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Structural and Compositional Changes Associated with Chlorine Inactivation of Polioviruses

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Chlorine inactivation of polioviruses resulted in the loss of viral ribonucleic acid, converting the viruses from 156S particles to 80S particles. However, it was found that virus inactivation occurred before the ribonucleic acid was released from the virions. Extraction of ribonucleic acid from partially inactivated virus suspensions indicated that chlorine inactivation was due to degradation of the ribonucleic acid before release and that ribonucleic acid loss was a secondary event. The empty 80S capsids had the same isoelectric point and ability to attach to host cells as infective virions. Thus, no major capsid conformational changes occurred during chlorine inactivation.

The viricidal effects of halogen compounds, particularly chlorine, have been studied extensively (4, 9, 10, and others). In general, the results show that viruses are irreversibly inactivated by chlorine, although the rates of inactivation are affected by pH, temperature, chlorine concentration, and virus aggregation. In contrast to the extensive literature on inactivation rates, little is known about the mechanisms of virus inactivation by chlorine, although some evidence of virus structural and compositional changes has been obtained. Sharp et al. (10) reported that ribonucleic acid (RNA) was lost from reoviruses after bromine treatment, and Floyd et al. (4) showed that stainability of chlorine-inactivated polioviruses with phosphotungstic acid was increased. Data on the nature of chlorine inactivation of viruses are needed to account for these changes and also to aid in explaining the basis for virus resistance to halogens. In this report, data which show that the primary inactivating event for chlorine-treated polioviruses is the cleavage of the viral RNA and that loss of RNA from the viruses occurs subsequently are presented.

MATERIALS AND METHODS

Virus preparation and assays. Poliovirus type 1 strain Mahoney was used throughout this study. Viruses were propagated, and infective titers and adsorption were assayed on HeLa cells as described previously (7). For radioactive labeling with either [^{3}H]-uridine or [^{3}H]leucine and [^{3}H]valine, 20 μCi of the radioactive compounds per ml was added to cultures at 2 h after infection of the HeLa cell monolayers. The cultures were grown in modified Eagle minimum essential medium containing either 1/10 the amount of nutrients for [^{3}H]uridine labeling or 1/100 the amount of [^{3}H]leucine and [^{3}H]valine for capsid labeling of the viruses. Radioactivity was determined by liquid scintillation spectrometry with Aquasol (New England Nuclear Corp., Boston, Mass.) as the cocktail.

Virus preparations were initially purified as described previously (7). Further purification was accomplished by centrifuging the partially purified virus preparations in 15 to 30% glycerol gradients (12) at 30,000 rpm in an SW41 rotor at 4°C for 3 h. The gradients were fractionated, and the virus-containing fractions were pooled and centrifuged at 30,000 rpm for 3 h to remove the glycerol. Finally, the virus preparations were further purified by equilibrium density centrifugation in CsCl at 65,000 rpm for 23 h at 4°C in an SW65 rotor. The CsCl gradients were fractionated, and the virus-containing fractions were dialyzed against phosphate-buffered saline for 24 h at 4°C. The purified viruses were stored in phosphate-buffered saline at 4°C.

Chlorine inactivation of viruses. Commercial household bleach was diluted with glass-distilled water to the desired concentration (1 mg liter^{-1}) for each experiment. Chlorine as sodium hypochlorite was determined spectrophotometrically by the orthotoludine method (1). The final pH of the chlorine solutions was 6.8 to 7.0. Chlorine determinations were made at the beginning and at the end of each experiment. We arbitrarily decided to disregard the results from experiments in which the chlorine concentrations decreased by more than 10% during the experiment.

Appropriate dilutions of radioactively labeled viruses were added to 1 ml of chlorine solution, and at appropriate intervals 0.1-ml samples were withdrawn and transferred to 0.9 ml of 0.01% sodium thiosulfate solution in phosphate-buffered saline to inactivate the chlorine. Controls were treated in an identical manner except that no chlorine was added. The chlorine-treated viruses were immediately assayed for infectivity as described above or were subjected to further analyses.

Determination of virus sedimentation charac-

teristics. The sedimentation characteristics of the viruses were determined in 15 to 30% glycerol gradients as described above. Untreated viruses of the opposite label served as markers for intact viruses (156S), and viruses heated at 56°C for 5 min served as markers for empty capsids (80S). The gradients were fractionated through a hole in the bottom of the tube, and the positions of the viruses were determined by counting the fractions in a liquid scintillation spectrometer.

