for 3 h at 4°C in an SW-41 rotor to sediment the viruses. The virus pellet was resuspended in up to 1.0 ml of phosphate buffered saline (PBS) and kept in the refrigerator overnight. The CsCl density gradient centrifugation technique described by Bachrach and Friedmann (3) proved to be a fast method to accomplish final purification of the viruses. The method consisted of preparing three solutions of CsCl which had densities of 1.1, 1.3, and 1.5 g/ml, respectively. Densities were calculated by using refractive indices obtained from a refractometer. In preparing the gradients, 1.3-ml aliquots of each solution were sequentially layered into 5-ml centrifuge tubes. Up to 1.0 ml of virus preparation could be layered on top of the preformed gradient which was then centrifuged at 33,000 rpm in an SW-65 rotor for 60 to 90 min at 4°C. This procedure allowed the formation of a linear gradient (1.1 to 1.6 g/ml), and the viruses banded approximately in the middle since polioviruses have a density of 1.34 g/ml. Detection of the virus band was performed by fractionation and liquid scintillation spectrometry as described above. Purified virus preparations contained from $5x10^8$ to $1x10^9$ plaque-forming units (PFU)/ml, ($5x10^5$ to $1x10^6$ counts per minute (cpm)/ml).

Preparation and analyses of halogen solutions

Chlorine stock solutions were prepared by diluting a 5% NaOCl solution in glass-distilled water and concentrations were determined by the orthotoluidine method (1).

Chlorine dioxide was generated by mixing 25.0 ml of a 16% (w/v) solution of sodium chlorite with 50.0 ml of 4% (w/v) potassium persulfate at 25°C according to the method of Roller et al. (47). The chlorine

dioxide gas generated was swept from solution with nitrogen gas and passed, first through a column packed with sodium chlorite powder, and then through an empty vessel to remove sodium chlorite dust. The chlorine dioxide gas was collected in 75.0 ml of glass-distilled water at 4°C. It was observed that even though a clean, all-glass apparatus was utilized, the chlorine dioxide demand (organic material that reacts with the halogen) of the system was considerable, which resulted in a low chlorine dioxide yield (less than 10 mg/l). To eliminate this problem, the initial reaction was allowed to occur for approximately 3 h. At this time, no chlorine dioxide could be detected in the collecting vessel. Then, the sodium chlorite-potassium persulfate mixture was decanted and replaced with a fresh mixture which was allowed to react for 30 min. Therefore, the first trial satisfied the chlorine dioxide demand of the system and the yield of chlorine dioxide was increased by a factor of 100 since stock solutions containing up to 1000 mg/l were obtained in the second run. Chlorine dioxide determinations were made spectrophotometrically at 357 nm as described by Roller et al. (47). Chlorine dioxide stock solutions could be kept in foil-wrapped, glass-stoppered bottles at 4°C for up to one month with no significant loss of concentration.

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 (DPD) method (25) which was modified for small volumes by the addition of 0.04 g of total halogen reagent to appropriately diluted iodine solutions in a final volume of 5.0 ml of glass-distilled water.

Halogen concentrations used in inactivation experiments are indicated in the text.

Virus inactivation experiments

Halogen demand-free (HDF) 0.05 M phosphate buffer (pH 6.0) and HDF 0.05 M borate buffer (pH 10.00) 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.

Isoelectric focusing of viruses

Isoelectric focusing of virus samples was based on the method of Korant and Lonberg-Holm (30). Glass tubes (19.5 by 0.7 cm) were carefully washed, soaked in a 1% (v/v) Photo-Flo 200 solution and dried overnight at 50°C. The tubes were then sealed at the bottom with a piece of dialysis membrane held in place with a short piece of rubber tubing. Using a disposable 1-ml pipette, 0.2 ml of a 48% sucrose solution containing 1% concentrated sulfuric acid was dispensed into the bottom of the sealed tubes. Sucrose gradients (15-30% sucrose in glass-distilled water containing 1% pH 3.5 to 10.0 LKB ampholine) were formed in the tubes using a density gradient former (Beckman Instruments) at 91% travel, fast settings. Gradient formation was interrupted after approximately two thirds of the tube was filled with sucrose solution, and 0.1 ml of capsid-labeled virus suspension adjusted to 20% sucrose and 1% ampholine was layered on the gradient. Formation of the gradient was

resumed and the tubes were filled to within 0.5 cm of the top. The tubes were then filled to the top with 2% ethanolamine solution. The bottom reservoir of the electrophoretic apparatus (anode) contained 48% sucrose and 1% sulfuric acid, whereas the top reservoir consisted of a 2% ethanolamine solution in glass-distilled water. Focusing of the viruses was carried out at constant voltage (400 V) for 4 h. Then the voltage was increased up to 700 V in 50 V increments every 15 min. The gradients were fractionated through the bottom, and 0.6-ml glass-distilled water added to each fraction. The pH of every other fraction was determined to assure that a pH gradient was formed in each case. All the fractions were then transferred to scintillation vials and counted by liquid scintillation spectrometry to locate the virus band.

Binding of ^{36}Cl and ^{125}I to poliovirus

^{36}Cl-labeled hypochlorous acid was prepared by adding 7.9 g of potassium permanganate to 50.0 ml of a 0.1 N HCl solution containing approximately 10^6 cpm/ml ^{36}Cl-HCl (New England Nuclear) at 38°C. The chlorine gas generated was swept out of solution with nitrogen and collected in 100.0 ml of distilled water at 4°C. After 40 to 70 min, the reaction was terminated by adding 50.0 ml of a 1.0 M ferrous sulfate solution. Stock solutions containing approximately 10.0 mg/l chlorine (20,000 cpm/ml) were obtained.

Elemental iodine ^{125}I was purchased from New England Nuclear and diluted in unlabeled iodine stock solutions containing 25 mg/l iodine.

Virus inactivation experiments were performed by adding 0.1 ml of stock halogen solution to 0.9 ml of buffer containing 10^7 PFU followed by sedimentation analysis as described above.

Virus adsorption and penetration/uncoating

The adsorption of halogen inactivated viruses to HeLa cells was determined by infecting HeLa cell monolayers (approximately 14 cm^2) with 0.2 ml of control or of halogen-inactivated, capsid-labeled virus suspensions. After a 30 min adsorption period at 37°C, the monolayers were washed five times with PBS. The cells were removed with trypsin-EDTA (Grand Island Biological Co.) and the cell suspensions cooled to 4°C by adding an equal volume of PBS. The cells were sedimented by centrifugation at 8,000 rpm for 20 min, the pellets resuspended in 0.4 ml of 0.01% sodium dodecyl sulfate (SDS), and the cells disrupted by sonication at 30 w for 10 s. The disrupted cell suspensions were added to 10.0 ml of scintillation cocktail and the samples counted in a liquid scintillation spectrometer. Viruses adsorbed to cells were determined from the 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 SW-41 rotor at 4°C. The gradients were fractionated and the fractions analyzed by liquid scintillation spectrometry.

RNA synthesis by chlorine dioxide-inactivated poliovirus

HeLa cell monolayers (14 cm^2) were infected with 0.5 ml of control or chlorine dioxide-inactivated samples. After 30 min, 5.0 ml of MEM containing 5% newborn calf serum (NCS) were added and the cultures incubated at 37°C for 90 min. The media was then replaced with 5.0 ml of the same base medium containing 5.0 µg/ml of actinomycin D and 0.5 µC/ml

of ^{14}C uridine (53 mC/mmole). The cultures were incubated for 3 h at 37°C to allow for the incorporation of the uridine into new viral RNA molecules. The uridine-containing medium was decanted, the monolayers washed five times with PBS, the cells removed with trypsin, and centrifuged as described above. After resuspending the pellet in 0.3 ml of buffer (0.005 M TRIS, 0.0005 M EDTA, 0.05 M NaCl, pH 7.5), SDS to a final concentration of 0.01% was added. Since the cells were lysed by this treatment and to avoid possible breakage of the newly synthesized viral RNA molecules, the sonication step was omitted. The lysed cells were layered onto preformed 5 to 30% glycerol gradients and centrifuged at 40,00 rpm for 5 h at 4°C in an SW-41 rotor. The gradients were fractionated and analyzed by liquid scintillation spectrometry.

CHAPTER III

THE MECHANISM OF INACTIVATION OF POLIOVIRUS BY CHLORINE DIOXIDE AND IODINE

Results

Inactivation of poliovirus by chlorine dioxide and iodine was studied at pH 6.0 and 10.0. The results presented in Fig. 1 show that chlorine dioxide at a concentration of 1.0 mg/l or iodine at 2.5 mg/l were more virucidal at pH 10.0 than at pH 6.0. These results are the reverse of those previously reported for chlorine since chlorine was found to be more virucidal at pH 6.0 than at pH 10.0 (19).

Sedimentation analyses of inactivated viruses revealed that inactivation of poliovirus by 1.0 mg/l chlorine dioxide or 2.5 mg/l iodine at pH 10.0 resulted in the separation of the RNA from the capsids converting the virions from 156S particles to 80S structures (Fig. 2). The RNA released from chlorine dioxide and iodine-inactivated viruses was also analyzed by sedimentation. It can be observed in Fig. 3 that the RNA released from chlorine dioxide and iodine-inactivated viruses was not degraded and sedimented at the same position as intact, 35S poliovirus RNA.

It was suggested earlier that separation of the RNA from the capsids of chlorine-inactivated poliovirus was a secondary event to virus inactivation (42). To study this possibility, radioactively labeled viruses were exposed to different concentrations of halogens at the optimum pH for a constant period of time (15 min). The results are shown in Fig. 4 for chlorine at pH 6.0 and in Fig. 5 for iodine at pH 10.0. It can be seen in Fig. 4 that at chlorine concentrations up to 0.4 mg/l

