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Mechanism of Chlorine Inactivation of DNA-Containing Parvovirus H-1

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An investigation was undertaken to determine the effect of chlorine on a small DNA-containing enteric virus. Parvovirus H-1 was exposed to sodium hypochlorite in a phosphate-buffered saline solution at pH 7. Then, the whole virion, the protein capsid, or the nucleic acid was subjected to analysis. The sedimentation rate of the chlorine-treated whole virus decreased from 110S to 43S. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the virus demonstrated the formation of higher-molecular-weight aggregates resulting from covalent cross-linking of the capsid proteins. Electron microscopic examination revealed that the DNA was extruded as a taillike structure which remained attached to the virus particle. Furthermore, the DNA was intact and still capable of in vitro replication. The adsorption of the chlorine-treated virions to host cells was inhibited, presumably due to the effect of chlorine on the particular spatial arrangement of the capsid proteins required for adsorption. Specific sites on these proteins had become highly reactive, indicating that the initial action of chlorine on parvovirus H-1 was on the viral capsid.

In 1968, a waterborne pathogen, later determined to be a parvovirus-like agent, caused an outbreak of gastroenteritis in Norwalk, Ohio (3). A recent epidemiological report on waterborne outbreaks of disease since then (4) has implicated a similar agent as being responsible for five outbreaks of waterborne gastroenteritis during the period between 1971 and 1978. Four of the five outbreaks were traced to inadequately disinfected water supplies. Because it is standard practice to treat water supplies with chlorine for disinfection, researchers have studied the mechanism of chlorine inactivation of human enteric viruses. The elucidation of inactivation mechanisms perhaps will aid in the optimization of chlorine disinfection of drinking water supplies. Previous studies have dealt, however, with only the RNA-containing poliovirus (1, 7, 13, 18).

The structural simplicity of enteric viruses relative to other viruses accommodates them to one of three possible mechanisms of inactivation: (i) an alteration of the protein capsid, (ii) an alteration of the nucleic acid, or (iii) a combination of both modes. Tenno and co-workers (18) and, more recently, Alvarez and O'Brien (1) have determined that the mode of action of chlorine on poliovirus is at the protein level. The RNA extracted from the treated virions was found to retain its infectivity. Whether this same mechanism will be true for the chlorine inactivation of all enteric viruses, including those which contain DNA, remains to be ascertained.

The experiments described herein were conducted with the small DNA-containing parvovirus H-1. This enteric virus is a putative human virus (19) that contains single-stranded DNA in a protein capsid that is 18 to 22 nm in diameter. Chlorine inactivation rates for the parvoviruses H-1 (C. C. Churn, G. D. Boardman, and R. C. Bates, Water Res., in press) and Kilham rat virus (6) have been determined. This investigation describes the effect of chlorine on the whole virion, the viral protein, and the extracted viral nucleic acid.

MATERIALS AND METHODS

Viruses and cells. Parvovirus H-1 was obtained from S. Rhode (Institute for Medical Research of Bennington, Bennington, Vt.) and propagated in the NB (simian virus 40-transformed newborn human kidney) cell line. The NB cells were grown to confluency in minimum essential media (Flow Laboratories, Inc., McLean, Va.) to which was added 0.625 μg of amphotericin B (Flow Laboratories) ml^{-1}, 584 mg of glutamine (Sigma Chemical Co., St. Louis, Mo.) $liter^{-1}$, 10% (vol/vol) fetal bovine serum (Flow Laboratories), 100 U of penicillin (Sigma) ml^{-1}, and 100 μg of streptomycin (Sigma) ml^{-1}.

Virus stocks were obtained by infecting partially confluent monolayers of NB cells at an approximate multiplicity of infection of 0.1 PFU per cell. At 10 to 12

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h postinfection, the virus was radioactively labeled in either the protein capsid or the nucleic acid by adding to the media 10 μCi of [^{3}H]methionine (Met) or [^{3}H]thymidine (TdR) (New England Nuclear Corp., Boston, Mass.) ml^{-1}, respectively. The cell material and lysate were collected when the cytopathic effect was complete, usually by 72 to 96 h postinfection.

Virus purification. Parvovirus H-1 was purified by a method similar to that previously described for bovine parvovirus (J. T. Patton, Ph.D. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1980). Cell material and lysate were collected, centrifuged (27,000 rpm, 2 h, 10°C, SW27 rotor), and suspended in a small volume (<20 ml) of 50 mM Tris-hydrochloride, pH 8.0. The cell material was then digested with 50 μg of DNase I (Sigma) ml^{-1} and 100 μg of RNase A (Sigma) ml^{-1} in the presence of 5 mM $MgCl_2$ at 37°C for 30 to 60 min. Then, 250 μg of trypsin (Sigma) ml^{-1} and 25 μg of chymotrypsin (Sigma) ml^{-1} were added, and the samples were incubated for an additional 15 min. A high-speed (50,000 rpm, 60 min, 4°C, Ti50 rotor) pellet was obtained from the supernatant of a low-speed (10,000 rpm, 10 min, 20°C, JA-17 rotor) centrifugation of the digested cell material. The high-speed pellet containing the virus was suspended in a small volume (<2 ml) of 50 mM Tris-hydrochloride at pH 8.0. This sample was then loaded onto a preformed cesium chloride (EM Reagents, Darmstadt, Germany) gradient consisting of, from bottom to top of the tube, 2.0 ml of 40% CsCl, 4.0 ml of 35% CsCl, 2.0 ml of 30% CsCl, and 2.0 to 2.8 ml of 1 M sucrose (EM Reagents). All solutions were made with 50 mM Tris-hydrochloride buffer at pH 8.0. The gradients were centrifuged at 34,000 rpm for 18 h in an SW41 rotor at 15°C.