For determining the state of viral RNA, the RNA was extracted from [^{3}H]uridine-labeled viruses as described elsewhere (8) and analyzed in 5 to 30% glycerol gradients (12) centrifuged at 65,000 rpm for 90 min at 4°C. The gradients were fractionated, and the position of the RNA in the gradients was determined by liquid scintillation spectrometry.

Isoelectric focusing techniques. The conformation of chlorine-treated polioviruses was analyzed by isoelectric focusing as described by Korant and Lonberg-Holm (5). Appropriate volumes (usually 75 μl) of virus preparations were adjusted to 20% sucrose with a 48% sucrose solution containing 1% ampholyte (pH 3 to 10; Bio-Rad Laboratories, Richmond, Calif.) and layered onto 10 to 40% sucrose gradients containing 1% ampholyte at the 20% sucrose position. The gradients were generated in glass tubes (20 by 0.6 cm) sealed at the bottom with a dialysis membrane. For focusing, the lower reservoir contained 48% sucrose in 1% (vol/vol) sulfuric acid, and the top reservoir contained 2% (vol/vol) ethanolamine. The voltage schedule was 250 V for the first 5 h, and then the voltage was increased by 50 V at 15-min intervals until 500 V was attained. The gradients were fractionated from the bottom of the tubes, and the fractions were analyzed for radioactivity and pH.

RESULTS

Chlorine inactivation of polioviruses has been studied extensively and has been shown to be temperature, pH, and chlorine concentration dependent (4). We confirmed these results in preliminary experiments although the data are not shown here. However, with highly purified virus preparations, the preliminary experiments showed that at pH 6.8 to 7.0, chlorine concentrations of 1 mg liter^{-1} were sufficient to result in approximately a $\log_{10}$ minute^{-1} reduction of infectivity at 25°C, and all experiments were done under these conditions. In agreement with Sharp et al. (10), we found that at chlorine concentrations higher than 1 mg liter^{-1}, the poliovirus particles were extensively degraded. Even at 1 mg of chlorine liter^{-1}, the viruses tended to disaggregate with time. Consequently, experiments to determine structural and compositional virus changes were done immediately after chlorine inactivation.

The effects of chlorine on polioviruses were first examined by suspending capsid-labeled viruses in chlorine for various times and then determining the sedimentation characteristics of the viruses in glycerol gradients. Results of these experiments are shown in Fig. 1, where it can be seen that there was a rapid shift of the sedimentation coefficient from 156S for intact viruses to 80S, which is characteristic of empty capsids (2). This result suggested, then, that chlorine inac-

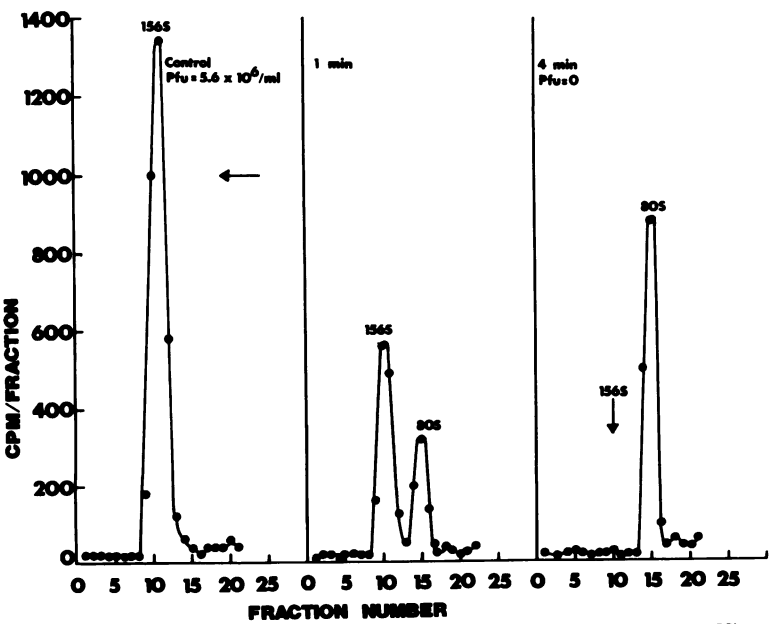

FIG. 1. *Effect of chlorine on sedimentation coefficients of capsid-labeled polioviruses. Viruses were exposed to chlorine for indicated times and then centrifuged at 30,000 rpm in 15 to 30% glycerol gradients for 3 h. The direction of sedimentation is indicated by the horizontal arrow. Pfu, Plaque-forming units.*

tivation of polioviruses caused the release of the viral RNA. This possibility was confirmed in identical experiments with RNA-labeled viruses. Results of these experiments (Fig. 2) confirmed that RNA was released from chlorine-treated viruses since the RNA radioactivity did not sediment to the position in the gradient typical for intact 156S virus particles.