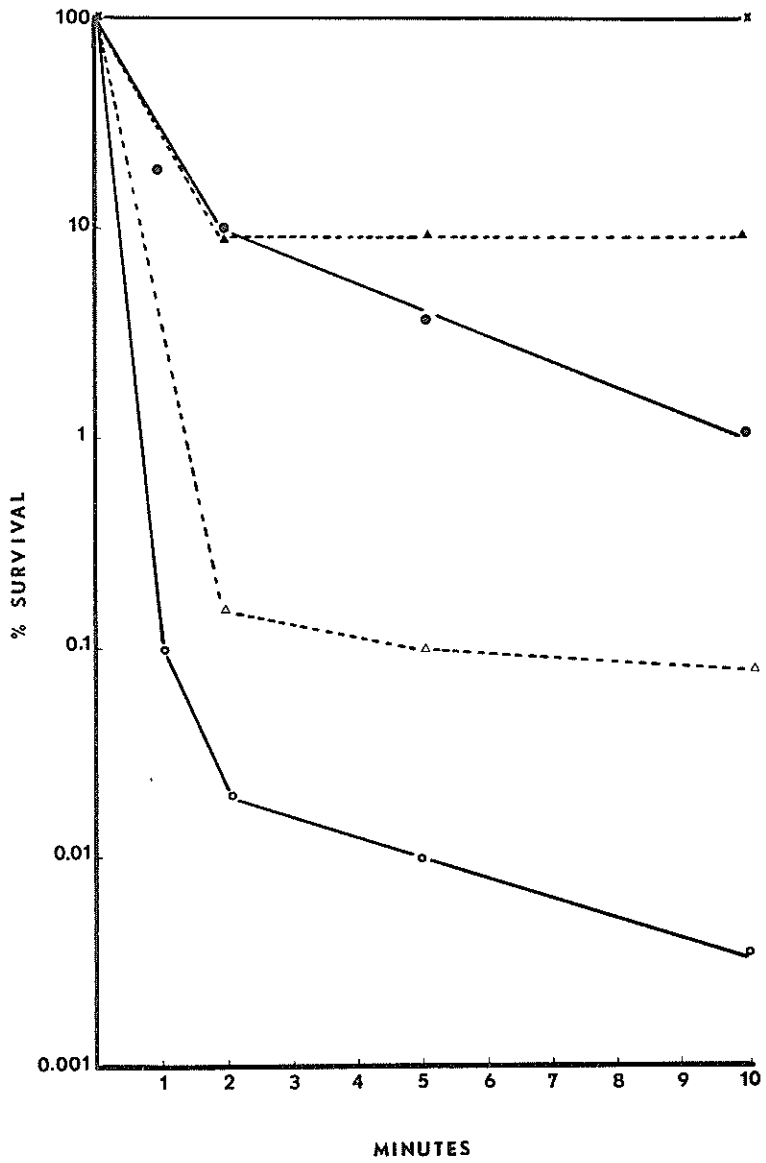

Fig. 1. Poliovirus inactivation by 1.0 mg/l chlorine dioxide or 2.5 mg/l iodine. Chlorine dioxide at pH 6.0 (●—●) and at pH 10.0 (○—○), Iodine at pH 6.0 (▲--▲) and at pH 10.0 (△--△). Untreated virus control (X—X). Chlorine dioxide or iodine was added to 3.0 ml of buffer containing viruses, and 0.1 mg samples were taken at the indicated time intervals and assayed on HeLa cell monolayers.

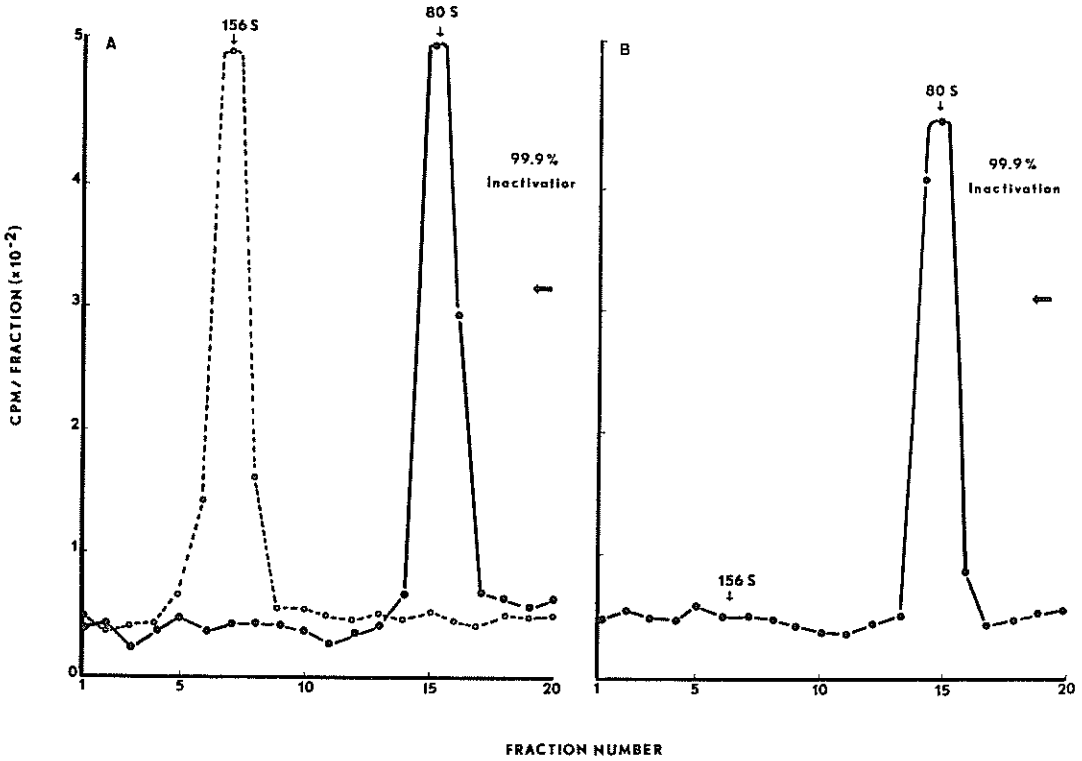

Fig. 2. Effects of chlorine dioxide or iodine on the sedimentation coefficients of capsid-labeled poliovirus. Viruses were exposed to 1.0 mg/l chlorine dioxide (A) or 2.5 mg/l iodine (B) at pH 10.0 and then centrifuged at 30,000 rpm in 15 to 30% glycerol gradients for 3 h. Control virus profile is shown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

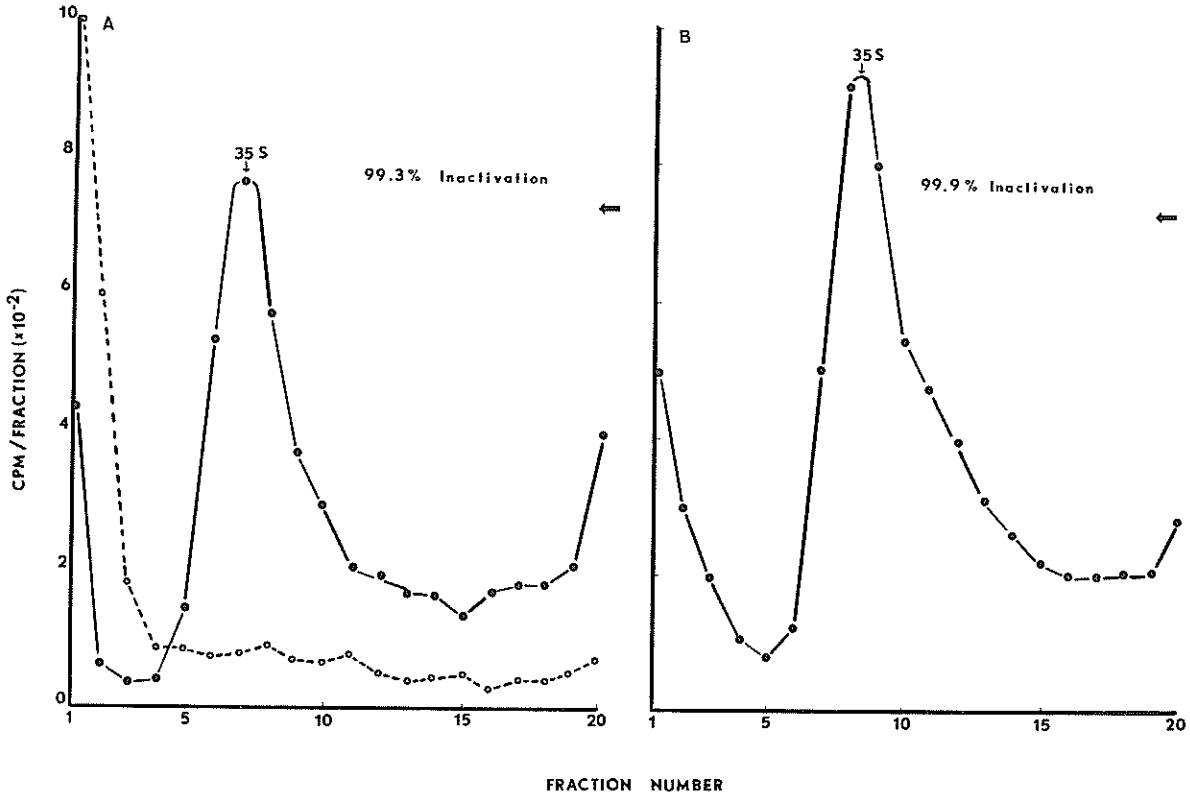

Fig. 3. Effects of chlorine dioxide or iodine on the sedimentation characteristics of poliovirus RNA. RNA-labeled viruses were exposed to 1.0 mg/l chlorine dioxide (A) or 2.5 mg/l iodine (B), and then the virus suspensions were centrifuged at 65,000 rpm for 90 min. in 5 to 30% glycerol gradients. Control virus profile is shown by the dotted line in panel A. Direction of sedimentation is indicated by the horizontal arrows.

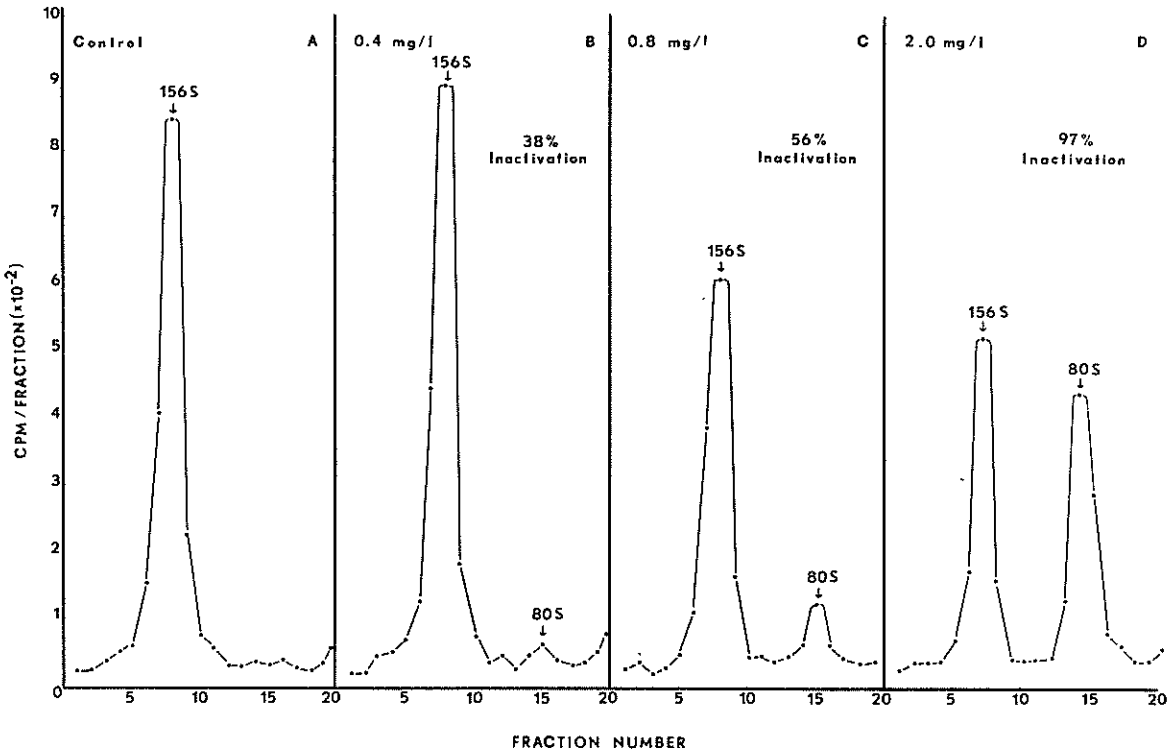

Fig. 4. Effects of chlorine concentration on sedimentation coefficients and infectivity of capsid-labeled polioviruses. Viruses were exposed to the indicated chlorine concentrations at pH 6.0, assayed for infectivity, and analyzed on 15 to 30% glycerol gradients. The direction of sedimentation is from right to left. As the chlorine concentration increased (B, C, and D), the percent inactivation values increased at a faster rate than the appearance of empty capsids (80S).