The opaque virus band was located by light scattering, collected by aspiration, and dialyzed against 50 mM Tris-hydrochloride buffer at pH 8.0 for a minimum of 1 to 2 h. The virus was then centrifuged into a 5 to 30% neutral sucrose gradient (50 mM Tris-hydrochloride, 0.5 mM EDTA, pH 8.7) for 2 h at 41,000 rpm and 4°C in an SW41 rotor. The gradients were fractionated from the bottom, and the virus was located by liquid scintillation spectrometry (model LS7500; Beckman Instruments, Inc., Silver Spring, Md.). The virus was dialyzed overnight against chlorine-demand-free phosphate-buffered saline (PBS) at pH 7.0, collected, and stored at 4°C.

Preparation of reagents and utensils. A PBS solution at pH 7.0 (0.01 M sodium phosphate, 0.14 M sodium chloride) was prepared with reagent-grade chemicals (Fisher Scientific Co., Pittsburgh, Pa.). Stock solutions (1 liter) of both 1 M sodium monobasic phosphate (NaH_2PO_4) and 1 M sodium dibasic phosphate (Na_2HPO_4) were prepared with either deionized, glass-distilled water or water that was treated by reverse osmosis and then deionized.

To exhaust the demand of the buffer for chlorine, household bleach (5.25% sodium hypochlorite [NaOCl]) was added to a final concentration of 2.1 mg liter^{-1} and allowed to react for 24 h. The solution was irradiated with a submersible quartz UV light (UV Products, Inc., San Gabriel, Calif.) until all remaining chlorine was quenched. This solution was then considered to be chlorine-demand free. The pH was determined with a standardized Fisher Accumet pH meter and was always pH 7.0 ± 0.05.

Polypropylene microcentrifuge tubes (Brinkmann Instruments Inc., Westbury, N.Y.) were soaked in 10% (vol/vol) HCl, washed with 5.25% (wt/vol) NaOCl, and then rinsed several times with distilled water.

Chlorine solutions were freshly prepared for each experiment from household bleach (5.25% [wt/vol] NaOCl) by dilution with PBS. Chlorine solutions were designed to provide NaOCl dosages of 1 to 5 mg liter^{-1} when mixed with either 50 or 100 μl of virus stock. A final volume of 300 μl was used in the experiments.

The sodium thiosulfate ($Na_2S_2O_3$) used to quench the residual chlorine in the samples was also made fresh for each experiment as a 10% (wt/vol) solution in PBS.

Plaque assay. Parvovirus H-1 was enumerated by plaque assay as described by Ledinko (10). Confluent monolayers of NB cells grown in plastic tissue culture dishes (60-mm diameter) were rinsed with Dulbecco phosphate buffer and infected with 0.25 ml of virus dilution. After a 45-min adsorption period, an overlay solution containing minimum essential media, 0.6% agarose (Bethesda Research Laboratories, Gaithersburg, Md., or FMC Corp., New York, N.Y.), and 10% fetal bovine serum (Flow Laboratories) was added. After 5 to 6 days of incubation, the cells were stained with a 0.1% (wt/vol) crystal violet stain.

Sucrose gradient analysis. Sedimentation rate analysis of control and chlorine-treated whole virions was by rate zonal centrifugation. Samples were layered onto 5 to 30% neutral sucrose gradients (50 mM Tris-hydrochloride, 0.5 mM Na_2EDTA, pH 8.7). These were centrifuged in an SW41 rotor for 2 h at 41,000 rpm and 4°C. The radioactivity associated with each 0.5-ml fraction was determined by liquid scintillation spectrometry.

Rate zonal centrifugation of the DNA from both control and chlorine-treated whole virions was with 5 to 30% alkaline sucrose gradients (0.3 M NaOH, 0.7 M NaCl, 1 mM Na_2EDTA, 0.15% Sarkosyl, pH 12.1). Samples were adjusted to 0.25 M NaOH and incubated for 10 min at 37°C before centrifugation. The 5 to 30% alkaline sucrose gradients were centrifuged in an SW41 rotor for 15 h at 30,000 rpm and 20°C. Gradients were fractionated into 0.4-ml fractions. Before the radioactivity associated with each fraction was measured, either the entire fraction or a sample of 10% of the total volume was acidified with glacial acetic acid into the pH range of 5 to 7.

Polyacrylamide gel electrophoresis. Proteins were isolated from both untreated and chlorine-treated samples by ethanol precipitation in the presence of 0.3 M sodium acetate, pH 5.2, at −80°C. Pellets were dried under N_2 and resuspended in 20 to 40 μl of Laemmli application buffer (9).

Polyacrylamide (7.5% acrylamide, 0.193% bisacrylamide) gels were run by the discontinuous gel system of Laemmli (9). Electrophoresis was performed with a constant current of 10 mA for stacking the proteins and 25 mA for resolution. The gels were prepared for fluorography by the method of Bonner and Laskey (2). Gels were dried onto Whatman filter paper and exposed to Kodak X-Omat R film with the aid of a Du Pont Cronex Lightning-Plus intensifying screen at −80°C.

