

A STUDY OF THE KINETICS AND MECHANISM OF INACTIVATION OF A
DNA-CONTAINING ENTERIC VIRUS BY CHLORINE,

by

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DEDICATION

In loving memory, to my father,
Cecil Calvert Churn, Jr.

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I. INTRODUCTION

In 1974 the World Health Organization (WHO) Conference in Mexico City established guidelines for the criteria of a virologically safe drinking water. Based on the well-accepted premise that the minimum infective dose required for the possible expression of a viral disease was one infective particle, their recommendation was that a finished drinking water should not contain greater than one infective viral particle per 100 gallons or 378.5 liters (Melnick, 1976). Since this conference, the concern over possible waterborne transmission of enteric viruses has waned to a point where it is presently overshadowed by a strong emphasis on the priority pollutants. Perhaps two major reasons can be offered as an explanation for this current attitude. First, the recommendation by the WHO seemed impractical, especially considering the detection methodology available at that time. Secondly, the limited research during the late-1960's through the mid-1970's demonstrated, a priori, that the proper operation and maintenance of water treatment processes, including disinfection, provided effective removal or inactivation of enteric viruses.

However, the effectiveness of treatment processes at reducing or eliminating a substance that is present in very minute quantities, whether it is a priority pollutant or an enteric virus, can be determined with an efficiency based solely on the merits of the available detection methodology. Although far from being optimal, concentration, detection, and enumeration methodologies for enteric

viruses have consistently improved over the past few years. Coupled with the discovery and detection of some new enteroviral pathogens, researchers are now beginning to give credence to the somewhat tenuous epidemiological evidence that enteric viruses can, and do, find their way into finished drinking water supplies.

The first U.S. study to detect enteric viruses in a finished drinking water that had been determined to be bacteriologically safe was reported by Hoehn and his coworkers (1977). Initially refuted by some as merely being sample contamination, further study revealed the viruses to be natural isolants. Later, two of the isolants, identified as polioviruses, were subcultured and subjected to inactivation trials (Schaffer et al., 1980). These natural isolants were found to be more resistant to inactivation by chlorine than laboratory strains. These somber facts dispute the argument that potable water supplies are, and will continue to be, virologically safe, especially when the argument is based on laboratory conditions utilizing laboratory strains of enteric viruses.

In a recent review paper (Melnick and Gerba, 1980) it was reported that viruses are usually detected in about one-third of the freshwater samples tested. The enteric virus concentrations in these samples have ranged from 0.1 to 280 infectious viral particles per liter. It is plausible that this amount of virus could be entering a water treatment facility. In a 1977 study at a Canadian water treatment plant, Metcalf et al. (1979) discovered polioviruses in the finished water. Melnick

and Gerba (1980) reported that researchers have also isolated viruses from the drinking water supplies of both Paris and Moscow. To the environmental virologist, these discoveries of viruses in freshwater samples and in the finished water, indicate the inadequacies of water treatment. Furthermore, it is indicative of how little is actually known about the fate of the more than 100 serotypes of enteric viruses that could possibly be entering a water treatment facility.

Currently, a newly discovered enteric virus has been definitely associated with large outbreaks of waterborne gastroenteritis (CDC, 1982; Paver and Clarke, 1976; Schreiber et al., 1974). Most commonly referred to as the Norwalk agent, this virus appears to be morphologically and biophysically similar to the parvoviruses. Presently, there is very little known about the fate of parvoviruses in environmental systems. Englebrecht and his coworkers (1978; 1980) subjected the parvovirus, Kilham rat virus (KRV), to chlorine and found that it was as resistant as representative picornaviruses. However, this needs further study because KRV hemagglutination titers were being compared to plaque assay titers of the picornaviruses. In this study the parvovirus H-1, a putative human virus, was used as a model virus for chlorine inactivation experiments. Morphologically, the H-1 parvovirus has two equally infectious particle types which are distinguishable by their bouyant density: heavy-full (banding at 1.46 to 1.48 g cm⁻³ in cesium chloride) and light full (banding at 1.39 to 1.42 g cm⁻³). This characteristic is primarily due to differences in the configuration and

distribution of polypeptides in the viral protein coat (Tattersall, 1978). Both particle types can be enumerated by plaque assay.

The research described herein was undertaken with two primary objectives:

- 1) To study the inactivation kinetics of parvovirus H-1 exposed to low doses of free chlorine at pH 7.0, and at 5, 10, 20, and 30°C; and,
- 2) To determine the mechanism by which free chlorine inactivates parvovirus H-1 at pH 7.0.

II. LITERATURE REVIEW

Of the several viruses implicated in acute enteritis, only rotaviruses and the Norwalk agent have been determined to be the causative agents of epidemic gastroenteritis as defined by Koch's postulates. The Norwalk agent is very similar to the parvoviruses in both morphology and biophysical characteristics, e.g. buoyant density in cesium chloride. There have been a number of other parvovirus-like viruses associated with epidemic gastroenteritis worldwide, all exhibiting various antigenic properties as shown by cross challenge studies and immune electron microscopy.

In 1972, the causative agent of a 1967 waterborne epidemic of acute gastroenteritis in a primary school in Norwalk, Ohio, was identified as a virus-like particle (Kapikian et al., 1972), and appropriately referred to as the Norwalk agent. Since then, similar virus-like particles, 25 to 27 nm in diameter, have been isolated from the stools of patients associated with other outbreaks of gastroenteritis. These particles include the Montgomery County agent, the Hawaii agent (Schreiber et al., 1974), the Ditchling and "W" agents, the Cockle agent, and the Parramatta agent (Banatvala, 1981). The development of radioimmunoassay tests for diagnosis aided in detecting the agent responsible in later outbreaks. There have been cases associated with swimming water (CDC, 1979) as well as drinking water (CDC, 1978), and most recently with a contaminated drinking water in Georgia (CDC, 1982). After a 24 to 36 hour incubation period, symptoms include nausea,

vomiting, vertigo, diarrhea, and abdominal pain, all of which may last 24 to 72 hours (Banatvala, 1982). Sera studies of all age groups show that antibody titers increase with age but only approximately 50 percent of adults have immunity (Banatvala, 1982).

Rotaviruses, also implicated in acute enteritis, are 65 to 70 nm in diameter, with a double capsid surrounding double-stranded RNA. The host range has been found to be quite extensive among neonates of several mammalian species, including man, mice, pigs, calves, apes, lambs, horses, and rabbits. The inner capsid appears to have group-specific antigens common to all the rotaviruses. The outer capsid has species-specific antigens associated with it (Banatvala, 1982). The symptoms are similar to those caused by the parvovirus-like particles, and occur most frequently in children 6 to 24 months old. Symptoms occur after a one to three day incubation period and persist for about a week. Examination of patient's feces show a maximum of 10^{10} virus particles per gram. There is evidence for at least four human serotypes of rotaviruses (Banatvala, 1982).