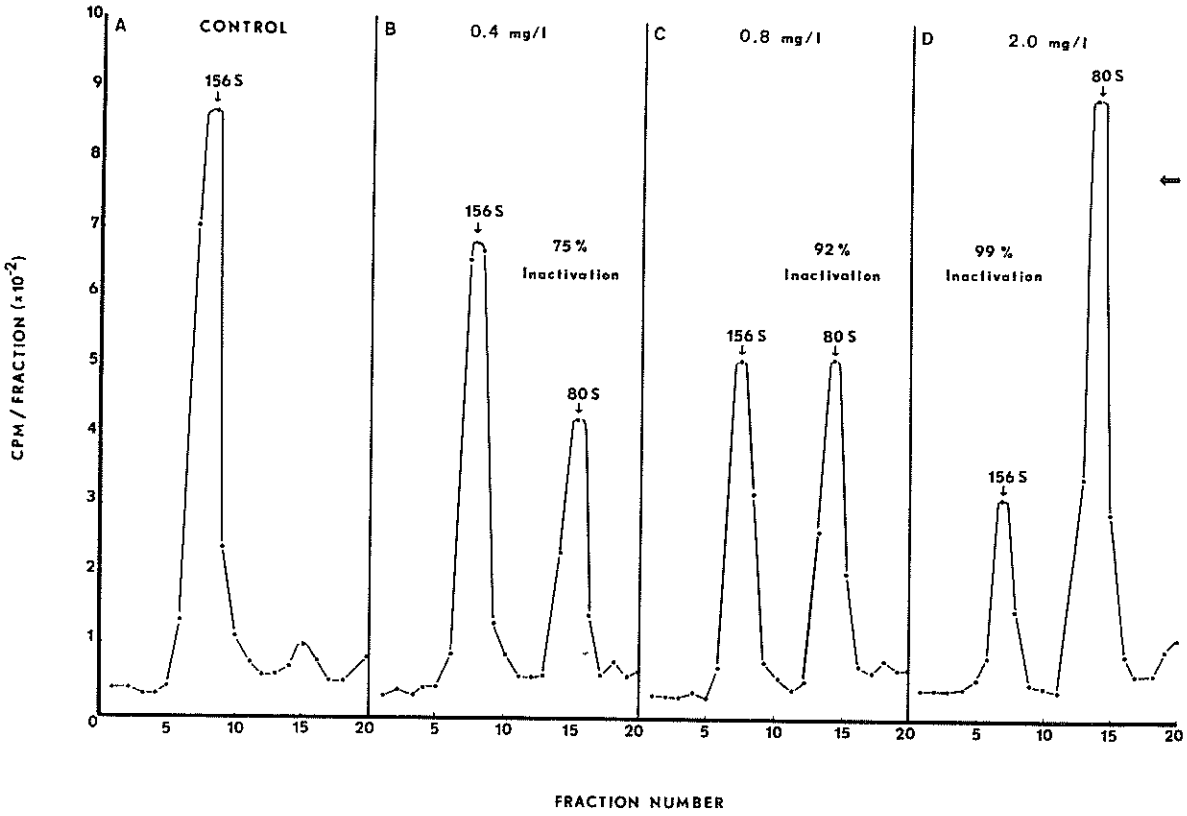

Fig. 5. Effects of iodine concentration on sedimentation coefficients and infectivity of capside-labeled polioviruses. Viruses were exposed to the indicated iodine concentrations at pH 10.0, assayed for infectivity, and analyzed on 15 to 30% glycerol gradients. The direction of sedimentation is from right to left. As the iodine concentration increased (B, C, and D), the percent inactivation values increased at a faster rate than the appearance of empty capsids (80S). The small peak corresponding to the 80S position in (A) is within the normal range of variation for untreated virus preparations observed in these experiments.

there were no major changes in the poliovirus sedimentation coefficients although substantial virus inactivation was obtained. At chlorine concentrations of 0.8 mg/l or higher, there was a shift of the virus sedimentation coefficients, from the 156S value associated with intact particles to 80S structures lacking RNA. The percent of the total recovered radioactivity associated with empty, 80S structures did not correlate with the percent inactivation values. For example, at chlorine concentrations of 0.8 mg/l 56% inactivation was obtained, but only 13% of the total radioactivity was associated with empty capsids. A similar pattern was observed with iodine at pH 10.0 (Fig. 5), although in this case a greater percentage of empty capsids was present at all concentrations, suggesting that the high pH increased the susceptibility of polioviruses to lose RNA. Nonetheless, it is evident that the percentage of radioactivity associated with empty capsids did not correlate with percent virus inactivation values. Similar experiments were not performed with different concentrations of chlorine dioxide at pH 10.0 since it was observed that exposure of poliovirus to concentrations as low as 0.5 mg/l for 1 min resulted in the complete conversion of viruses to empty, 80S capsids. In addition, it was difficult to determine low chlorine dioxide concentrations accurately due to the extreme volatility and reactivity of the compound. It is of interest to note, however, that when virus inactivation by 1.0 mg/l chlorine dioxide was carried out at pH 6.0, no significant amount of empty capsids was present although virus inactivation was 90% complete (Fig. 6). These results show that separation of the RNA from the capsids of poliovirus is a secondary event to virus inactivation by chlorine, chlorine dioxide, and iodine. Apparently, the extent of RNA separation

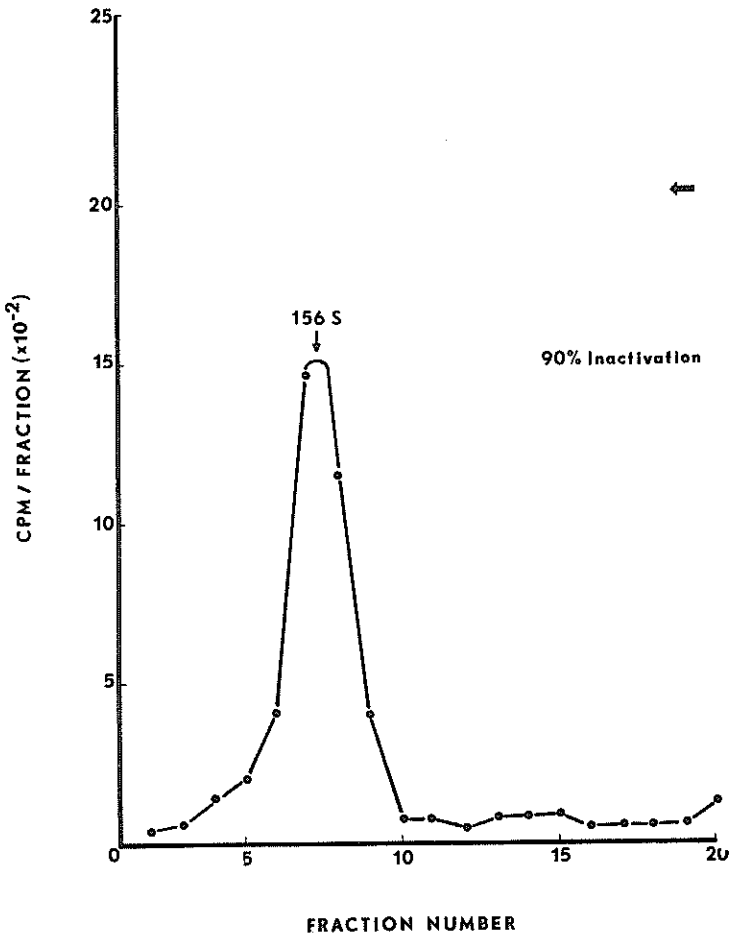

Fig. 6. Effect of chlorine dioxide on the sedimentation coefficient of capside-labeled poliovirus. Viruses were exposed to 1.0 mg/l chlorine dioxide at pH 6.0 and then centrifuged in 15 to 30% glycerol gradients. The inactivated viruses sedimented as 156S structures although virus inactivation was 90% complete. Direction of sedimentation is indicated by the horizontal arrow.

is highly dependent on the initial halogen concentration, the rate at which virus inactivation occurs, and the pH of the environment.

Since it was observed that separation of the viral components did not correlate with percent virus inactivation values, experiments on the nature of virus inactivation by chlorine dioxide or iodine were done under conditions which minimized virus structural damage. For iodine, the experiments were done in solutions containing less than 1.0 mg/l halogen at pH 10.0. In the case of chlorine dioxide, 1.0 mg/l at pH 6.0 was appropriate since under these conditions no significant structural damage of virus particles was obtained. In addition, the inactivated virus preparations were centrifuged through glycerol gradients and the fractions containing only 156S particles used in subsequent experiments. A sensitive method for detecting capsid alterations is isoelectric focusing. Accordingly, the isoelectric points (pI) of inactivated viruses were compared to those of infective viruses. It was shown earlier that the pI value of intact and of chlorine-inactivated viruses was 7.0. The results of chlorine dioxide and iodine-inactivated viruses are shown in Fig. 7, where it can be seen that the pI of chlorine dioxide and iodine-inactivated viruses was shifted from pH 7.0 to pH 5.8. The inactivated particles that focused at pH 5.8 contained RNA.