DNA isolation, replication, and electrophoresis. A solution of [^{3}H]TdR-labeled H-1 whole virions was

adjusted to 0.3 M NaOH (pH 12.1) and incubated at 37°C for 10 min. The labeled DNA was banded in 5 to 20% alkaline sucrose gradients as described previously. Peak fractions were pooled and dialyzed overnight against 50 mM Tris-hydrochloride–0.5 mM EDTA (pH 8.0) and then ethanol precipitated.

The resulting pellets were then suspended in 100 μl of a reaction mixture for replication. This mixture consisted of the following: 50 mM Tris-hydrochloride, pH 8.0; 50 mM NaCl; 10 mM $MgCl_2$; 80 μM of dGTP; 80 μM of dATP; 80 μM of dTTP; 20 μM of dCTP; 25 μg of bovine serum albumin; [α-^{32}P]dCTP (20,000 cpm pmol^{-1}); and 1 U of *Escherichia coli* DNA polymerase I (large fragment, Klenow). After incubation for 1 h at 37°C, the reaction was stopped by the addition of EDTA to 10 mM. Unincorporated nucleotides were removed by chloroform-isoamyl alcohol extraction followed by spin dialysis. Spin dialysis columns were prepared and utilized as described previously by Neal and Florini (12). The samples were made 0.3 M sodium acetate (pH 5.2), and the DNA was ethanol precipitated.

Pellets were dried under N_2 and suspended in electrophoresis application buffer (20 μl of distilled water, 4 μl of Ficoll, 1.25 μl of bromphenol blue, and 0.1 μl of 20% [wt/vol] sodium dodecyl sulfate). Samples were loaded onto a 1.4% agarose (Bethesda Research Laboratories) gel and subjected to electrophoresis at 90 V for 2.75 h. The gel was blotted dry and exposed to Kodak X-Omat R film at −80°C with the aid of a Du Pont Cronex Lightning-Plus intensifying screen. Both single- and double-stranded DNAs from bovine parvovirus were used as markers.

Restriction endonuclease treatment. Restriction endonucleases were used to digest double-stranded replicated DNA by the conditions specified by the supplier (Bethesda Research Laboratories). Analysis of the resulting fragments was performed by electrophoresis on 1.4% agarose gels as described above.

Adsorption experiments. Confluent monolayers of NB cells were grown in 100-mm-diameter plastic tissue culture dishes. The medium was removed, and the cell monolayer was rinsed twice with Dulbecco phosphate buffer. The [^{3}H]Met-labeled virus samples (300 μl), both chlorine treated and untreated, were diluted with PBS to a final volume of 0.5 ml. The entire sample was placed onto the rinsed monolayers and allowed to adsorb for 45 to 60 min at 37°C with agitation at 15-min intervals. Then the cells were scraped into a small volume (<5 ml) of PBS, and the resulting cell suspension was filtered through Whatman glass-fiber filters. Replicate samples were thoroughly washed with either PBS or PBS-EDTA solution. The filters were dried and then counted by liquid scintillation spectrometry.

RESULTS

Effect of chlorine on sedimentation rate, adsorption ability, and infectivity of parvovirus H-1. The effect of chlorine on parvovirus H-1 was investigated by exposing [^{3}H]Met-labeled virions to doses of NaOCl ranging from 1 to 5 mg liter^{-1} for various exposure times, and the change in sedimentation rate of the treated virus was determined by rate zonal centrifugation with 5 to 30% neutral sucrose gradients. Treatment of the protein capsid-labeled virus with 1 mg of NaOCl liter^{-1} for 60 min revealed the formation of a lighter particle type located in fraction 18 (Fig. 1B). By increasing the NaOCl dosage to 5 mg liter^{-1}, the enhancement of the formation of the

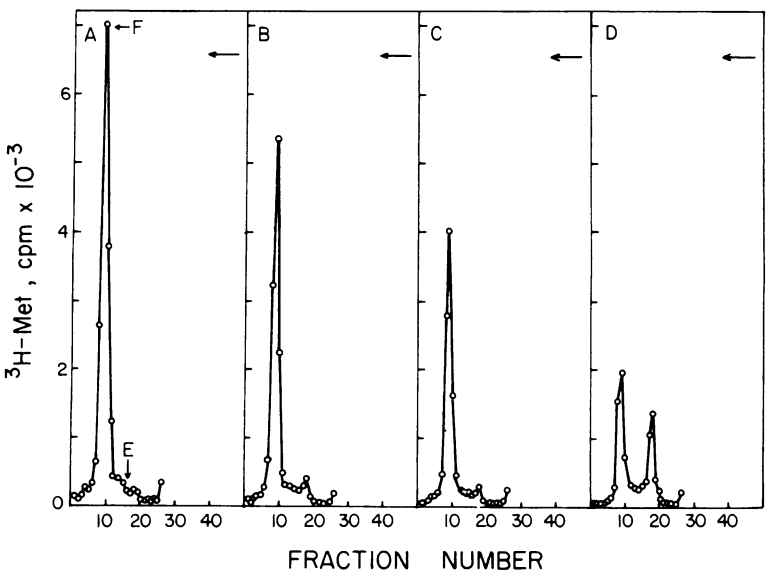

FIG. 1. Sedimentation rate analysis of chlorine-treated, [^{3}H]Met-labeled whole virus in a 5 to 30% neutral sucrose gradient. (A) Control. F indicates the position of the full virus (110S); E indicates the position of the empty capsids (70S). (B) NaOCl (1 mg liter^{-1}), 60-min exposure. (C) NaOCl (5 mg liter^{-1}), 5-min exposure. (D) NaOCl (5 mg liter^{-1}), 60-min exposure. The arrow indicates the direction of sedimentation.