Although the evidence is somewhat speculative, several other viruses have been implicated in non-bacterial gastroenteritis. Astroviruses were found in the feces of babies with acute enteritis (Kurtz et al., 1977). Caliciviruses were detected in the stools of school children after an outbreak of winter vomiting disease (McSwiggan et al., 1976). Another group, the coronavirus, is known to cause diarrhea in pigs and calves and has been detected in human patients with

severe diarrhea (Caul et al., 1975). Adenoviruses have also been linked to outbreaks of acute gastroenteritis in hospitals (Richmond et al., 1979). Representatives from all subgroups of enteroviruses have at some time or another been associated with gastroenteritis cases. The evidence is very tenuous for most of them but strongly implicates some echoviruses, the polioviruses, some reoviruses, and some from both subgroups of coxsackieviruses.

The need to study the inactivation kinetics of enteric viruses exposed to a disinfectant in a water treatment system was first realized in the late 1940's to early 1950's. Because chlorine is the popular disinfectant in treatment systems, the first section in the following review of the literature will present inactivation studies utilizing chlorine in a finished water or buffered-water system.

Secondly, this literature review will examine the information available on how various disinfectants inactivate enteric viruses. The research into the mechanism of inactivation of viruses, regardless of the inactivating agent, reveals many similarities in experimental procedures. Therefore, this section is initially divided into subsections presenting results obtained by the investigators utilizing a particular procedure. The final subsection presents the conclusions that were made by the various investigators based on their results.

Kinetics of Viral Inactivation by Chlorine

It is generally accepted that current treatment processes, including disinfection, for both wastewater and water neither remove nor inactivate all enteric viruses that may be present. Many individuals may argue, however, that the reduction of viruses by current treatment procedures has minimized the possibility of the waterborne transmission of enteroviral diseases to an acceptable level of risk. This attitude is somewhat misleading for there is still much to be learned about the transmission and the inactivation of enteric viruses, especially those that cause gastroenteritis. Furthermore, the potential for concentrating enteric viruses to levels never before encountered in environmental systems, comes with the impending need for water recycling and reuse. Also, the creation of toxic by-products from the use of chlorine has warranted investigations into the effectiveness of alternative disinfectants.

This section of the literature review will deal primarily with the inactivation kinetics of enteric viruses by chlorine. Inasmuch as the control of enteric viruses is most important in finished drinking waters, studies utilizing those conditions associated with effluents from water treatment facilities will be presented. These studies, however, will make evident the complexity of the disinfection process, especially when complicated by the variety of chlorine compounds created, each exhibiting a different degree of viral inactivation capability. The hydrolysis of chlorine produces hypochlorous acid

(HOCl) which is currently thought to be the most potent virucidal form. Other forms of varying virucidal activity are also present in a finished water. The relative amounts of these forms--hypochlorite ion (OCl^-), ammonia chloramines, and organic chloramines--vary with pH, water composition and quality, and temperature. To reduce the complexity of disinfection, researchers have attempted to study inactivation in a controlled, laboratory situation. Usually this involves controlling the chlorine species by buffering the water and controlling the temperature, and chlorine dosage. However, no standard procedure has been adopted thus far, making the comparison of results among the various studies somewhat difficult. Nevertheless, it will be possible to illustrate whether or not the current EPA recommendation of 0.2 mg L^{-1} free chlorine throughout water distribution systems (EPA, 1976) is justified when considering the virological quality of the water. Also, one can become informed of the relative resistance of various viruses studied to inactivation by chlorine.

Early Studies

One of the earliest studies involved the testing of a grossly contaminated water supply that had been implicated in the outbreak of infectious hepatitis. Neefe and his coworkers (1947) had human volunteers drink samples of this water, some of which had been subjected to various water-processing treatments along with different dosages of chlorine. They found that 83 percent of the controls contracted

infectious hepatitis from water which had neither been treated nor disinfected. Treatment of the water--consisting of coagulation, settling, and diatomaceous earth filtration--reduced the incidence of disease by one-half. Treatment of this treated water with enough chlorine to produce a 1.1 and 0.4 mg L⁻¹ residual of combined and free chlorine, respectively, after 30 minutes of contact time, apparently inactivated all the hepatitis virus. These same residuals, however, in water which had not undergone treatment did not inactivate all the virus, because 40 percent of the volunteers contracted with hepatitis. Little information on the minimum amount of chlorine and required contact time was achieved by this study, although it was a beginning. Also, the use of human volunteers was obviously not a simple and economical way to assay for virus.

Another early study involved treatment of coxsackievirus A2 by chlorine. Clarke and Kabler (1954) designed experiments to determine the time required to reduce 500 LD₅₀ of virus to 2 LD₅₀ (99.6 percent reduction). The LD₅₀ was determined by infecting virus suspensions into suckling mice. The results obtained illustrated the dependence of inactivation on pH, temperature, time of exposure, and the dosage of chlorine. For instance, analysis of the data by the van't Hoff equation showed that as the pH decreased and the temperature increased, conditions became more favorable for lower concentrations of chlorine to effect proportionally more inactivation than higher chlorine concentrations. At pH 9 and 3 to 6°C, twelve times the chlorine and

twice the time were required to effect the same inactivation as at pH 6. Clarke and Kabler (1954) also reported that it appeared to take seven to forty-six times as much chlorine to inactivate coxsackievirus A2 as compared to E. coli. Interestingly, the higher the pH, the less the difference in the chlorine residuals needed for comparable percentages (i.e., 99.9 percent) of inactivation for E. coli and coxsackievirus A2.

Clarke and his coworkers (1956 and 1954) did inactivation experiments utilizing adenovirus 3. At pH 7, 25°C, and 0.5 mg L⁻¹ it took free chlorine less than eight seconds to achieve better than a 99.8 percent reduction. Adenovirus was inactivated at a rate similar to E. coli and at approximately twenty-five times the rate for coxsackievirus A2.

Although not the first to inactivate poliovirus with chlorine, Weidenkopf (1958) was the first to attempt to rid experiments of the high amount of chlorine-demanding organic material in the virus preparation, and he enumerated the virus by plaque assay rather than LD₅₀ or TCID₅₀ end points. For these reasons his results are often quoted as being the beginning of kinetic studies. Experiments were performed utilizing poliovirus type 1 suspended in 0.05 M phosphate buffered saline (0.11 M NaCl). The usual inactivation curves at pH 6 and 7 were biphasic, but at pH 8.5 the curves were linear throughout the time course of the experiment. Analysis by the van't Hoff equation produced coefficients of dilution, n, of less than one for all pH

levels. The constant, k , obtained from this same equation was smaller at pH 6 than at 9, thereby indicating a more potent chlorine species at pH 6. Reaction rates were found to increase with either decreasing pH or increasing chlorine residuals. At pH 7, 0°C, 0.12 and 0.23 mg L⁻¹ of available chlorine, the times required for 99.9 percent reduction in virus titer were 10.5 and 18.5 minutes, respectively.