According to Mandel (34), poliovirus with a pI of 4.5 cannot adsorb to host cells. Therefore, the adsorption of viruses with altered pI values was compared to adsorption of control virions. Results of virus adsorption experiments are summarized in Table 1. It was found that chlorine dioxide-inactivated virions adsorbed to HeLa cells as efficiently as control viruses. In contrast to these results, the data

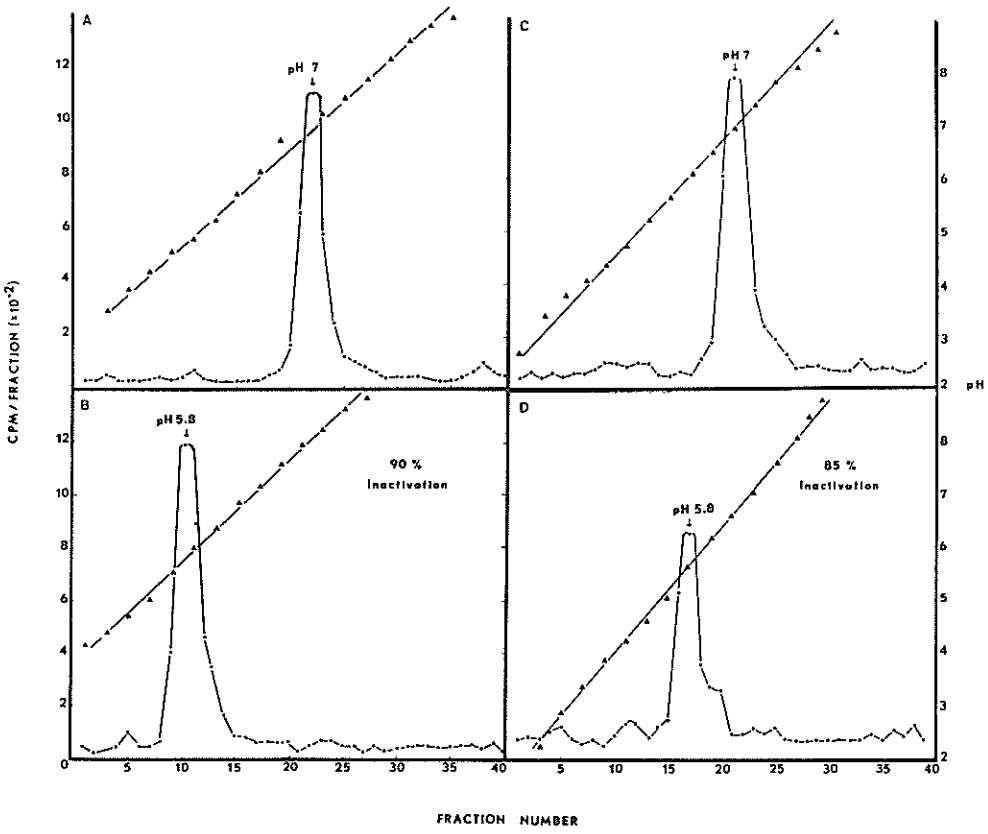

Fig. 7. Effects of chlorine dioxide or iodine on the isoelectric point of poliovirus. Capside-labeled viruses were exposed to 1.0 mg/l chlorine dioxide at pH 6.0 (B) or 0.8 mg/l iodine at pH 10.0 (D). The virus suspensions were than centrifuged through 15 to 30% glycerol gradients. The gradient fractions containing 156S virus particles were pooled and focused in sucrose gradients. Control virus isoelectric points are shown in panels A and C. Direction of migration during isoelectric focusing was from right to left. The pH gradient is shown in each panel (Δ--Δ).

Table 1. Effect of chlorine dioxide or iodine on poliovirus adsorption to HeLa cells.[a]

Virus Treatment	Halogen concentration (mg/l)	Relative % adsorption to HeLa cells[b]	% Reduction[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 capsid-labeled viruses. Adsorption was determined from viral radioactivity associated with the host cells.

[b] $\frac{\text{\% inactivated viruses adsorbed to HeLa cells}}{\text{\% control viruses adsorbed to HeLa cells}}$

[c] (relative % control virus adsorbed to HeLa cells)-(relative % inactivated viruses adsorbed to HeLa cells).

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

clearly showed that iodine-inactivated viruses had impaired adsorption capabilities, and that the reduced adsorption correlated with the amount of inactivation.

In view of the effects of iodine on virus adsorption to host cells, it was of interest to determine if iodine became associated with the viruses. For this purpose, ^{125}I was added to iodine solutions. After the viruses were exposed to the labeled halogen solution, they were analyzed in 15 to 30% glycerol gradients. Results of these experiments are shown in Fig. 8. It is evident that no ^{125}I became associated with the viruses. In contrast, similar experiments done with ^{36}Cl showed that approximately 63% of the amount of ^{36}Cl added was associated with the virus particle (Fig. 9).

Since chlorine dioxide-inactivated viruses adsorbed to HeLa cells as efficiently as controls, experiments to determine the ability of chlorine dioxide-inactivated viruses to penetrate HeLa cells and initiate the uncoating process were done. The gradient profiles of lysed cells that had been infected with control or chlorine dioxide-treated viruses are presented in Fig. 10. No differences were observed between control and inactivated viruses. In these experiments the early modified (M) and further modified (C) particles described by DeSena and Torian (14) were observed in the gradient profiles of cells infected with controls of chlorine dioxide-treated viruses.

In summary, no differences were observed in the adsorption, penetration, or uncoating of chlorine dioxide-treated viruses as compared to controls. Therefore, the possibility that chlorine dioxide reacted with the RNA was next considered. Although the RNA released from chlorine dioxide-inactivated viruses was found to cosediment with intact

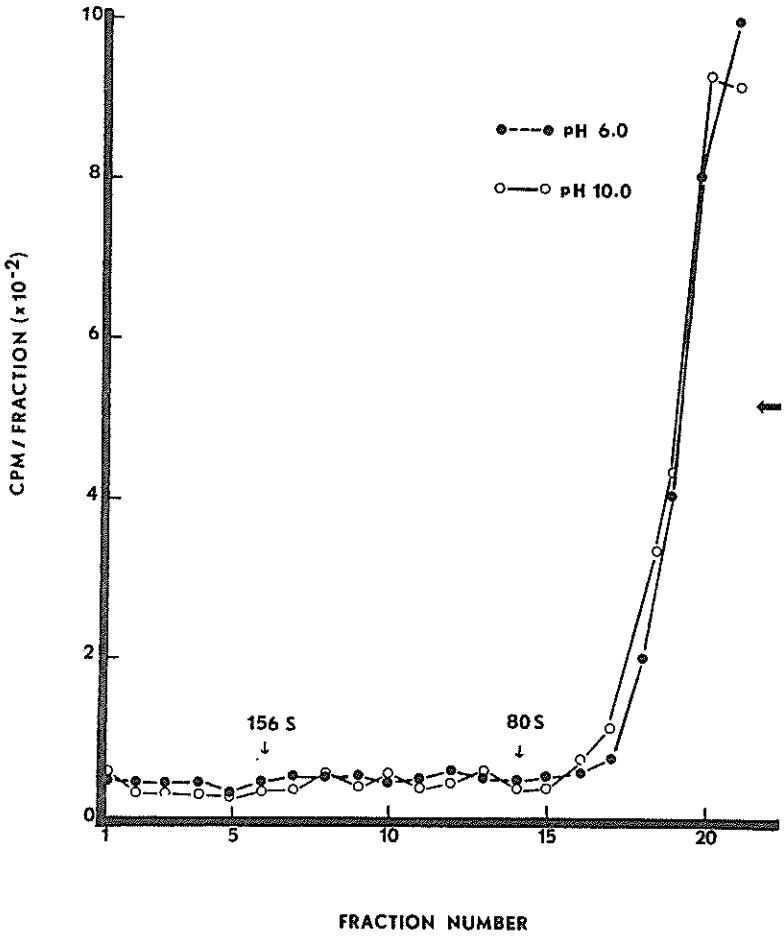

Fig. 8. Binding of ^{125}I by iodine-inactivated polioviruses. Viruses were resuspended in 2.5 mg/l iodine solution containing radioactive iodine. Virus suspensions were centrifuged through 15 to 30% glycerol gradients. Positions of 156S and 80S particles are shown. Direction of sedimentation is indicated by the horizontal arrow.

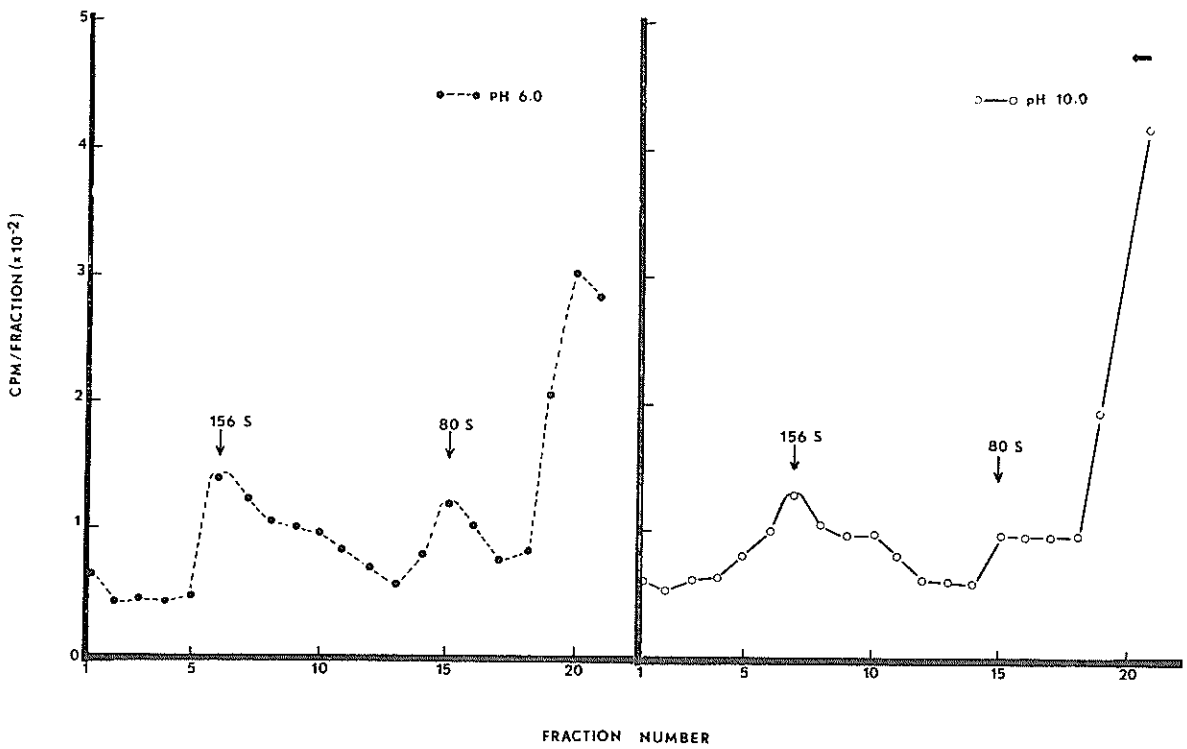

Fig. 9. Binding of ^{36}Cl by chlorine-inactivated polioviruses. Viruses were resuspended in 1.0 mg/l chlorine solution containing radioactive chlorine. Virus suspensions were then centrifuged through 15 to 30% glycerol gradients. Positions of 156S and 80S particles are shown. Direction of sedimentation is indicated by the horizontal arrow.