lighter particle type with increasing exposure times was readily apparent (Fig. 1C and D). Total conversion of the full virion to this new particle could be achieved by chlorinating at 5 mg of NaOCl liter $^{-1}$ for 60 min (Fig. 2). The sedimentation coefficients for full virions, empty particles, and the new particle type were estimated to be ca. 110, 70, and 43S, respectively, by the method of Griffith (8). The sedimentation rate coefficients for the full virions are in agreement with previously reported values (16). Chlorine, at the levels used in experiments with full virions, caused no alteration in the sedimentation rate of empty H-1 capsids (data not shown).

Because these experiments reveal only what occurs with the protein component of the virus, the same protocol was used to determine the effect of chlorine on [^{3}H]TdR-labeled virus. Figure 3 shows that the same sedimentation pattern was obtained, indicating that the 43S peak contained not only protein but also nucleic acid.

The ability of the chlorine-treated virus to adsorb to host cells was investigated (Table 1). The chlorine-treated, [^{3}H]Met-labeled virus was allowed to adsorb to host cells for 45 to 60 min at 37°C. This time period is adequate for adsorption but minimizes penetration. Each virus-cell mixture was collected and filtered and then subjected to washing by PBS or PBS containing EDTA. The purpose of the EDTA was to eliminate any nonspecific binding of damaged virions (11). On the basis of radioactivity, the levels of chlorine-treated virus remaining bound to host cells after washing with PBS were similar to those of the control. The PBS-EDTA wash, however, revealed that ca. 50% of the treated virus had nonspecifically adsorbed to the host cell.

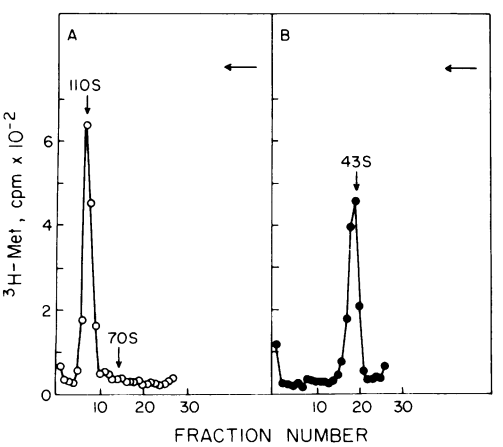

FIG. 2. Sedimentation rate analysis of chlorine-treated, [^{3}H]Met-labeled whole virus in a 5 to 30% neutral sucrose gradient. (A) Control, showing location of 110S and 70S (empty capsid) particles. (B) NaOCl (5 mg liter^{-1}), 60-min exposure; the sedimentation coefficient for the chlorine-treated virions was 43S.

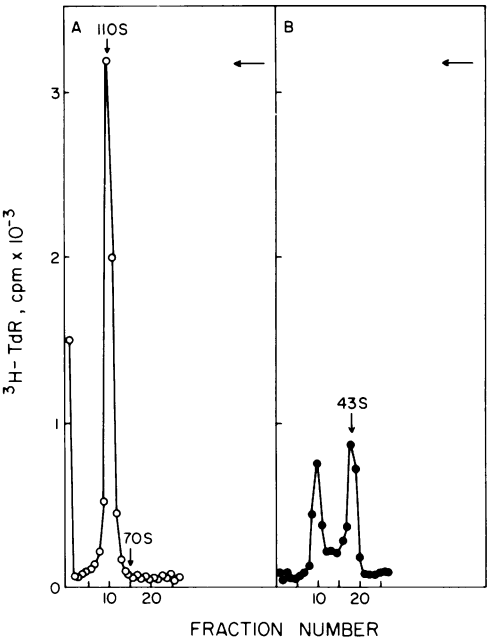

FIG. 3. Sedimentation rate analysis of chlorine-treated, [^{3}H]TdR-labeled whole virus in a 5 to 30% neutral sucrose gradient. (A) Control. (B) NaOCl (5 mg liter^{-1}), 60-min exposure.

The fractions corresponding to peak radioactivity from the sedimentation rate analyses were assayed for infective particles. A 3-log decrease in virus titer was observed when the virus was treated with 1 mg of NaOCl liter^{-1} for 60 min.

TABLE 1. Adsorptive ability of chlorine-treated parvovirus H-1

Sample treatment[a]	Input cpm	Adsorbed cpm	% Input	% Control
Full virus	70,700[b]			
PBS wash				
Control		21,360	30.0	
NaOCl (5 mg liter^{-1}, 60-min exposure)		20,349	29.1	95.2
Blank filter		68		
PBS + EDTA wash				
Control		18,308	25.7	
NaOCl (5 mg liter^{-1}, 60-min exposure)		8,594	12.1	46.9
Blank filter		164		

[a] Samples were prepared as described in the text.
[b] [^{3}H]Met-labeled virus.