A more extensive study of enteroviral inactivation by chlorine utilized five strains of poliovirus, including two wild-type strains isolated from wastewater, and coxsackieviruses B1 and B5. Kelly and Sanderson (1958) inactivated poliovirus type 1 (strains Mahoney, MK500, and 487), poliovirus type 2 (MEF₁), and poliovirus type 3 (Saukett) to observe any differences between types of poliovirus as well as strains within type 1. Interestingly, they did find that poliovirus type 3 was more resistant to the same amount of free chlorine than poliovirus type 1 at 1 to 5°C but not at 25 to 28°C. Moreover, it seemed that resistance to inactivation may also have been a function of strain, as the wild-type poliovirus type 1 (MK500) remained viable from two to four times longer than the laboratory strain (Mahoney) of poliovirus type 1. They concluded that exposure to a free residual chlorine concentration of 0.3 mg L⁻¹ for 30 minutes of contact time would inactivate enteric viruses in water at 25°C and pH 7. Also, at pH 8 the contact time would have to be doubled, and with a 20°C decrease in temperature, the rate of inactivation would slow by a factor from two to fifteen.

Kelly and Sanderson (1960) also studied the inactivation of these same viruses by combined chlorine. They reported that, at pH 7 and 25°C, 9 mg L⁻¹ of combined chlorine was required to inactivate greater than 99.7 percent of the most resistant virus, poliovirus type 1 (MK500). Six mg L⁻¹ caused the same amount of inactivation in one hour, but 0.5 mg L⁻¹ required more than seven hours of exposure time. The combined chlorine in this study was produced by passing a stream of chlorine gas through a solution of ammonium chloride. Unfortunately, Kelly and Sanderson (1960) did not determine if the ammonium ion produced caused any inactivation of the viruses. They reported that raising the pH decreased the rate of inactivation by combined chlorine; however, they didn't mention that the higher pH also converted ammonium ion into ammonia, which was later demonstrated to be virucidal for poliovirus type 1 (CHAT) by Ward (1978).

The major problem of research into the inactivation of viruses is the lack of an established standard procedure which makes a comparison of data from different researchers very difficult. Liu et al. (1971) have performed the most comprehensive study utilizing the same conditions for determining the relative resistance of twenty enteric viruses to free chlorine. A large volume of river water taken from one mile downstream of the sewage treatment plant was coagulated and filtered through a sand filter. The river water had a small chlorine demand that was satisfied by maintaining the desired residual of 0.5 mg L⁻¹ throughout the experiment. The pH was adjusted to 7.8 and the water

temperature maintained at 2°C. The time required to inactivate 99.99 percent of the initial virus titer, based on experimental evidence and also on graphical extrapolation of the inactivation data, is presented in the following table for simplicity of comparison (Table 1). The data in the table show the reovirus group to be the least resistant to chlorine, while both polioviruses and coxsackieviruses were the most resistant of the twenty viruses tested. The few differences in the observed and calculated times for a four log decrease in virus titer was explained as being either protective aggregation of the virus particles or the presence of a significant amount of virus particles that are genetically more resistant.

Buffered Water Systems

The complexity of the reactions between chlorine and various constituents in the water used in the earlier inactivation studies prompted investigators to more closely control such variables. The employment of a chlorine-demand-free buffered water in later studies became a necessity for minimizing those variables as well as, differences between testing procedures. Previous studies showed hypochlorous acid, HOCl , to be a more effective virucide than hypochlorite ion, OCl^- , which is the chlorine species favored by decreases in hydrogen ion concentration. One of the first and more significant studies utilizing a buffered water system found, however, that high pH values actually enhanced viral inactivation. The research,

Table 1. Relative Resistance of Twenty Human Enteric Viruses to 0.5 mg L⁻¹ Free Chlorine in Potomac River Water (pH 7.8 and 2°C)^a.

Comparison Based on Experimental Data		Comparison Based on First Order Reaction	
Virus	Min ^b	Virus	Min ^b
Reo 1	2.7	Reo 1	2.7
Reo 3	4.0	Reo 3	4.0
Reo 2	4.2	Reo 2	4.2
Adeno 3	4.3	Adeno 3	4.8
Cox A9	6.8	Cox A9	6.8
Echo 7	7.1	Echo 7	7.1
Cox B1	8.5	Cox B1	8.5
Echo 9	12.4	Echo 9	12.4
Adeno 7a	12.5	Adeno 7a	12.5
Echo 11	13.4	Echo 11	13.4
Polio 1	16.2	Adeno 12	13.5
Echo 29	20.0	Echo 12	14.5
Adeno 12	23.5	Polio 1	16.2
Echo 1	26.1	Cox B3	16.2
Polio 3	30.0	Polio 3	16.7
Cox B3	35.0	Echo 29	20.0
Cox B5	39.5	Echo 1	26.1
Polio 2	40.0	Cox A5	33.5
Cox A5	53.5	Cox B5	39.5
Echo 12	60.0	Polio 2	40.0

^aAdapted from Liu *et al.* (1971).

^bMinutes required to inactivate 99.99 percent of the virus.

performed by Scarpino et al. (1972), showed that OCl^- , predominating at pH 10, inactivated poliovirus type 1 (Mahoney) at a rate 7 times faster than HOCl (pH 6) at a temperature of 5°C . E. coli exhibited the reverse effect, being inactivated faster at pH 6 than pH 10. Compared to the poliovirus, E. coli was inactivated 130 times faster by HOCl , but three-fold slower by OCl^- . This apparent contradiction to both earlier and later studies was found to be a function of the amount of salt, in this case, potassium chloride, in the higher pH buffer (Englebrecht et al., 1978, and 1980, Jensen et al., 1980; Sharp et al., 1980).

Sharp and coworkers (1980) concerned themselves with the effect of sodium chloride, NaCl , on the 20°C inactivation rates of poliovirus type 1 (Mahoney) by chlorine at pH 6, 7.8 and 9. They found that 0.1 M NaCl in 0.05 M phosphate buffer increased the reaction rates by a factor of two to four times, depending on the pH. At pH 10, the rate difference was enhanced by an order of 50 to 100 times faster. This enhancement, however, occurred with chlorine concentrations of up to 0.5 mg L^{-1} at pH 10, as the rate changed very little at 2.1 mg L^{-1} . Increasing the concentration of NaCl from 0.1 M to 0.2 M had little effect on the inactivation rates.