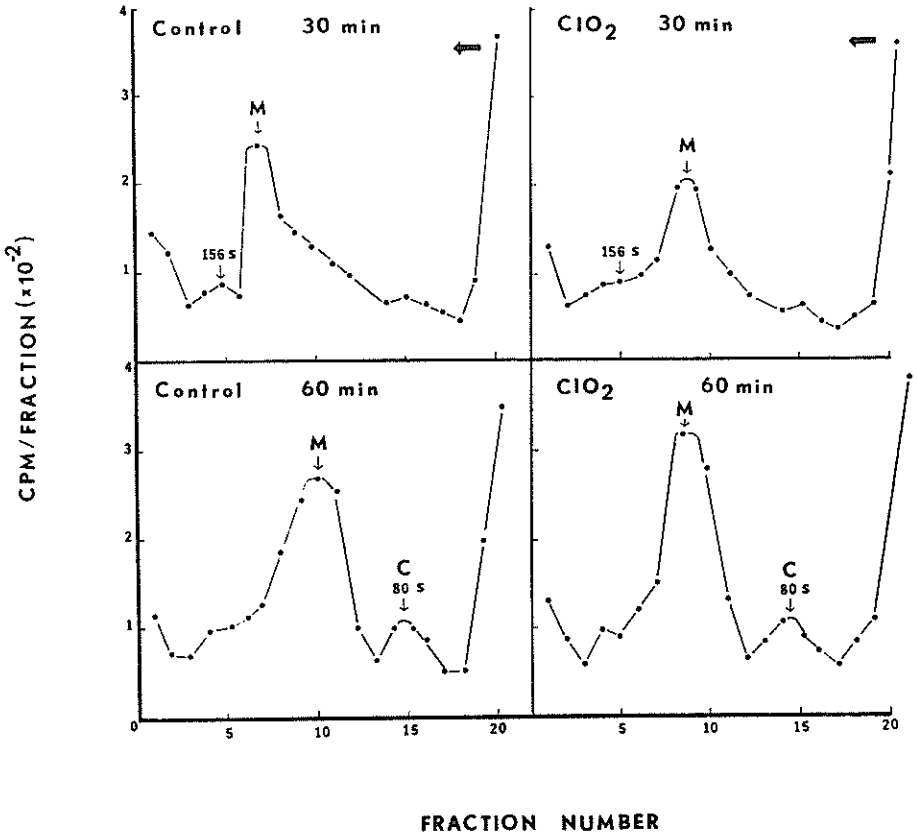

Fig. 10. Effects of chlorine dioxide on uncoating of capsid-labeled poliovirus in HeLa cells. Virus suspensions were 90% inactivated in 1.0 mg/l chlorine dioxide at ph 6.0. HeLa cells were infected with control or inactivated viruses for the time periods indicated and the cells analyzed after lysing on 15 to 30% glycerol gradients. The positions of M and C particles are shown. Direction of sedimentation is indicated by the horizontal arrows.

viral RNA (Fig. 3), the possibility still remained that the molecule had suffered less obvious damage not detectable by sedimentation analysis. Accordingly, experiments were done to determine the ability of the RNA from inactivated virions to incorporate ^{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 (2 h after infection). The actinomycin D was added 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 ^{14}C-labeled viral RNA was then analyzed by rate zonal centrifugation and liquid scintillation spectrometry. The results showed that there was a reduction in the incorporation of the ^{14}C-uridine into new RNA molecules by chlorine dioxide-treated samples as compared to controls (Fig. 11). Furthermore, the percent reduction (determined by the total amount of radioactivity incorporated into viral RNA) correlated reasonably well with the percent inactivation value (Table 2).

Discussion

The results presented in this section show that separation of the RNA from the capsids of poliovirus occurred under optimum conditions for inactivation by chlorine dioxide or iodine. The same phenomenon has also been reported for poliovirus inactivation by chlorine and bromine chloride (28, 42). Therefore, under appropriate conditions the separation of viral components appears to be a generalized phenomenon in enterovirus inactivation by halogens. However, in no case does separation of viral components appear to be the cause of inactivation.

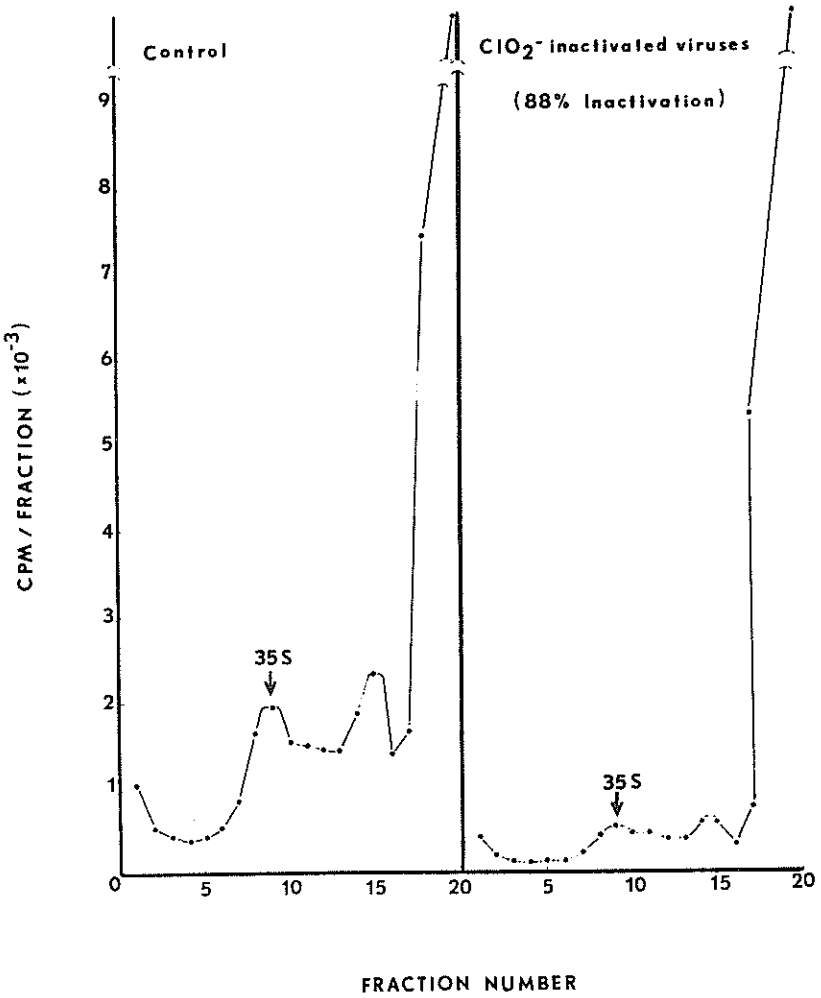

Fig. 11. Incorporation of ^{14}C uridine in viral RNA in HeLa cells infected with control of chlorine dioxide-inactivated polioviruses in the presence of actinomycin D. Virus controls or virus suspensions inactivated by 1.0 mg/l chlorine dioxide were used to infect HeLa cells. Radioactively labeled uridine was added 2 h after infection. The cells were analyzed after lysing on 5 to 30% glycerol gradients. The RNA that sedimented at the 15S through the 35S position was of viral origin. Sedimentation was from right to left.

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

HeLa cell treatment	% ^{14}C uridine incorporation[c]	% reduction of ^{14}C uridine incorporation[d]	% virus inactivation[e]
None	0	-	-
Infective virus	8	0	0
Inactivated virus	2.5	69	88

[a] Poliovirus suspensions were inactivated with chlorine dioxide prior to infecting HeLa cells.

[b] 5 µg/ml actinomycin D added to infected cell cultures at the same time as ^{14}C uridine.

[c] Determined by the ratio of:

$$\frac{\text{cpm } ^{14}C \text{ uridine incorporated into RNA}}{\text{total } ^{14}C \text{ uridine cpm added}}$$

[d] $$\frac{\%\ [^{14}C]\ \text{uridine incorporated by cells infected with inactivated viruses}}{\%\ [^{14}C]\ \text{uridine incorporated by cells infected with infective viruses}}$$

[e] % PFU reduction by chlorine dioxide.

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 in disinfection by these compounds has not been studied in detail. However, chlorine dioxide has been reported to remain undissociated in aqueous solutions at pH values from 4.0 to 8.4 (5). In alkaline solution it disproportionates to chlorite (ClO_2^-) and chlorate (ClO_3^-) according to the following reaction:

$$2ClO_2 + 2OH^- \rightleftharpoons H_2O + ClO_3^- + ClO_2^- \quad (20)$$

Since the end product formed as a result of the oxidation of organic matter by undissociated chlorine dioxide is the ClO_2^- ion (16, 40), it is likely that ClO_3^- is the most active species in inactivation of poliovirus at alkaline pH levels.

In iodine solutions at pH 10.0, more than 88% is in the form of hypoiodous acid (HIO) whereas, at pH 6.0, 90% is in the form of elemental iodine (8). Thus, it appears that with iodine the most active species in virus inactivation is the HIO molecule.

It is not surprising, then, that chlorine dioxide and iodine inactivated poliovirus by different mechanisms. The present results indicate that when iodine is the inactivating agent, the ability of the viruses to adsorb to host cells is impaired. Since the percent inactivation values correlate well with the percent reduction in adsorptive capabilities, it is suggested that iodine reacts primarily with the protein coat of poliovirus instead of the RNA. This proposal is in agreement with the findings of Hsu (26), who reported that poliovirus RNA and coliphage f2 RNA were fully resistant to iodine. The reactions of iodine with the protein coat of poliovirus appear to be oxidative rather than substitutive since no evidence was found of ^{125}I binding to

the viruses. 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 (24).

Brigano et al. (9) suggested that chlorine dioxide inactivated viruses by denaturing the protein coat. However, this hypothesis was based on thermodynamic analyses of inactivation curves rather than on structural and functional analyses of inactivated viruses. The data reported here indicate that, although chlorine dioxide reacts with the protein coat and changes the pI of poliovirus, the critical target is the RNA, which is impaired in its ability to serve as a template for viral RNA replication.

It has been suggested that the most effective virucidal compound used for water disinfection should inactivate the genome of viruses (58). These results, then, indicate that chlorine dioxide may be a good primary or secondary disinfectant since viral RNA appears to be the critical target. Clearly, further studies on the nature of virus inactivation by halogen compounds are needed if more effective virucidal agents are to be developed.

CHAPTER IV

THE EFFECTS OF KCl AND $MgCl_2$ ON POLIOVIRUS INACTIVATION

Results

The ionic environment, especially the presence of cations, has been shown to affect the rate of inactivation of enteroviruses in several environments (15, 18, 44, 48, 53). In this section, the effects of 0.1 M KCl and 0.1 M $MgCl_2$ on the inactivation of poliovirus by chlorine, iodine, or chlorine dioxide are presented. The effects of these salts on virus structure and composition are also included.