TABLE 2. Infectivity assay of peak fractions from sucrose gradient analysis of control and chlorine-treated parvovirus H-1

Sample	Gradient fraction	PFU ml^{-1}
Control	8[a]	1.8×10^8
NaOCl (1 mg $liter^{-1}$, 60-min exposure)	9	8.0×10^4
	18	0
Control	7[b]	1.6×10^7
	8	4.4×10^6
	9	2.8×10^6
NaOCl (5 mg $liter^{-1}$, 60-min exposure)	7	0
	8	0
	9	0
	18	0
	19	0
	20	0

[a] Fractions from experiment shown in Fig. 1A and B.

[b] Fractions from experiment shown in Fig. 2A and B.

There were no infective particles associated with the fraction corresponding to the peak of radioactivity at the 43S position (Table 2). The infective virus titer of the individual fractions 7, 8, and 9 of the control shown in Fig. 2 ranged from 10^6 to 10^7 PFU ml^{-1}. There was no infectivity in the corresponding fractions of the virus that had been treated with 5 mg of NaOCl $liter^{-1}$ for 60 min. Also, there were no infective particles associated with the 43S peak, even though both the capsid protein and the nucleic acid were present in this peak.

Effect of chlorine on parvovirus H-1 as observed by electron microscopy. To determine what morphological changes occurred during the 60-min exposure to an NaOCl concentration of 5 mg $liter^{-1}$, we examined the virus at various time intervals by electron microscopy. Figure 4 shows electron micrographs taken of the control (A), the virus exposed for 10 min (B), and the virus exposed for 60 min (C). The micrograph of the virus exposed for 10 min (Fig. 4B) revealed that the protein capsid had been altered such that the uranyl acetate stain entered the particle and made it appear dark. Conspicuous, however, were the taillike extrusions from the ruptured particles (indicated by the arrow). Although there was some aggregation after 10 min of exposure, after 60 min the aggregation was so extensive that the taillike extrusions were no longer discernable.

Effect of chlorine on capsid proteins as analyzed by electrophoresis. [^{3}H]Met-labeled parvovirus H-1 was treated with various doses of NaOCl ranging from 1 to 5 mg $liter^{-1}$ for exposure times of less than 5 min. These initial experiments indicated that as the chlorine dose increased or the exposure time increased, several new, higher-molecular-weight polypeptides were being

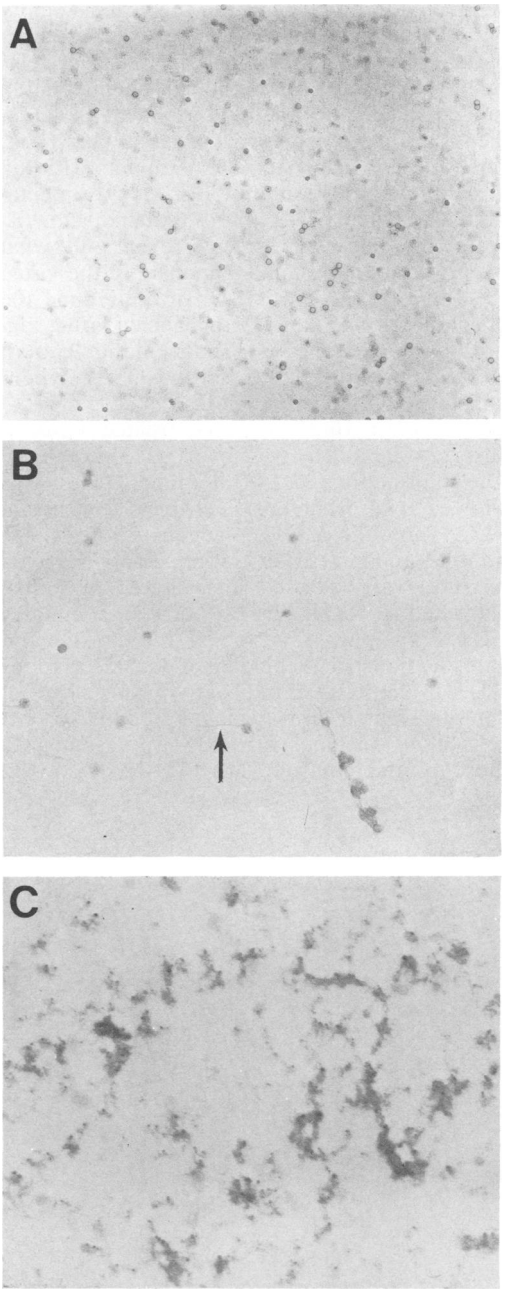

FIG. 4. Electron micrographs of negatively stained untreated parvovirus H-1 (A) and virus treated with NaOCl (5 mg $liter^{-1}$) for 10 min (B) or 60 min (C). The virus particles in (A) are 18 to 22 nm in diameter. Of particular interest are the taillike structures (arrow) extruded from the virus particles (B).

formed. Enhancement of this effect was achieved by 5 mg of NaOCl liter^{-1} at exposure times of up to 60 min (Fig. 5A). Lane 1 was the control, and the remaining lanes represented increasing exposure times and showed how chlorine treatment enabled the viral proteins to aggregate in a gradual, but definite and consistent, pattern. The transformation of the capsid proteins appears to have gone through an intermediate stage composed of two or three major aggregate sizes. A lower-percentage polyacrylamide gel revealed the formation of 12 to 15 different higher-molecular-weight aggregates (Fig. 5B). After 30 min of exposure time, both the original capsid proteins and the intermediate aggregates had simultaneously disappeared. Because the sample was boiled in the presence of a strong detergent (sodium dodecyl sulfate), only covalently cross-linked polypeptides remained intact.