Jensen et al. (1980) continued to examine this salt effect by determining if the rate increase also occurred with coxsackievirus B3 and B5. They studied the effects of KCl and cesium chloride, CsCl , as well as NaCl , on the inactivation rates of these two viruses. Coxsackievirus B5 was not inactivated any faster at pH 6 and 20°C by the

1.0 mg L⁻¹ chlorine with or without 0.1 M NaCl. Increasing the pH to 10 slowed the inactivation rate, however, upon addition of 0.1 M NaCl the time required for 99 percent inactivation was reduced from 7.9 minutes to 17 seconds. The effect of KCl was greater than for NaCl. The cesium chloride was not as effective as either of the other two salts. Similar results were obtained with coxsackievirus B3 under the same conditions. The addition of 0.1 M NaCl to the buffer reduced the time for 99.99 percent inactivation from 15.5 minutes to 25.5 seconds. Jensen and coworkers also found that coxsackievirus B5 aggregated readily at pH 3 to 10 and thus explains why it has been found to be so resistant to chlorine (Liu et al., 1971). Furthermore, the significant increase in the activation rate at pH 10 versus pH 6 found by Scarpino and coworkers (1972) can be attributed to the presence of KCl in the pH 10 buffer but not the pH 6 buffer. Jensen and his coworkers (1980) suggested that the monovalent cations, Na⁺, K⁺, and Cs⁺, form ion pairs with OCl⁻ (NaOCl, KOCl, and CsOCl). These ion pairs are seemingly just as effective virucides at alkaline pHs as HOCl is at acidic pHs.

Thus far, apparently only one small study has been conducted on the effect of water hardness, that is polyvalent cations, on the rate of inactivation. Jensen et al. (1980) claimed that the study by Kenyon and Schaub (1978) indicated that 250 mg L⁻¹ of calcium chloride, CaCl₂, added to the buffer system increased the rate of inactivation at alkaline pH. However, the authors actually stated that inactivation

rates for f2 and poliovirus type 1 in hard water were approximately 25 percent slower than in soft water (Kenyon and Schaub, 1978).

In a study on the effects of pH on chlorine, Kott et al. (1975) attempted to repeat the findings of Scarpino et al. (1972). This was truly a feeble attempt, for they used poliovirus type 2 which was shown by Liu et al. (1971) to be almost 2.5 times as resistant to chlorination as the poliovirus type 1 used in the study by Scarpino et al. (1972). Although Kott and coworkers (1975) could not duplicate the finding that OCl^- was seven times more effective at pH 10 than HOCl at pH 6, they did show that the inactivation rates were nearly the same for both chlorine species. They also showed poliovirus type 2 to be more resistant than E. coli at pH 6 and 10. Furthermore, reanalyzing their graphical data, a line of best fit constructed by straight-line linear regression for HOCl (pH 6) will show that OCl^- could be as much as 1.5 times more effective at reducing the poliovirus titer by 99 percent (10 seconds versus 15 seconds of contact time).

Young and her coworkers (1977) felt that it was important to observe inactivation rates of a monodispersed virus in the first few seconds thereby avoiding the departures from linearity usually obtained due to aggregation. Utilizing a special sampling device, they determined that 1 mg L^{-1} free chlorine at pH 6 and 20°C caused a 98 percent reduction of echovirus type 1 in a 0.01 M phosphate buffer containing 0.1 M NaCl . Curiously, inactivation of the virus ceased for the next 14 seconds at which time rapid inactivation continued, causing

a 99.99 percent reduction in titer by 24 seconds of total contact time. There was no significant depletion of HOCl, no observation of aggregates by electron microscopy, and no resistant fraction of virus was isolated. Reference was made to a study by Fujioka and Ackermann (1975) who determined that poliovirus existed in four interchangeable conformational states, all retaining infectivity. Young et al. (1977) propose that echovirus may exist in at least two conformational states, one of which is temporarily more resistant to inactivation by HOCl. Further study by Young and Sharp (1979) into the inactivation of echovirus revealed that a decrease in pH caused partial reactivation of the inactivated virus. By acidifying samples of chlorine-treated echovirus to pH 4.5 they could induce aggregation which in turn apparently enhanced the efficiency of infection over that of a single, damaged virion through a mechanism of mutual aid between the damaged virions comprising the aggregate. The effect was dramatic, causing an 800 to 2500-fold increase in titer of chlorine-treated virus.

Floyd et al. (1979) reexamined the inactivation kinetics of poliovirus type 1 (Mahoney) at pH 6 (0.01 M phosphate buffer with 0.1 M NaCl) and at pH 10 (10^{-4} M NaOH). The presence of NaCl at the lower pH inhibited aggregation. At pH 6, concentrations of 0.07 to 2.8 mg L^{-1} NaOCl caused 99.99 percent inactivation in less than one minute but not until after an initial lag period. Reaction rates were obtained from the straight line portion of the inactivation curves and plotted against chlorine concentrations. These graphs were biphasic in nature with the

slope of the first sections decreasing rapidly in relation to decreases in temperature. The second portion of the curve did seem to show increases in reaction rates with increases in chlorine concentrations. However, the rate increase was so slight that the maximum reaction efficiency was at the lower chlorine concentrations. The reaction rates at pH 10 were approximately one-tenth those at pH 6. Floyd and his coworkers (1979) found that the monodispersed poliovirus in these experiments resulted in inactivation rates at pH 6 that were 6.4 times those reported by Scarpino et al. (1972). Similarly, the rates were three times faster than reported by Weidenkopf (1958). At pH 10, Scarpino et al. (1972) had rates that were 3.1 times as fast as Floyd et al. (1979) due to the presence of KCl.

Sharp and Leong (1980) did a study with another poliovirus strain for comparison to the results of Floyd et al. (1979). They used poliovirus type 1 (Brunhilde) under the same conditions and found it to be about twice as resistant as the Mahoney strain. Rates of inactivation were faster at pH 6 than at pH 10, until 0.1 M NaCl was added, increasing the rate at pH 10 by 31-fold. This salt enhancement made $OC1^-$ at pH 10 greater than three times as effective as HOCl at pH 6.