The influence of 0.1 M KCl or 0.1 M $MgCl_2$ on inactivation of poliovirus by chlorine, iodine, or chlorine dioxide is shown in Figs. 12, 13, and 14, respectively. The results presented in Fig. 12 show that when chlorine was the inactivating agent, the presence of KCl or $MgCl_2$ significantly enhanced the inactivation rate of poliovirus both at pH 6.0 and 10.0. In contrast, the presence of either cation did not alter the inactivation rate of poliovirus by iodine (Fig. 13). The small differences in the rates of inactivation at either pH 6.0 or 10.0 in the presence and absence of KCl or $MgCl_2$ are within the range of variation observed in these experiments and are not considered significant. The results obtained with chlorine dioxide were surprising since at pH 10.0 the viruses were significantly protected from inactivation when KCl or $MgCl_2$ were present (Fig. 14). On the other hand, at pH 6.0, inactivation of poliovirus by chlorine dioxide was not affected by the presence of either KCl or $MgCl_2$ since the small variation observed between the three curves is not considered significant (Fig. 14).

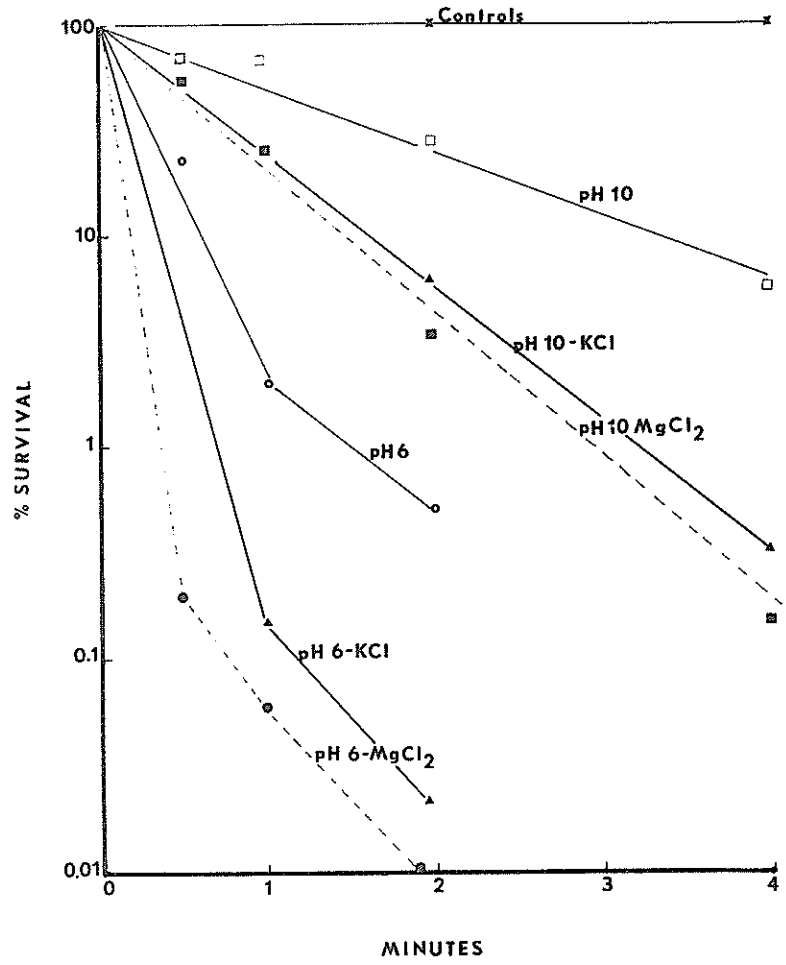

Fig. 12. Poliovirus inactivation by 2.0 mg/l chlorine in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$. Chlorine was added to 3.0 ml of buffer containing viruses, and 0.1 ml samples were taken at the indicated time intervals and assayed for infectivity on HeLa cell monolayers.

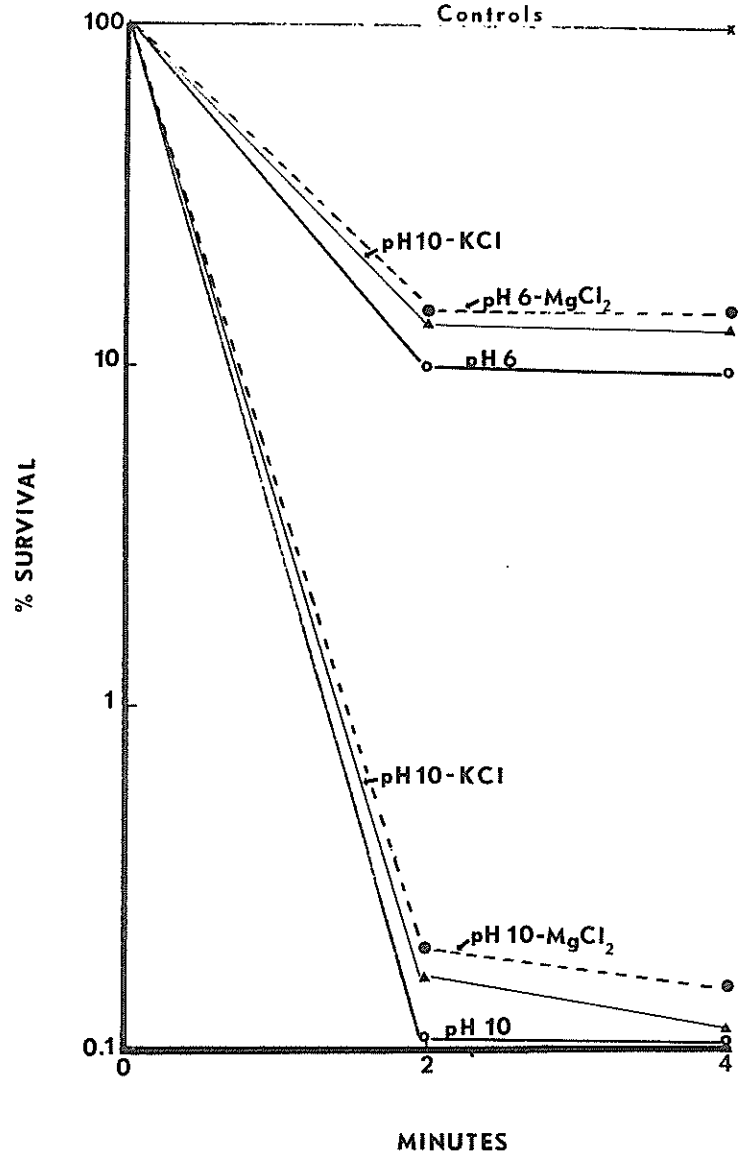

Fig. 13. Poliovirus inactivation by 2.5 mg/l iodine in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$. Iodine was added to 3.0 ml of buffers containing viruses, and 0.1 ml samples were taken at the indicated time intervals and assayed for infectivity in HeLa cell monolayers.

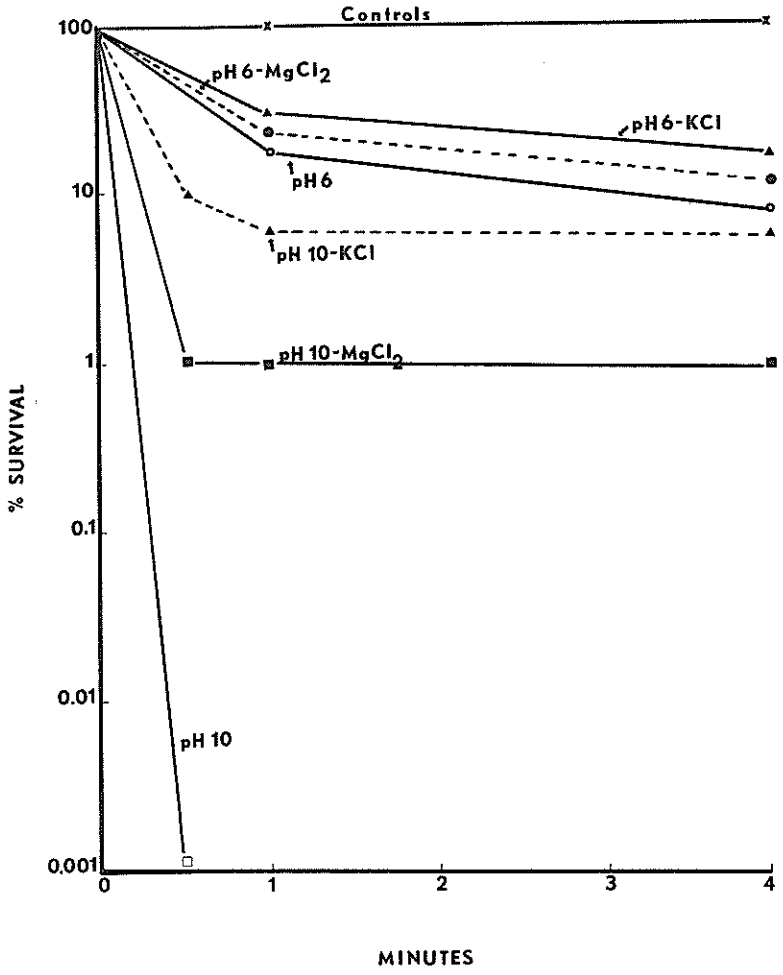

Fig. 14. Poliovirus inactivation by 1.0 mg/l chlorine dioxide in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$. Chlorine dioxide was added to 3.0 ml of buffers containing viruses, and 0.1 ml samples were taken at the indicated time intervals and assayed for infectivity on HeLa cell monolayers.

In view of the differences observed on the effects of KCl and $MgCl_2$ on the kinetics of inactivation of poliovirus by chlorine, iodine, and chlorine dioxide, it was of interest to study the effects of these salts on the structure and composition of halogen-inactivated viruses. Accordingly, radioactively labeled polioviruses which had been inactivated by chlorine, iodine, or chlorine dioxide in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$ were analyzed by rate zonal centrifugation in glycerol gradients.