Pairs of replicate samples were exposed to 5 mg of NaOCl liter^{-1} for 10, 30, and 60 min to determine whether there was any protein-DNA cross-linking caused by chlorination. One sample of the pair was radioactively labeled with [^{3}H]Met, and the other was labeled with [^{3}H]TdR. The protocol described previously for precipitating the virus was used for both samples. After electrophoresis, staining with Coomassie blue showed similar protein patterns for both samples in the pair. A fluorograph of the gel (Fig. 6), however, revealed that the DNA was not associated with the protein and had remained in the stacking gel.

Effect of chlorine on intactness and biological activity of nucleic acid. NaOCl treatment (5 mg liter^{-1}) of H-1 virions containing a full complement of DNA caused a complete loss of infectivity. To determine if the loss of infectivity was associated with damage to the nucleic acid, the intactness of the DNA was investigated. This was initially determined by digesting any associated protein in the sample under highly alkaline conditions. This alkaline state was maintained on 5 to 30% sucrose gradients during analysis of the intactness of the DNA. An example (Fig. 7) of these fractionated gradients illustrates that, up to a maximum of 60 min of exposure time, the DNA of chlorinated virions sedimented to the same position in the gradient as the control DNA

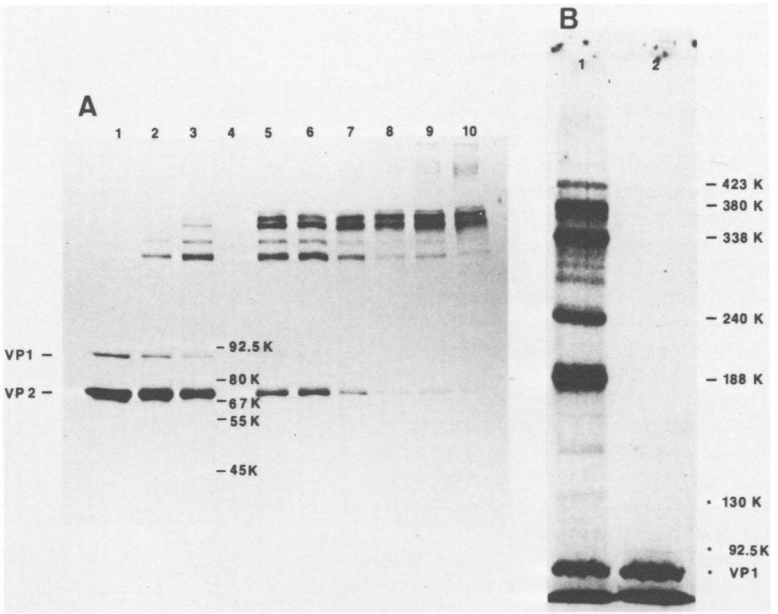

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of the capsid proteins extracted from whole parvovirus H-1, both untreated and chlorine treated. All treated samples were exposed to NaOCl (5 mg liter $^{-1}$) for various exposure times. (A) Gel (7.5%). Lane 1, Control. Lane 2, 3 min. Lane 3, 5 min. Lane 4, Standard markers, relative positions, and the individual molecular weight values are indicated. Molecular weights were determined by the method of Shapiro et al. (17). Marker proteins included phosphorylase A (92,500 [92.5 K]) human transferrin (80 K), bovine serum albumin (67 K), catalase (55 K), and ovalbumin (45 K). Lane 5, 10 min. Lane 6, 10 min; sodium sulfite was used to quench the reaction rather than sodium thiosulfate. Lane 7, 15 min. Lane 8, 30 min. Lane 9, 30 min; sodium sulfite. Lane 10, 60 min. VP, Viral protein. (B) Gel (5.0%). Lane 1, 30-min exposure. Lane 2, Control; an additional marker protein was β-galactosidase (130 K). Asterisks indicate the positions of marker proteins.

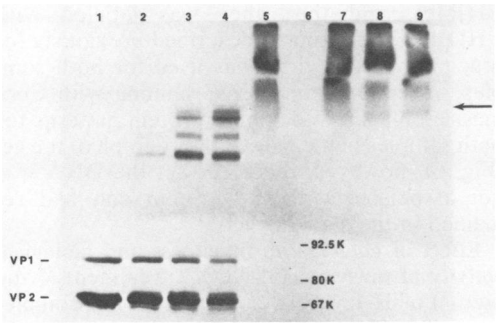

FIG. 6. Sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis and fluorography of a dual experiment in which whole H-1 virions radioactively labeled in either the protein or the nucleic acid were exposed to NaOCl (5 mg liter^{-1}) for various times. The viral protein was extracted from all samples and electrophoresed. Staining by Coomassie blue produced identical protein patterns in lanes 1 and 5, 2 and 7, 3 and 8, and 4 and 9. The fluorograph presented above shows the protein ([^{3}H]Met) pattern in lanes 1 to 4. Lanes 5 and 7 to 9 indicate that the viral DNA ([^{3}H]TdR) was not associated with the high-molecular-weight aggregates produced by chlorination. Lane 1, Control, [^{3}H]Met; lane 2, 10-min exposure; lane 3, 30 min; lane 4, 60 min; lane 5, control, [^{3}H]TdR; lane 6, standard markers, relative positions, and molecular weight values indicated correspond to those in Fig. 5; lane 7, 10 min; lane 8, 30 min; and lane 9, 60 min. The arrow indicates the interface of the stacking and resolving gels. VP, Viral protein.