In a comparative study by Englebrecht and his coworkers (1978 and 1980) six enteric viruses were tested for their relative resistance to chlorine at pH 6 and 10, using a 0.05 M sodium phosphate buffer and a 0.05 M borate buffer, respectively. The borate buffer did not contain

KCl. Pairs of viruses from three groups were inactivated at 5°C. They included poliovirus 1 and 2, echovirus 1 and 5, and coxsackievirus A9 and B5. The time required for 99 percent inactivation was 5 to 192 times slower at pH 10 than at pH 6 depending on the virus. Interestingly, the relative resistance between the two poliovirus types as well as between the two echovirus types was reversed with the change in pH. For example, poliovirus 1 and echovirus 5 were more resistant than their counterpart in the pair at pH 6. At pH 10 however, the reverse was true thereby indicating that the structural integrity of the virus at various pH levels is important in its inactivation. This pH effect was better illustrated by a more rapid inactivation of poliovirus type 1 at pH 7.8 than at pH 6. Englebrecht et al (1980) also performed inactivation experiments of a few viruses at pH 7.8 and 5°C for comparative purposes with the data of Liu et al. (1971). The five to six fold increase of the virus inactivation rates for the buffer compared to those for Potomac River water suggests that the river water may somehow retard the virus-chlorine reaction.

Of particular interest in the study by Englebrecht et al. (1980) was the inactivation of Kilham rat virus, a parvovirus containing single-stranded DNA. At pH 6, 0.5 mg L⁻¹ free chlorine, and 5°C, it required less than 20 seconds to reduce the hemagglutination (HA) titer of the parvovirus by 99 percent. Similarly, Simian virus 40 (SV40), a papovavirus containing double-stranded DNA, was 99 percent inactivated in 10 seconds as demonstrated by plaque assay.

One of the causative agents of non-bacterial gastroenteritis is rotavirus. A recent study by Berman and Hoff (1982) using a simian rotavirus, SA-11, showed that there was a 99 percent reduction of the virus in 33 seconds by 0.5 mg L^{-1} free chlorine at pH 6. The same chlorine residual at pH 10 took 1.4 minutes to inactivate 99 percent of the virus.

An important aspect to consider is the possibility of creating a chlorine-resistant strain of enteric virus which will in turn be cause to reexamine the current practices of disinfection. Studies by Bates and his coworkers (1977 and 1978) revealed that repeated exposure of poliovirus type 1 (LSc) to sublethal dosages of chlorine at pH 7 or 9 resulted in either the selection of, or creation of, a more chlorine resistant virus population. For example, at pH 9 there was almost a two-fold increase in the time required for 99 percent inactivation after five cycles of repeated chlorination of surviving populations. After ten cycles the time required had increased by 3.5 times that of the original virus stock. Although the times for inactivation were faster at pH 7, the resistance effect incurred had the same magnitude as at pH 9. There was no effect at pH 5, perhaps indicating a difference in the mechanism of inactivation at various pH levels.

The implications of chlorine resistant viruses in a drinking water are obvious. In fact, wild-type polioviruses that were isolated from a drinking water supply (Hoehn *et al.*, 1977) were cultured and subjected to inactivation studies in comparison to laboratory strains of

polioviruses (Schaffer et al., 1980). Interestingly, the two natural isolants were much more resistant to chlorination at pH 7.2 and 22°C than either the Mahoney or LSc strains of poliovirus type 1. After 10 minutes of exposure to free chlorine residuals of 0.11 to 0.15 mg L⁻¹, the Mahoney and the LSc strains were reduced in titer by 99.99 and 99.999 percent, respectively. The natural isolants were reduced by only 98.65 and 97.75 percent after 30 minutes of exposure to the same chlorine residuals. This significant amount of wild-type virus remaining could be of great consequence if disinfection processes are to be designed and operated on data obtained by utilizing laboratory strains rather than wild-type strains of enteric viruses.

Mechanisms of Inactivation

With the development of viral vaccines came investigations into the mechanism by which viruses were inactivated. It was of utmost importance that the chemical or physical agent used to achieve inactivation create a product that was rendered non-pathogenic but retained its antigenicity. Whereas the mode of action of chemical agents was initially speculative, recent investigations, with newly developed techniques, have more clearly defined the mechanisms of viral inactivation.

Both the ubiquitous nature of viruses and the epidemiological implications of the waterborne transmission of an increasing number of viruses have turned the attention of several investigators to a more

applied and environmental approach to the question of how viruses are inactivated. The focus of their research has been on the determination of the fate of viruses in environmental systems such as wastewater and water treatment facilities. The disinfection process has been studied the most for it is the last process providing safeguards against the spread of viral disease. The viruses of importance in this respect are the relatively stable enteric viruses. Narrowing the scope further, the enteric virus of choice has come primarily from the picornaviridae group. Although poliovirus has been extensively studied, echovirus, coxsackievirus, and even some bacteriophages have been used in studies.

As mentioned, an applied approach has been undertaken, with inactivating agents normally being either those currently employed, or those under consideration as potential drinking water disinfectants. Chlorine, being the disinfectant of choice in most treatment facilities, has been used in a majority of the research. Other physical or chemical agents have included iodine and bromine compounds, chlorine dioxide, ozone, UV light, and high-energy ionizing rays (gamma). The following will be an account of the research into the mechanism of inactivation of enteric viruses treated with these various agents.

The structural simplicity of the majority of enteric viruses accommodates three obvious modes for the inactivation of the individual virion. A particular chemical agent or physical condition can affect either, 1) the protein capsid, or 2) the nucleic acid, or 3) both

sites. However, as straight-forward as this may seem, inactivation mechanisms are complex, being determined by the chemical and physical nature of the virion in its entirety.

Sedimentation Rate Analysis

Initial attempts to elucidate the inactivation mechanisms have dealt with changes in the physical nature of the inactivated virion. One method of detecting subtle changes in the whole virion was comparison of sedimentation rate coefficients as determined by rate zonal centrifugation. Both treated and control virus were centrifuged in linear sucrose or glycerol gradients. The use of virus radioactively labeled in either the protein or nucleic acid increases the sensitivity of sedimentation rate analysis by indicating the loss of any portion or all of either viral component.

Cooper (1962) reported that the sedimentation rate of poliovirus treated with 7.2 M urea was slightly slower but not seriously affected. His methodology however, did not compare this slower sedimenting material with that of empty poliovirus particles, even after he stated that the RNA was released during treatment. Although a small portion of viral protein was solubilized and lost, he did not correlate this with the slight decrease in sedimentation rate.

Similarly, the inactivation of cosackievirus A13 by low ionic strength conditions (Cords et al., 1975) just slightly decreased the sedimentation rate as compared to intact virions (150S). There was no

evidence of the release of RNA nor the presence of empty capsids at the 80S position. Ward and Ashley (1976) found that anaerobically digested sludge caused inactivation of poliovirus without significantly affecting the sedimentation coefficient. Aerosolization of the phage ØX174 into clean air caused inactivation, and gradient analysis revealed that the DNA was released from the phage (de Mik and de Groot, 1977). These same researchers aerosolized ØX174 phage into atmospheres containing either ozone or a reaction product of ozone, ozonized cyclohexene. Gradient analysis showed ozone to effect a two-step process in which the biological activity of the DNA was inactivated secondarily to the protein and at a slower rate as compared to the whole virion. The secondary air pollutant, ozonized cyclohexene, inactivated ØX174 by a rapid and seemingly simultaneous reaction with both the protein and DNA of the phage. Interestingly, cyclohexene by itself caused relatively little inactivation or damage.