The gradient profiles of viruses inactivated by chlorine at pH 6.0 in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$ are shown in Fig. 15. It was mentioned earlier in this report that poliovirus inactivation by chlorine at the optimum pH in the absence of K^+ or Mg^{++} ions resulted in the formation of empty, 80S capsids which lacked RNA. It can be seen in Fig. 15 that poliovirus inactivation by chlorine at pH 6.0 in the presence of KCl also resulted in the formation of 80S structures. On the other hand, when $MgCl_2$ was present, chlorine inactivation of poliovirus at pH 6.0 did not result in the formation of 80S capsids. Instead, particles with sedimentation coefficients of 120 to 130S, and 156S structures were observed. Chlorine inactivation of the viruses at pH 6.0 in the presence of $MgCl_2$ resulted in the loss of 50 to 70% of the total radioactivity added to the gradient, which indicated that the presence of Mg^{++} ions increased the adsorption of inactivated viruses to the walls of the cellulose nitrate centrifuge tubes. Inactivation of viruses in other systems has also resulted in the loss of radioactivity due to nonspecific attachment of inactivated virions to various surfaces (49). Due to the significant losses of radioactivity in experiments carried out at pH 6.0, it was decided to carry out similar experiments at pH 10.0. The results

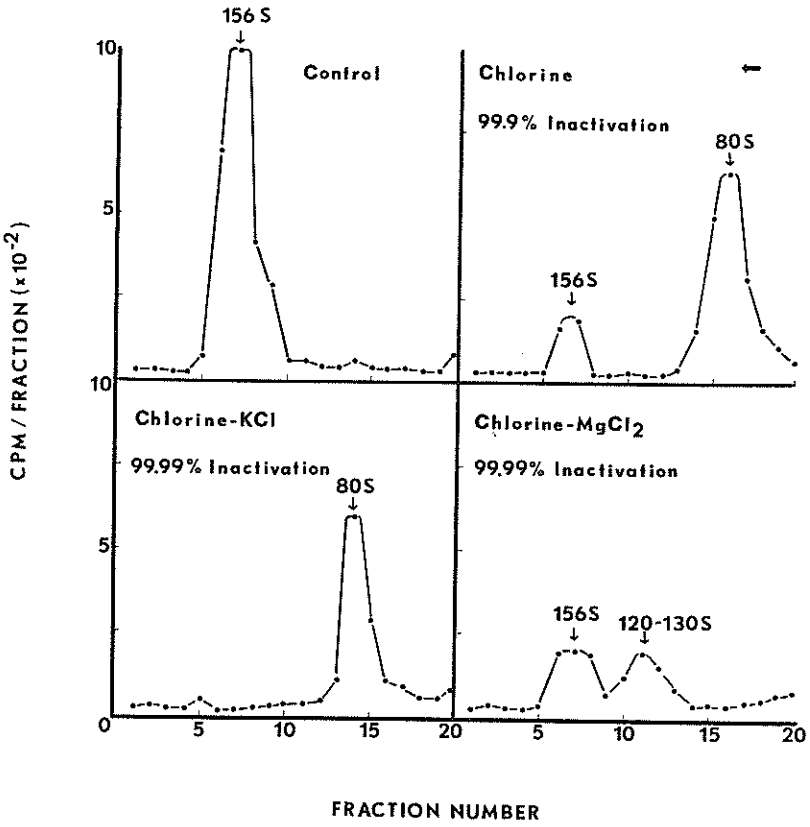

Fig. 15. Effects of 0.1 M KCl or 0.1 M $MgCl_2$ on the sedimentation coefficient of chlorine-inactivated poliovirus. Capsid-labeled viruses were exposed to 2.0 mg/l chlorine in pH 6.0 buffer with or without 0.1 M KCl or 0.1 M $MgCl_2$ and then assayed for infectivity and analyzed in 15 to 30% glycerol gradients. Direction of sedimentation is indicated by the horizontal arrow.

are shown in Fig. 16 and it can be seen that at this pH, the recovery of radioactivity was greatly increased. It is also evident in Fig. 16 that intermediate 120 to 130S particles were formed when poliovirus was inactivated by chlorine in the presence of Mg^{++} at pH 10.0. Moreover, the intermediate particles were found to contain RNA.

Although it was shown earlier that the presence of 0.1 M KCl or 0.1 M $MgCl_2$ did not influence the rate of inactivation of poliovirus by iodine, the sedimentation characteristics of viruses inactivated by iodine in the presence and absence of both salts at pH 10.0 were also analyzed (Fig. 17). The presence of KCl did not affect the sedimentation characteristics of iodine-inactivated viruses and only 156S or 80S particles were seen. Although no intermediate 120 to 130S particles were detected in gradient profiles of viruses inactivated by iodine in the presence of $MgCl_2$, it was noticed that the percentage of empty 80S structures was diminished as compared to that of samples inactivated in the absence of the cation (Fig. 17). This suggested that, although Mg^{++} did not alter the rate of inactivation of poliovirus by iodine, the separation of the viral components was reduced. The gradient profiles of viruses inactivated by iodine in the presence of KCl or $MgCl_2$ at pH 6.0 also showed only 156S or 80S structures and no intermediate particles were detected (results not shown).

The sedimentation characteristics of poliovirus inactivated by chlorine dioxide at pH 10.0 in the presence and absence of 0.1 M KCl or 0.1 M $MgCl_2$ are shown in Fig. 18. It can be observed that the presence of KCl reduced the separation of the viral components, and only 156S or 80S structures were present. Addition of $MgCl_2$, however, resulted in the formation of intermediate particles (120 to 130S) similar to those found

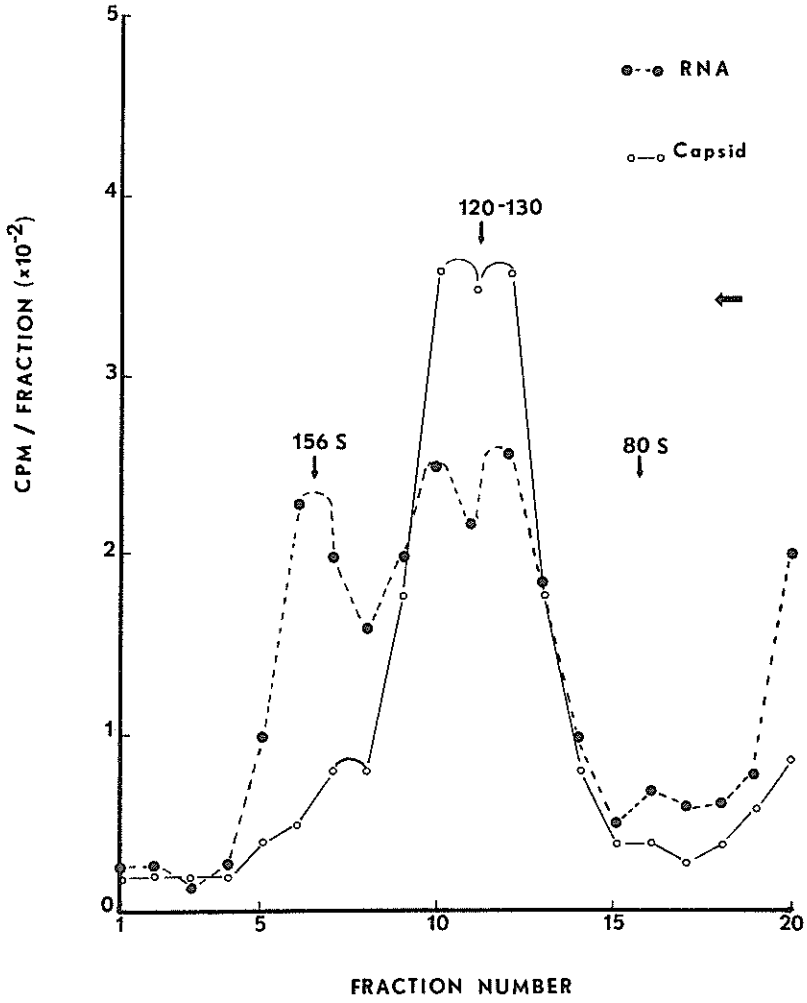

Fig. 16. Effect of 0.1 M $MgCl_2$ on the sedimentation coefficient of chlorine-inactivated poliovirus. Capsid or RNA-labeled viruses were inactivated by 2.0 mg/l chlorine in pH 10.0 buffer with 0.1 M $MgCl_2$. The samples were then analyzed in 15 to 30% glycerol gradients. Virus inactivation was 99.9% complete. Direction of sedimentation is indicated by the horizontal arrow.

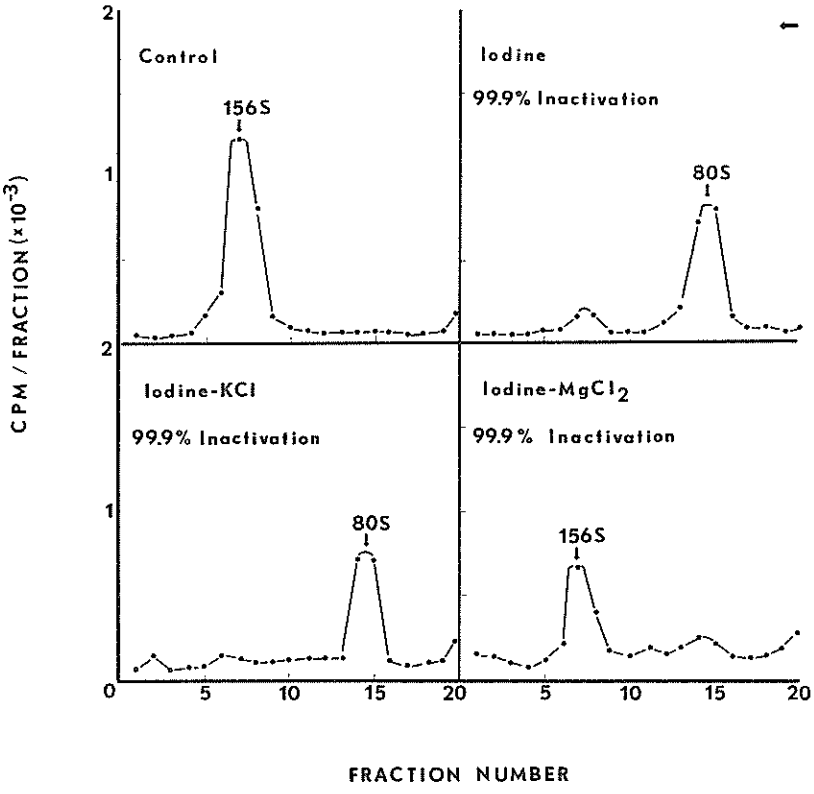

Fig. 17. Effects of 0.1 M KCl or 0.1 M $MgCl_2$ on the sedimentation coefficients of iodine-inactivated polioviruses. Capsid-labeled viruses were exposed to 2.5 mg/l iodine in pH 10.0 buffer with or without 0.1 M KCl or 0.1 M $MgCl_2$ and then assayed for infectivity and analyzed in 15 to 30% glycerol gradients. Direction of sedimentation is indicated by the horizontal arrow.