The preceding experiments showed that RNA was lost from polioviruses during chlorine inactivation. However, the conditions of the gradient centrifugation used in the analysis of intact viruses were such that the 35S poliovirus RNA genome remained at or near the top of the gradient. Thus, the question remained as to the nature of the released RNA. This question was addressed in experiments in which chlorine-inactivated viruses were subjected to sedimentation analysis in 5 to 30% glycerol gradients for 90 min at 65,000 rpm. Under these conditions, the 35S poliovirus RNA sedimented to the position indicated by the arrow in Fig. 3, and RNA-containing viruses sedimented to the bottom of

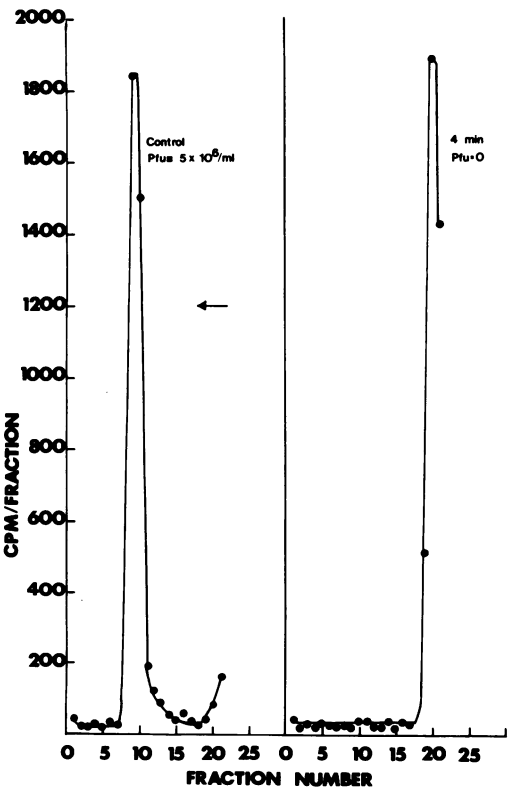

FIG. 2. *Effect of chlorine on RNA content of polioviruses. RNA-labeled viruses were exposed to chlorine for the indicated time. The viruses were centrifuged in glycerol gradients as described in the legend to Fig. 1. Pfu, Plaque-forming units.*

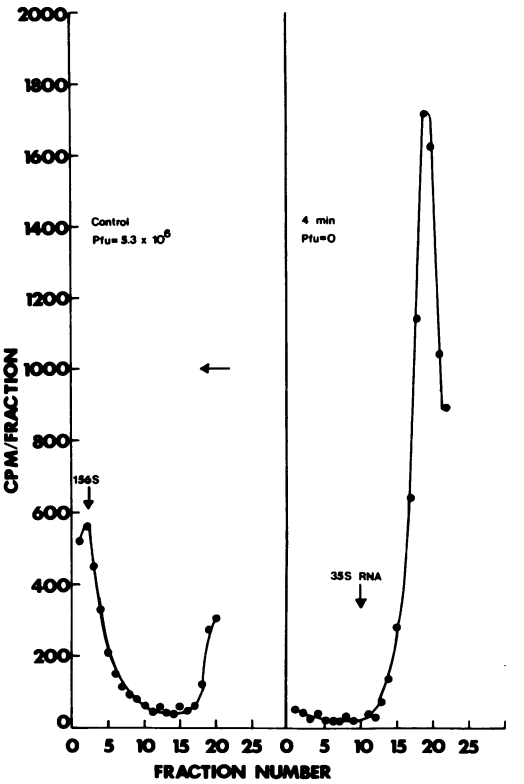

FIG. 3. *Sedimentation characteristics of RNA released from chlorine-inactivated polioviruses. RNA-labeled polioviruses were exposed to chlorine for the indicated time. The virus suspensions were centrifuged on 5 to 30% glycerol gradients at 65,000 rpm for 90 min. The direction of sedimentation is indicated by the horizontal arrow. Pfu, Plaque-forming units.*

the gradient. However, as shown in Fig. 3, the RNA released from chlorine-inactivated viruses remained at the top of the gradient, indicating that the released viral RNA was degraded into fragments of 5S or less. Although not shown here, the RNA extracted from polioviruses and dissolved in 1-mg liter^{-1} chlorine solutions was not altered, as evidenced by sedimentation characteristics.