Treatment of the bacteriophage f2 with 0.09 mg L^{-1} ozone for 5 seconds created a degradation of the protein capsid which significantly reduced the sedimentation rate (Kim et al., 1980). Electron microscopy revealed the capsid to be broken down into the protein subunits of the phage f2 which have a sedimentation rate of approximately 11S. The use of bacteriophage as enteric virus models may be misleading, as the treatment of poliovirus with 0.32 mg L^{-1} residual ozone for 30 seconds caused no change in the sedimentation rate of 158S which is characteristic of intact single virions (Roy et al., 1981). The graphic

data presented by these researchers is, however, somewhat lacking. If ozone caused a release of RNA, the resulting empty capsids were not evident in the gradient because [^3H]-uridine labeled virions were used. Also, any released radioactive RNA was not indicated as the data for the upper seven fractions of the gradient were omitted from the graphs.

Breindl (1971) studied the mechanism of heat inactivation of poliovirus and found that the resulting particles still contained a full complement of RNA but had a sedimentation rate similar to empty capsids. Sedimentation rate analysis also revealed that some intact RNA (35S) as well as a capsid protein, identified as VP4, was completely dissociated from the heat inactivated poliovirions. By altering the salt concentration during heat inactivation, Breindl (1971) could control the relative amounts of both the 80S ribonucleoprotein and the 35S free RNA.

Sedimentation to the bottom of a tube by ultracentrifugation of both poliovirus and coxsackievirus B1 which had been inactivated by river water failed to leave behind any solubilized protein in the supernatant (O'Brien and Newman, 1977). The researchers concluded that inactivation was not caused by any major conformational change of the viral capsid, which may have resulted in loss of protein. Yeager and O'Brien (1979) studied the effect of dessication of poliovirus in a soil filtrate. The RNA was released, leaving a virus particle that had the same sedimentation coefficient as empty virions (80S). Although there was a significant amount (approx. 15 percent) of radioactive protein at the top of the gradient, they made no attempt to explain this as the

possible loss of viral capsid protein. The effect on the sedimentation coefficient of poliovirus inactivated by ammonia was minimal, indicating only minor alteration of the protein capsid (Ward, 1978). Further evidence of only slight modification of the proteins was established from the similarity of the isoelectric points of treated versus non-treated poliovirions. Ward and Ashley (1980) found that treatment of rotavirus with sodium dodecyl sulfate (SDS) did not alter the sedimentation rate, but treatment with ethylenediaminetetraacetate (EDTA) caused the virus to sediment at a rate characteristic of noninfectious particles lacking the outer protein shell. Ward and Ashley (1979) determined by gradient analysis that the site of inactivation of poliovirus by detergents was the viral capsid proteins, and that the inactivating reaction was pH dependent. Sharp and coworkers (1975) inactivated reovirus with bromine and found that the density of the virus was decreased from 1.36 g cm^{-3} to 1.25 g cm^{-3} . The sedimentation rate of the treated reovirus also decreased compared to control virus, however, they did not report any measurements.

Keswick and coworkers (1981), using isopycnic centrifugation to analyze the density of poliovirus which had been inactivated with bromine chloride (BrCl) reported no change in the structural integrity of the virion. The virus remained intact even after 15 minutes of exposure to 5 mg L^{-1} of BrCl. However, extensive exposure to BrCl (10 mg L^{-1}) did degrade the virus into a more dense ribonucleoprotein complex.

Tenno and coworkers (1979) showed that a 10 minute exposure to 0.2 mg L^{-1} HOCl caused no significant change in the sedimentation rate of poliovirus even though it was 99.999 percent inactivated. However, after 30 minutes of exposure to the same chlorine dose, some of the poliovirus particles sedimented at a rate similar to empty particles. No attempt was made to determine if the RNA was released from the virion. O'Brien and Newman (1979) found that poliovirus exposed to 1 mg L^{-1} NaOCl lost its RNA and had a sedimentation rate similar to that of an empty capsid.

Antigenic and Adsorption Studies

A method to detect whether inactivation is effected by damage to the capsid proteins is to determine if the virus retains the ability to adsorb to host cells, and if its antigenic properties remain the same. In a study of the inactivation of several myxoviruses, Franklin and Wecker (1959) determined that hydroxylamine did not affect either the complement-fixing activity, hemagglutinating activity, or the enzymatic activity of the viral proteins. Breindl (1971) determined that heat inactivation of poliovirus created a ribonucleoprotein complex that was unable to adsorb to HeLa cells, and had antigenic C reactivity in contrast the antigenic D reactivity of untreated poliovirions. Cooper (1962) reported that some of the poliovirions treated with high concentrations of urea lost the ability to adsorb to host cells and became more sensitive to heat. The treated poliovirus however, was not

affected in its response to neutralizing antibody. Ward and Ashley (1980) found that treatment of rotavirus with either SDS or EDTA caused a loss of adsorptive ability.

Cords and coworkers (1975) found that low ionic strength solutions induced inactivation of coxsackievirus A13 by a structural alteration of the protein capsid which prevented adsorption to host cells. Likewise, the ability of poliovirus to adsorb and penetrate host cells was reduced by 75 percent at the 95 percent inactivation level when treated with high concentrations of ozone (Riessler et al., 1977).

Kim et al. (1980) found that the ability of the bacteriophage f2 to adsorb to the pili of its host was significantly reduced after treatment with ozone. Because of the inability of the ozone treated phage to non-specifically bind to the pili of a non-host strain of bacteria, these investigators felt that there was little, if any non-specific binding of the ozone treated phage to normal host pili. Ozone treated poliovirus which was [³H]-uridine labeled was allowed to adsorb to host cells (Roy et al., 1981). After washing of the cell monolayer to remove any unbound virus, the measurement of radioactivity associated with the cells revealed no change in the ability of the virus to adsorb to a host cell. The questions of non-specific binding of damaged virions and adsorption of empty capsids were not addressed in the investigation. The inactivation of poliovirus with ammonia caused only a slight decrease in the ability of the virus to attach to host cells (Ward, 1978). The retained adsorptive ability of the poliovirus indicated that

there was no major conformational change in the capsid proteins. O'Brien and Newman (1977) indirectly found that neither poliovirus type 1 nor cosackievirus B1 had lost the ability to adsorb to host cells after being inactivated by exposure to river water. Apparently, VP4, which is thought by some to be required for adsorption, was not cleaved from the viral capsid during inactivation. These same researchers showed that the treatment of poliovirus with chlorine (1 mg L^{-1} , pH 6.8-7.0) for up to four minutes did not affect the ability of the virus to adsorb to host cells. However, neither study attempted to control for non-specific binding of the virus to the host cell.