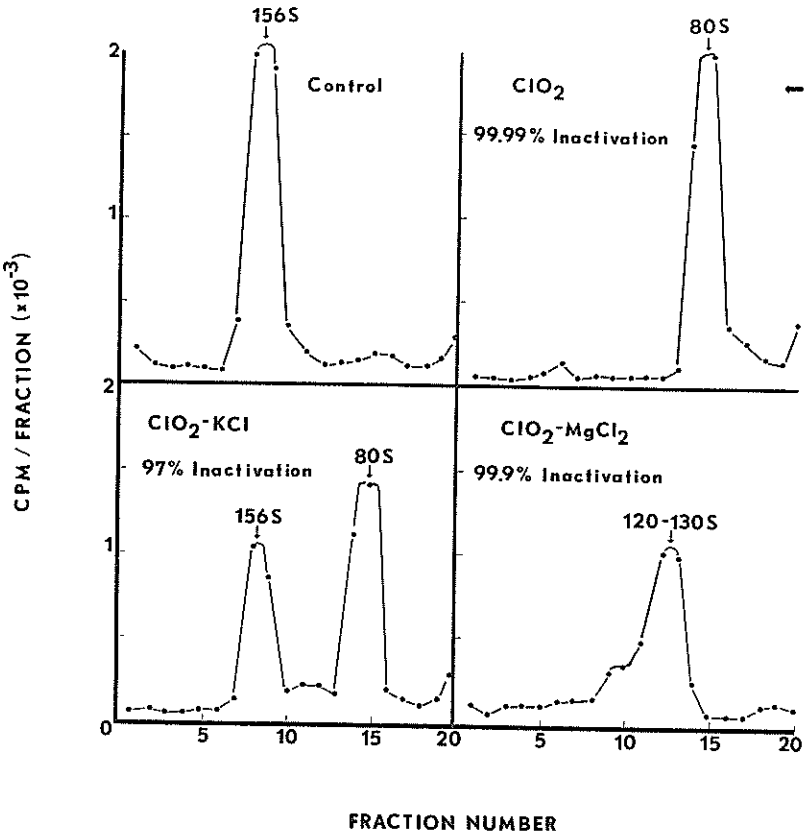

Fig. 18. Effects of 0.1 M KCl or 0.1 M $MgCl_2$ on the sedimentation coefficients of chlorine dioxide-inactivated polioviruses. Capsid-labeled viruses were exposed to 1.0 mg/l chlorine dioxide in pH 10.0 buffer with or without 0.1 M KCl or 0.1 M $MgCl_2$ and then assayed for infectivity and analyzed in 15 to 30% glycerol gradients. Direction of sedimentation is indicated by the horizontal arrow.

with chlorine and $MgCl_2$. In these gradient profiles all of the virus radioactivity recovered was associated with the intermediate particles. At pH 6.0, chlorine dioxide inactivation of poliovirus in the presence of 0.1 M KCl or $MgCl_2$ did not affect the sedimentation coefficients of inactivated viruses which sedimented as 156S particles (data not shown).

Discussion

The results presented in this section show that KCl and $MgCl_2$ have different effects on the inactivation of poliovirus by chlorine, iodine, and chlorine dioxide. The addition of 0.1 M KCl or 0.1 M $MgCl_2$ enhanced poliovirus inactivation by chlorine at both pH 6.0 and 10.0. In contrast to these results, the presence of either salt significantly protected poliovirus from inactivation by chlorine dioxide at pH 10.0, whereas no effect was seen at pH 6.0. When iodine was used as the inactivating agent, poliovirus inactivation curves in the presence and absence of KCl or $MgCl_2$ looked identical, indicating that poliovirus inactivation by iodine is not affected by the presence of these salts. The effects of the salts on inactivation rates are attributed to the presence of the cation (K^+ or Mg^{++}) rather than the chloride ion since experiments conducted using $MgSO_4$ gave similar results (results not shown).

The differences observed in the effects of cations on inactivation by the three halogen compounds cannot be explained definitively at this time. One possible explanation is that the positively charged K^+ and Mg^{++} ions can react electrostatically with negatively charged halogen species (OCl^-, ClO_2^-, ClO_3^-, OI^-) to form ion pairs (56). Jensen et al. (27) have calculated the extent of ion pairing between OCl^- and Na^+ and K^+ and found it to be 4.9% and 6.9%, respectively. Sugam and Helz (57)

have suggested that the ion pairs formed by OCl^- and cations may have greater penetrating power and thus be more effective than free OCl^-. If the extent of ion pairing between OCl^- and K^+ or Mg^{++} ions is considered significant, the results obtained in this investigation agree with this proposal since K^+ and Mg^{++} enhanced the rate of inactivation of poliovirus by chlorine.

The degree of ion pairing between K^+ or Mg^{++} and the ClO_2^- and ClO_3^- ions present when chlorine dioxide is the disinfectant is not known. However, if ion pairs do form, it would appear from the data presented in Fig. 14 that, in contrast to the OCl^--cation pair, the ClO_2^--cation and ClO_3^--cation pairs are less effective virucides since poliovirus was protected from inactivation when the cations were present. This difference could probably be due to the increased size of the ion pairs caused by the extra oxygen atoms present in ClO_2^- and ClO_3^- as compared to OCl^-.

According to Black et al. (8), no significant amount of OI^- ion is present when iodine is used as the inactivating agent at the pH range tested in this study. Thus, even if ion pairing would occur between OI^- and K^+ or Mg^{++}, their concentration would presumably not be significant. This would explain why the presence of K^+ and Mg^{++} ions did not affect inactivation rates of poliovirus by iodine.

Whether ion pairing is a factor in the rates of inactivation of viruses by halogens remains to be established. However, there is some evidence to indicate that other factors may be involved. One of these factors is the structure or conformation of the virus particle itself. Several virus groups within the Picornavirus family have been shown to display different susceptibilities to chlorine disinfection. It is now

becoming evident that the rates of inactivation of viruses by chlorine are affected differently by the presence of cations. For example, the rate of inactivation of two strains of poliovirus (Mahoney and Brunhilde) by comparable chlorine doses was enhanced by the presence of 0.1 M NaCl at both pH 6.0 and 10.0 (53, 54). On the other hand, the inactivation rates of coxsackieviruses B3 and B5 by chlorine were enhanced by the addition of 0.1 M NaCl at pH 10.0 but not at pH 6.0. (27). Since the concentrations of the ionic species were similar in those experiments, it is possible that the differences can be explained by taking into account that polioviruses and coxsackieviruses may exist in different conformational structures at pH 6.0 or 10.0. Cations could, then, affect the inactivation of viruses by affecting the conformational arrangement of the virus protein subunits making them more or less susceptible to halogen attack.

Another piece of evidence that contradicts the ion pair hypothesis comes from the influence of cations observed on the thermal inactivation of poliovirus. If the effects of cations on inactivation of viruses were primarily due to an effect on the ionic species involved in disinfection, one would expect to see no influence of the cations on inactivation by agents which do not involve ionic species such as heat. However, it has been reported that NaCl and $MgCl_2$ protect poliovirus from inactivation at temperatures higher than 44°C (15, 33). In both reports, an attempt was made to explain this phenomenon on the basis of an effect of the cations on the RNA-protein interactions within the virus particle.

Cations have been shown to bind to a variety of plant viruses (17). Also, rhinoviruses reversibly bind up to 5,000 Cs atoms per virus particle, which suggests that cations can also bind, presumably to the

RNA, of animal viruses (35). Although the protein coat of poliovirus is considered to be a very compact structure impermeable to ions (35, 50, 62), there is some evidence to indicate that the capsid can be loosened as a result of aldehyde treatment. It is possible, then, that the reactions of the halogen with the virus particle trigger a conformational rearrangement that allows the passage of cations which can, in turn, bind to the RNA or participate in RNA-protein interactions. This could result in the formation of a particle that would be more stable, less stable, or equally stable to the intact virion. The various effects of cations when different inactivating agents are used might then be explained on the basis of the mechanisms of inactivation displayed by the halogens, which presumably result in different conformational changes of the virus particle.

With respect to the virus compositional changes detected by sedimentation studies, the intermediate 120 to 130S, RNA-containing particles formed as a result of inactivation by chlorine or chlorine dioxide in the presence of Mg^{++} ions have not been reported before. Previous reports on the inactivation of poliovirus by several agents have only indicated the presence of 156S and 80S particles. The detection of intermediate particles, however, has been reported in other systems. For example, acid-treated rhinoviruses sediment as 135S particles which lack VP-4 (31). Also, noninfectious, 130S poliovirus particles containing RNA and lacking VP-4 have recently been detected in the virus progeny of cells infected with the Sabin type 3 vaccine strain (39). Moreover, the immediate precursors in the in vivo assembly of poliovirus have a sedimentation coefficient of 125S, contain the three procapsid proteins VP-0, VP-1, and VP-3 as well as 35S RNA (21, 22). These 125S particles,

which have been named provirions, upon incubation with EDTA lose the nucleic acid which results in the formation of 80S particles. This indicates that divalent cations are important in maintaining the provirion structure.

These findings suggest that the presence of intermediate particles can be detected under certain circumstances and that these particles represent a transition structure between intact 156S virions and empty 80S protein shells. It can also be speculated that the loss of RNA that results from halogen inactivation of viruses is a stepwise transition which occurs through conformational rearrangements that are the reverse of those involved in the in vivo assembly of enteroviruses. Apparently cations can, under certain circumstances, stabilize the structure of these conformational intermediates before empty 80S capsids are formed.

The complete characterization of the 120 to 130S intermediate particles formed as a result of poliovirus inactivation by chlorine or chlorine dioxide in the presence of Mg^{++} ions was beyond the scope of this study. Studies of this nature will undoubtedly provide valuable information in the future, not only on the mechanisms of inactivation of viruses, but also on virus structure.

CHAPTER V

SUMMARY AND CONCLUSIONS

The mechanism of inactivation of poliovirus by chlorine dioxide and that by iodine were found to differ. Iodine inactivated the viruses by reducing their adsorptive capabilities to host cells, whereas chlorine dioxide impaired the ability of poliovirus RNA to serve as a template for replication. However, some general similarities in the effects of the halogens on poliovirus became evident. Both halogen compounds were more effective disinfectants at pH 10.0 than at pH 6.0, although chlorine dioxide was a more effective virucide than iodine. Also, the effects of chlorine dioxide and iodine on gross structural and compositional changes of the viruses were similar. Poliovirus inactivation by chlorine dioxide or iodine resulted in the loss of the RNA from the capsids under optimum conditions, although it was shown that this phenomenon was not the cause of inactivation of viruses. Finally, the RNA released from chlorine dioxide or iodine-inactivated viruses was not degraded, and the isoelectric points of viruses inactivated by either chlorine dioxide or iodine were shifted from pH 7.0 to pH 5.8. These results illustrate that the analyses of gross compositional changes of viruses that occur as a result of halogen inactivation may not reveal the actual effect of the halogen that is responsible for loss of viability. Therefore, care must be taken to also analyze more meaningful virus properties such as their adsorptive and penetrative capabilities, and the ability of the viral RNA to replicate once inside the host cell.

The effects of cations on virus inactivation by halogen compounds were analyzed using 0.1 M KCl and 0.1 M $MgCl_2$. The results depicted a

very complex situation since no general pattern could be discerned. For example, K^+ and Mg^{++} ions enhanced the rate of inactivation of poliovirus by chlorine at both pH 6.0 and 10.0. Iodine inactivation of poliovirus was not affected by the presence of K^+ or Mg^{++} at either pH value. Inactivation of poliovirus by chlorine dioxide was reduced in the presence of K^+ or Mg^{++} at pH 10.0 but not at pH 6.0. The evidence presented indicated that those phenomena may be due either to an effect of the cations on the ionic species involved in disinfection or to an effect on the stability of the virus particle. Alternatively, the possibility that both mechanisms are operative cannot be ruled out.

Therefore, the inactivation of poliovirus by chlorine, chlorine dioxide, or iodine was found to be highly dependent on the pH of the environment and on the presence of cations. A review of the literature on virus disinfection by chlorine would appear to indicate that the virus type itself will determine whether inactivation occurs under a particular halogen concentration and ionic environment. All of these parameters must then be considered when applying data obtained in the laboratory to the disinfection of public water supplies.

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