(fraction 17). Also, none of the gradients had any extra peaks which would have been indicative of fragmentation. Although this gradient analysis showed the intactness of the nucleic acid, it does not demonstrate whether any subtle changes may have occurred in the DNA. For example, a chlorine-induced lesion may have been slight but still have been capable of preventing replication of the nucleic acid.

A reliable in vivo method for determining the biological activity or infectivity of parvovirus DNA is not yet available. Therefore, an experiment to test the in vitro replicability of the DNA from chlorine-treated virus was implemented. Again, whole virions were treated with 5 mg of NaOCl liter^{-1} for various exposure times. After extraction of the viral DNA as described above, the DNA in each sample was replicated in vitro with DNA polymerase (Klenow fragment) in a reaction mixture containing a ^{32}P-labeled deoxynucleotide. The ability of the polymerase to copy the single-stranded DNA partially or fully was determined by analysis of the replication products on an agarose gel (Fig. 8). The viral DNA extracted from treated virus could be fully replicated in vitro to a double-stranded form up to and including 60 min of exposure to chlorine. This suggests that the viral DNA was not affected by the chlorine treatment. To ensure that the DNA produced by in vitro replication was indeed double stranded and sensitive to restriction enzymes requiring double-stranded DNA templates, the samples of replicated DNA were cleaved with either *Eco*RI, *Pst*I or *Bcl*I. The expected fragment pattern was observed for H-1 viral DNA cleaved with *Eco*RI (Fig. 9) (15) and for *Pst*I and *Bcl*I cleavage (data not shown).

DISCUSSION

In a previous study of inactivation kinetics (in press), we indicated that there may be more than one inactivation site on parvovirus H-1. Graphic analysis of the inactivation data with the classic Arrhenius equation revealed that there was more than one reaction taking place during inactivation, especially at a free chlorine dose of 0.05 mg liter^{-1}. To determine the location of these sites on the virus, the whole virion containing a full complement of DNA was subjected to chlorination. After an initial analysis of the treated virus as a whole, the component parts of the virion, consisting of the capsid proteins and the nucleic acid, were analyzed separately.

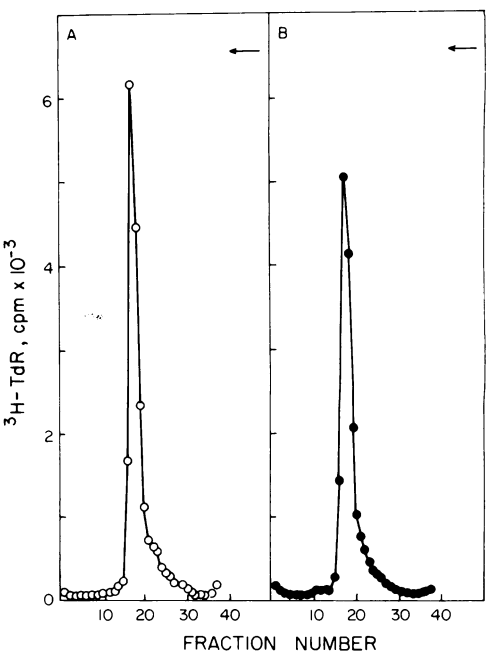

FIG. 7. Sedimentation rate analysis of [^{3}H]TdR-labeled DNA extracted from chlorine-treated whole virus and analyzed in a 5 to 30% alkaline sucrose gradient. (A) Control. (B) NaOCl (5 mg liter^{-1}), 60-min exposure.

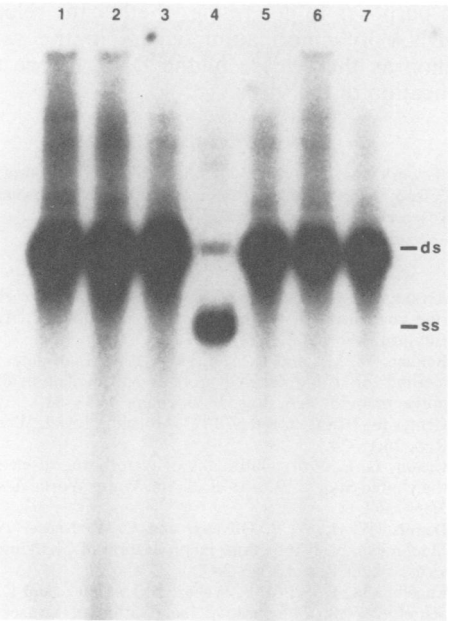

FIG. 8. Agarose (1.4%) gel electrophoresis and autoradiograph of in vitro-replicated DNA which had been extracted from whole parvovirus H-1 exposed for various times to NaOCl (5 mg liter^{-1}). Lane 1, Control without sodium thiosulfate; lane 2, control; lane 3, 1-min exposure; lane 4, bovine parvovirus single-stranded (ss) and double-stranded (ds) DNA markers; lane 5, 10 min; lane 6, 30 min; and lane 7, 60 min.

The effect of chlorine on the whole virion was first revealed in the sedimentation rate analysis. When [^{3}H]Met-labeled virus was exposed to 1.0 mg of NaOCl liter^{-1}, a small amount of virion-associated radioactivity was detected at a position different from that of normal viral species. An increase in the chlorine dosage to 5 mg of NaOCl liter^{-1} enhanced the development of this new peak with a simultaneous loss of radioactivity from the whole virion position. By using the calculation methodology of Griffith (8), we calculated the sedimentation rate coefficient of the [^{3}H]Met-labeled protein associated with the new peak to be 43S. Furthermore, by utilizing whole virions that were radioactively labeled in the nucleic acid instead of the protein, it was demonstrated that the 43S peak also contained DNA. Interestingly, there was no infectivity associated with this peak (Table 1) even though both viral components were present in a nucleoprotein complex.