Gel Electrophoresis

A better method for detecting whether the capsid proteins are involved in the inactivation mechanism, is to determine the change in the electrophoretic pattern of the virion's capsid polypeptides. Ward (1978) determined that there were no apparent differences in the gel electrophoretic pattern of poliovirus capsid proteins before and after inactivation of the whole virus with ammonia. Ward and Ashley (1976), however, found that structural alterations of poliovirus capsids by a component in anaerobically digested sludge was due to the breakdown of VP1 and VP2. Although there seemed to be no change in VP4, the breakdown products of VP1 and VP2 could have electrophoresed to the VP4 position, masking any loss of VP4. Analysis of poliovirus which had been treated with ozone concentrations sufficient to achieve seven logs

of inactivation in 20 minutes showed that it had been degraded to fragments so small they ran off the gel.

In the characterization of an 80S ribonucleoprotein complex created by heat inactivation of poliovirus, Breindl (1971) used acrylamide gel electrophoresis to show that the capsid protein, VP4, was missing from the complex. Similarly, Cords and coworkers (1975) demonstrated that inactivation of coxsackievirus A13 was also due to the loss of the viral capsid protein, VP4. Ward and Ashley (1980) reported that of the five polypeptides comprising the two protein shells of rotavirus, treatment with sodium dodecyl sulfate (SDS) removed only VP3 which presumably is the most exposed polypeptide. Treatment with ethylenediaminetetraacetate (EDTA) caused a more extensive alteration by removing both VP3 and VP5. The inactivation of poliovirus by 0.7 mg L^{-1} ozone for 60 seconds caused obvious damage to polypeptides VP1 and VP2, but no apparent change in VP4 which is the polypeptide thought by some to be required for adsorption to host cells (Roy et al., 1981). Again, the possibility of a degradation product from VP1 and VP2 coincidentally showing up in place of missing VP4 cannot be ruled out from the results presented by these investigators.

Analysis of Nucleic Acids

Other than the protein moiety, researchers have only the nucleic acid component to consider in the activation of most enteric viruses. Methodology utilized thus far for studies in this regard involve

analysis of either the intactness of the nucleic acid or its biological activity. Biological activity has been ascertained by plaque assays of those nucleic acids which are known to be infectious and will replicate in vivo. An early study of Franklin and Wecker (1959) determined that the site of inactivation of mouse encephalomyelitis virus by 1 M hydroxylamine was on the nucleic acid. This they deduced from experiments showing that the loss of infectious RNA paralleled the loss of infective whole virions. However, utilizing a variety of methods in a study of the effect of 1 M hydroxylamine on "model" RNA which had been extracted from rat liver and calf liver, they determined by analytical ultracentrifugation that there was no degradation of the RNA. Also, both paper chromatographic and paper electrophoretic studies revealed no changes in the RNA bases. This information indirectly suggested that hydroxylamine did not alter or degrade the RNA of mouse encephalomyelitis virus, fowl plaque virus, and swine influenza virus. O'Brien and Newman (1979) found that the inactivation of poliovirus with 1 mg L^{-1} NaOCl at pH 6.8-7.0 degraded the RNA into fragments smaller than 5S, then released the degraded RNA from the capsid. Kim and coworkers (1980) reported that the RNA extracted from the ozone treated bacteriophage f2 was more infectious than the treated phage but less infectious than naked RNA which had been ozonated. The RNA extracted from the ozone treated phage appeared to remain intact as a single molecule but unexplainably had lost radioactivity and had become less dense. Ozone treatment of poliovirus with subsequent extraction and

analysis of the RNA revealed that the nucleic acid had been fragmented into various lengths of short chains (Roy et al., 1981). The major difference between the conditions under which the study of Roy et al. (1981) and Kim et al. (1980) took place was the pH. Roy et al. (1981) used pH 4.3, while Kim et al. (1980) worked at pH 7.0.

Keswick and coworkers (1981) showed that the RNA extracted from poliovirus which had been inactivated by BrCl retained its biological activity. The RNA remained infectious as detected by plaque assay. Ward and Ashley (1976), however, found that a component of anaerobically digested sludge was able to nick the RNA of poliovirus, creating irreparable products with an average sedimentation rate of 25S. Intact and infectious poliovirus RNA has a sedimentation rate of 35S. In another study, Ward (1978) determined that, while ammonia had no effect on naked poliovirus RNA, it did degrade the RNA from the treated intact virion. The loss of RNA infectivity was proportional to the loss of whole virion infectivity. Further experimentation revealed that the damage to the RNA was sufficient to interrupt the virus infection cycle by preventing the suppression of host protein synthesis. Exposure to river water inactivated both poliovirus type 1 and coxsackievirus B1 by apparently altering capsid integrity and completely degrading the RNA (O'Brien and Newman, 1977). Naked RNA, extracted from these same two viruses, was rapidly degraded in river water. Yeager and O'Brien (1979) showed that poliovirus inactivated in both natural and sterile moist soils had lost its RNA in degraded fragments of less than 5S. Similar

results were obtained for natural dry soils. However, the RNA of viruses inactivated in dry sterile soil was released as intact molecules. The RNA extracted from poliovirus which had been exposed to 0.2 mg L^{-1} HOCl for intervals up to 30 minutes was found to be infectious for all exposure times (Tenno *et al.*, 1979).

Another early study (Cooper, 1962) showed that the treatment of poliovirus with 7.2 M urea caused a release of the RNA, making it susceptible to nucleases. When stabilized by the addition of glutathione, this RNA remained intact and had retained its infectivity as detected by plaque assay. Hsu (1964) showed that the iodine inactivation of f2 phage did not destroy the infectivity of the RNA, which had been extracted from the inactivated phage particles. Also, the biological activity of the naked RNA was not altered by treatment with iodine. Heat inactivation of poliovirus created an 80S ribonucleoprotein complex which contained intact and infectious RNA as determined by plaque assay (Breindl, 1971). It was also reported that heat inactivation liberated some intact and infectious free RNA. Interestingly, Cords and coworkers (1975) inactivated coxsackievirus A13 by merely subjecting the virions to low ionic strength conditions. The loss of infectivity under these conditions was not a result of either the loss of or damage to viral RNA.