The release of viral RNA resulted in the irreversible inactivation of polioviruses. However, it was not clear from the preceding experiments whether the loss of infectivity after chlorine treatment was due to the release of RNA or whether the loss of RNA was a secondary event to an as yet undetected change in viral conformation or composition which reduced infectivity. These questions were approached in three ways. First, the RNA contents of chlorine-treated viruses were compared with the percentage of infectivity remaining. The rationale for

these experiments was that if inactivation was due to the loss of RNA, then the loss of virus-associated RNA should correlate with the loss of infectivity. However, as shown in Table 1, the decrease in infectivity was not accompanied by a concomitant loss of RNA. Thus, the separation of RNA from the capsids apparently was not the primary virus-inactivating reaction of chlorine. A second series of experiments was done in which the ability of chlorine-inactivated viruses to attach to host cells was compared with that of infective viruses since it has been shown that the loss of certain capsid components by enteroviruses or conformational changes impairs the ability of the viruses to attach to host cells (3, 6). This possibility was eliminated by the results of the adsorption experiments (Table 1), which clearly show that the ability of chlorine-inactivated virus capsids to attach to HeLa cells was the same as that of infective virus preparations. The third approach to determine the nature of chlorine-induced poliovirus inactivation was to examine the state of viral RNA during inactivation but before release of RNA from the viruses. This was accomplished by extracting RNA from the viruses after brief (15- to 30-s) exposures to chlorine. Brief exposures to chlorine were necessary since when virus inactivation was an order of magnitude or greater, the RNA was extensively broken down and was being released from the capsids. A typical result of these experiments (Fig. 4) shows that there was a close correlation between the loss of [^{3}H]uridine label from the 35S RNA peak (65%) and the reduction of infectivity (70%). These results indicate, then, that the inactivation of polioviruses by chlorine was due to cleavage of the RNA while the RNA was still associated with the capsid and also suggest that the release

TABLE 1. *Relationships between chlorine inactivation of polioviruses and the viral RNA content and ability of the viruses to adsorb to host cells*

Min[a]	Initial PFU[b] ml^{-1} (%)	Initial RNA content (%)[c]	Viruses adsorbed to HeLa cells (%)[d]
0	100.0	91	31
2	1.2	62	32
4	0.2	36	31

[a] Time exposed to 1 mg of chlorine liter^{-1}.

[b] PFU, Plaque-forming units.

[c] Expressed as percentage of virus-associated [^{3}H]-uridine counts per minute after centrifugation of viruses in 15 to 30% glycerol gradients at 30,000 rpm for 3 h.

[d] Expressed as percentage of capsid-labeled viruses adsorbed to HeLa cells after 30 min.

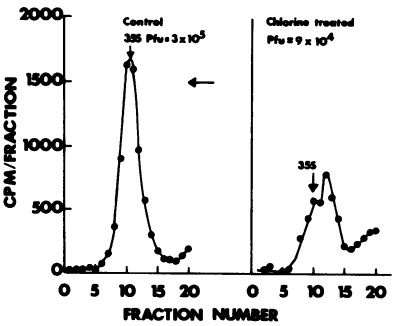

FIG. 4. *Effects of chlorine on poliovirus RNA. RNA-labeled viruses were exposed to chlorine for approximately 30 s. RNA was extracted from the viruses and analyzed in 5 to 30% glycerol gradients as described in the legend to Fig. 3. Pfu, Plaque-forming units.*

of the degraded RNA was an event secondary to virus inactivation. Apparently, for RNA to be degraded it was necessary that the RNA be associated with the capsids since the sedimentation coefficient of RNA extracted from infective viruses was not affected when suspended in a 1-mg liter^{-1} chlorine solution.