Further study into the effect of chlorine on the viral protein revealed that the two capsid polypeptides were not cleaved into small peptides but were covalently cross-linked into higher-molecular-weight aggregates. Presumably, the formation of the 43S nucleoprotein complex was due in part to the stepwise aggregation of the viral proteins. This apparently occurred through a series of intermediate reactions leading to dimers and trimers of the individual proteins, as well as various combinations of these with each other. Similar aggregates were observed when H-1 capsid proteins from empty particles were treated with chemical cross-linking agents (14). We found the empty capsids to be less reactive with chlorine than the full virus (data not shown).

The reactive sites were probably located on exposed R-groups of certain amino acids constituting the protein. This implies that the architecture of the entire capsid and the spatial arrangement of the two major polypeptides are important because they direct which capsid protein and which R-group is most exposed to the chlorine.

Observation of the chlorine-treated virus by electron microscopy revealed that visible mor-

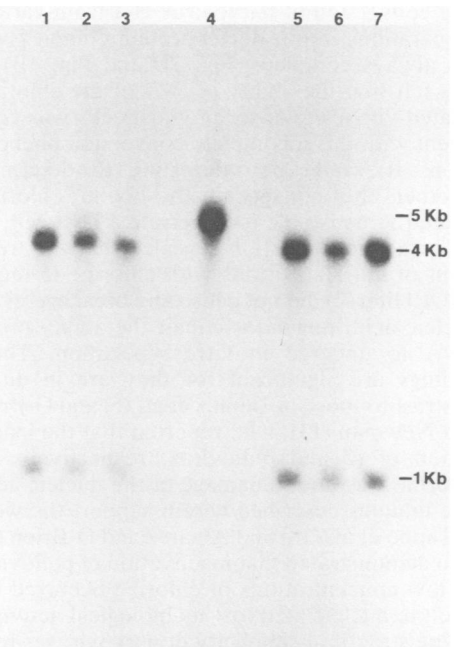

FIG. 9. Autoradiogram of *Eco*RI restriction endonuclease analysis of in vitro-replicated DNA. Whole virus was treated with NaOCl (5 mg liter^{-1}) for various exposure times and the single-stranded DNA was isolated. After in vitro replication of the DNA, aliquots of these samples were treated with *Eco*RI and analyzed by electrophoresis through a 1.4% agarose gel. Lane 1, Control without sodium thiosulfate; lane 2, control; lane 3, 1 min-exposure; lane 4, replicated control (double-stranded DNA) which was not treated with *Eco*RI; lane 5, 10 min; lane 6, 30 min; and lane 7, 60 min. Kb, Kilobase pairs.

phological changes had taken place. After 10 min of exposure to an NaOCl solution of 5 mg liter^{-1}, the viral capsid had apparently ruptured, thereby releasing the DNA as a taillike extrusion which remained attached to the capsid. After 60 min of exposure, there was a massive aggregation of these nucleoprotein complexes, presumably due to the continued reactivity of the viral proteins.

If inactivation of parvovirus H-1 is caused by an alteration of the protein capsid, then any steps in the infection process that require the protein capsid to be in its original state would be blocked. Initial experiments indicate that there was no difference in the adsorptive capabilities of chlorine-treated and control virus when the infected cells were washed with PBS. However, to eliminate any nonspecific binding of the virus to the host cell, a PBS solution containing 0.001 M EDTA (11) was used on replicate samples. The interaction of chlorine-treated viruses with host cells was then found to be considerably different through the chelation of metallic ions which may have aided in nonspecific binding. The conversion of parvovirus H-1 from various preparations into a nucleoprotein complex was not always complete (Fig. 1D and Fig. 3B). It was felt that the ability of 50% of the chlorine-treated viruses to adsorb to host cells was coincident with this incomplete conversion phenomenon. It would be interesting to determine whether the nonspecifically bound chlorine-treated virus is able to penetrate a host cell.

Further investigation revealed that the treatment of full H-1 virions with chlorine (5 mg of NaOCl liter^{-1}) did not cause any breakage in the nucleic acid, nor did it impair the ability of the DNA to undergo in vitro replication. These findings are significant for they are in direct contrast to those of Dennis et al. (5) and O'Brien and Newman (13), who reported that the inactivation of f2 and poliovirus, respectively, by chlorine was due to damage of the nucleic acid. The findings described herein support the work of Tenno et al. (18) and Alvarez and O'Brien (1), who demonstrated that inactivation of poliovirus by low concentrations of chlorine occurred before the nucleic acid lost its biological activity.

The structural simplicity of parvoviruses relative to enteroviruses aided in demonstrating that inactivation mechanisms vary with the virus under study, and the loss of a polypeptide is not required for the loss of adsorptive capabilities. In other words, an alteration of the integrity of the virion capsid by rearranging, not degrading, the major polypeptides may be all that is required for inactivation. Although the details of the infection process for parvoviruses are uncertain at this time, it may prove useful for disinfection purposes to determine whether the release of DNA by chlorination occurs in the same fashion as the release of the DNA before the replication of the virus.

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