The intraviral cleavage of RNA described above suggested the possibility that the interaction between chlorine and the viruses resulted in a conformational shift of the capsid. Preliminary sodium dodecyl sulfate-gel electrophoresis of capsid proteins showed that all four capsid proteins were present in inactivated viruses. These results prompted additional experiments to examine the conformational state of chlorine-inactivated viruses by isoelectric focusing. The results of these experiments are shown in Fig. 5, where the isoelectric properties of chlorine-inactivated viruses are compared with those of infective viruses and viruses heated at 56°C for 5 min. The latter treatment causes a shift of the isoelectric point from pH 7.0 to 4.5 and the loss of viral RNA and capsid protein VP-4 (2, 6). Heat-treated viruses had isoelectric points of approximately pH 4.5, whereas control viruses focused at about pH 7.5. Chlorine-inactivated viruses also focused at pH 7.5, indicating that the empty capsids had the same isoelectric points as did infective viruses. These results indicate that chlorine inactivation did not detectably alter the conformational state of the viruses.

DISCUSSION

The reaction by which polioviruses are inactivated by chlorine was shown to be degradation of the viral RNA genome. The results of this study also show that poliovirus RNA is appar-

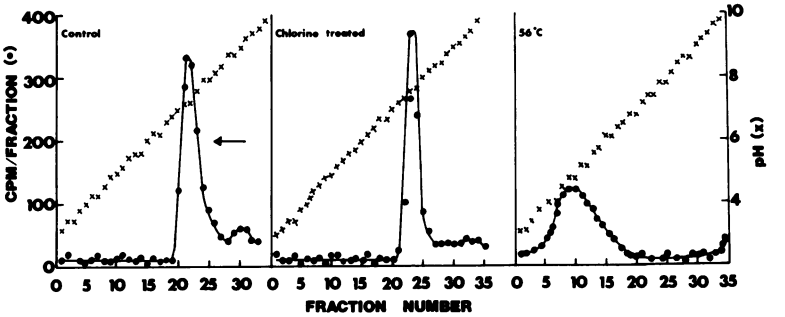

FIG. 5. *Effects of chlorine and heat on the isoelectric point of polioviruses. Viruses were either exposed to chlorine for 4 min or heated at 56°C for 5 min. Isoelectric focusing was done in sucrose gradients.*

ently sensitive to chlorine only while the RNA is associated with the capsid, at least at the concentration used here. The mechanism of chlorine inactivation of polioviruses is, in part, similar to the mechanism by which ammonia inactivates polioviruses (11) since ammonia also causes a breakdown of encapsulated RNA. However, the nature of the interactions of chlorine and ammonia with polioviruses is clearly different since with chlorine, the RNA is subsequently released from the capsid, whereas with ammonia, the RNA remains associated with the virions.

The loss of RNA after halogen treatment may be a generalized phenomenon for enteroviruses since Sharp et al. (10) reported that RNA was released from reoviruses after inactivation with bromine. However, it is not clear whether bromine inactivation of reoviruses was due to the loss of RNA per se or whether the loss of RNA was a secondary event. As shown here, caution must be used in attributing virus inactivation to gross virus compositional or structural changes in the absence of data relating such changes to the amount of virus inactivation.

The nature of the chlorine-induced breakdown of poliovirus RNA remains unknown at this time. Clearly, the capsid changes after chlorination are different from those caused by heating at 56°C. With chlorine, the capsids had the same isoelectric points as did infective viruses, whereas the isoelectric point of capsids produced by heating was changed to pH 4.5. Nevertheless, it must be presumed that chlorine caused a conformational or structural change which resulted in cleavage of the viral RNA. Evidence that capsids are altered by chlorine is also indicated by our finding that the empty capsids were fragile and tended to fall apart upon standing. The results of Floyd et al. (4) also indicate that capsid integrity is changed since polioviruses treated with chlorine at a concentration comparable to that used here were increasingly stainable with phosphotungstic acid. However, inactivation of the viruses preceded entry of phosphotungstic acid into the viruses, which is similar to our finding that viruses are inactivated before RNA is lost. It is possible that phosphotungstic acid stainability and RNA loss are related effects of chlorine treatment. The capsids of polioviruses, although fragile, maintain sufficient structural integrity to permit sedimentation studies when chlorine concentrations of 1 mg liter^{-1} or less are used; however, at higher chlorine levels, the capsids are extensively disaggregated (4).

More detailed studies on the nature of chlorine-virus interactions are necessary before speculating further on capsid effects. Hopefully, such studies will aid in explaining the cleavage of virus-associated RNA.

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