Inactivation Mechanisms

Franklin and Wecker (1959) suggested that hydroxylamine caused inactivation of several RNA viruses by cleaving an amino acyl ester bond and removing an extraneous amino acid which is essential to the structure of biologically active viral RNA but not to the RNA by itself. Because not all RNA viruses have this hypothetical amino acid, they are able to explain the susceptibility of some myxoviruses and the resistance of others. The similarities of urea inactivation and heat inactivation of poliovirus indicated to Cooper (1962) that the mild disruption of hydrogen bonds in the protein capsid caused a release of the RNA in a manner analogous to the uncoating process of the virus during infection. This would explain the expulsion of the nucleic acid out of the urea-treated virus.

Hsu (1964) offered an explanation as to the inconsistencies reported by others in the sensitivities of various RNA viruses to the effect of iodine. Different iodine concentrations in the reaction will affect the iodination reaction of the virus; i.e., the higher the iodide concentration, the slower the iodination reaction. Hsu demonstrated this by comparing the iodination of tyrosine to inactivation of both f2 phage and poliovirus at increasing iodide concentrations. The full explanation, however, eluded him, for under identical conditions the rate at which the infectivity of f2 phage was lost much faster than the rate at which tyrosine was iodinated. Therefore, the poor correlation

of these two reaction rates meant that the reaction mechanism was more complex than expected.

Breindl (1971) showed that heat inactivation of poliovirus produced a ribonucleoprotein complex which would not adsorb to host cells, but contained intact and infectious RNA. Also, the sedimentation rate for this complex had decreased to about 80S or approximately the same as empty poliovirus capsids. An explanation for these changes was based on the loss of a viral capsid protein, VP4. He suggested that the observed alterations may be part of the natural uncoating mechanism. Dewey (1972) showed that gamma irradiation of T4 phage in dilute aqueous solutions caused the formation of hydrogen atom radicals from solution which in turn possibly triggered the release mechanism of the phage DNA. The DNA was then susceptible to breakage by the gamma irradiation. Thomas et al. (1982) found that the small size, and single-stranded RNA of swine vesicular disease virus makes it most resistant to gamma irradiation.

Coxsackievirus A13 was subjected to low ionic strength conditions by Cords and coworkers (1975) and found to lose infectivity. These researchers discovered that the viral capsid was altered with a loss of VP4 but not the RNA, which remained intact and infectious. The loss of infectivity was attributed to the inability of the treated virus to adsorb to host cells. However, Cords et al. (1975) emphasized that there is no direct evidence indicating VP4 is the polypeptide responsible for adsorption.

The thermal stability of various enteric viruses was found to be affected by ionic detergents isolated from wastewater sludge (Ward and Ashley, 1978). It was established that ionic detergents, especially cationic types, reduced the heat required to inactivate reovirus, but protected two strains of poliovirus and a coxsackievirus. The inactivity effect of the detergents on a variety of viruses was found to be extremely pH dependent (Ward and Ashley, 1979). The anionic detergent, dodecyl sulfate, was more virucidal at low pH, while dodecyltrimethylammonium, a cationic detergent, was more virucidal at alkaline conditions. These investigators concluded that the variable susceptibility of viruses to the inactivating effects of detergents appeared to be a function of the pH dependence of the ionic states of the viral capsid proteins and not the nucleic acid.

In contrast, ammonia was found by Ward (1978) to damage the RNA of poliovirus but not the capsid proteins. Further study revealed that even though adsorption was still possible, the repression of host cell protein synthesis by the inactivated virus was prevented. O'Brien and Newman (1977) utilized indirect techniques to ascertain that the inactivation of both poliovirus type 1 and coxsackievirus B1 which had been exposed to river water was due to a rapid degradation of the RNA. The RNA was exposed by minor alteration of the protein capsid, which had no effect on the adsorption abilities of the virus. A study of the inactivation of poliovirus placed in either dry (non-sterile) or moist (non-sterile and sterile) soils revealed a similar mechanism, which

involved dissociation of the viral components and release of the RNA in a highly degraded form. Although loss of infectivity with exposure to dry, sterile soil was associated with separation of viral components, the RNA was released intact.

Electron microscopy of chlorine inactivated echovirus type 1, revealed particles whose appearance suggested protein capsid damage (Young and Sharp, 1979). The proportion of inactivated echovirus that appeared normal was sufficiently great to suggest that damage to the RNA caused inactivation. Regardless of the mechanism, Young and Sharp (1979) showed that, under proper acidic conditions, the resulting aggregation of inactivated echovirus enhanced the efficiency of plaque production, thereby increasing the surviving titers by as much as 40-fold. These researchers describe this as a random inactivation of any one of several critical sites on the virus, which prevents a single virion to be infectious by itself. However, in an aggregated state the summation of damaged virions may be able to act as a whole virion, with each virion contributing to the infection process. This is referred to as multiplicity reactivation and is probably a function of the virus type, the degree of aggregation, and, most importantly, the extent of damage to the virion (Young and Sharp, 1979). The inactivation of reovirus by bromine (Sharp et al., 1975) caused a decrease in both the density and sedimentation rate. Electron microscopy revealed particles that lacked both RNA and some structural material.

Dennis et al. (1979) investigated the mechanism of inactivation by examining the incorporation of radioactive chlorine during the disinfection of the bacteriophage f2. These researchers discussed the ability of a reducing agent such as sodium thiosulfate, to remove chlorine which had attached to the amino groups of proteins but not chlorine which had attached to the purine and pyrimidine bases of nucleic acids. Their results indicated that the radioactive chlorine was incorporated into the nucleic acid and they concluded that this was the site of inactivation of the phage f2. Since the inactivation reaction was quenched with sodium thiosulfate, they attempted to establish the possibility of a redox reaction between chlorine and protein by determining the loss of free chlorine in the reaction mixture. The decrease in free chlorine was explained as being caused either by extraneous reactions of contaminating material in the virus preparation or an increase in pH. Unfortunately, no further attempt was made to determine if any chlorine-protein interaction was taking place and if it could be the actual cause of inactivation. In direct contrast to Dennis et al. (1979) are the results of Tenno and coworkers (1979) which indicate that the inactivation of poliovirus by chlorine is at the protein level. They found that 0.2 mg L^{-1} HOCl caused 99.999 percent inactivation of poliovirus in 10 minutes. Neither sedimentation nor buoyant density analyses revealed any significant change in the virus treated for 10 minutes. After 30 minutes exposure there was some evidence of protein degradation when some of the treated virus had a

sedimentation rate similar to empty capsids. Regardless of whether or not the RNA was released from the capsid at this point in time, it was still infectious when plaque assayed.

O'Brien and Newman (1979) reported that treatment of poliovirus with 1 mg L^{-1} NaOCl caused complete conversion to empty particles which sedimented at a rate of 80S. The released RNA was found to be completely degraded into fragments smaller than 5S. Also, the release of the degraded RNA was a secondary event to inactivation. Although they determined that there was no loss of any of the four capsid proteins and the isoelectric point of the treated virus was the same as the control, they observed that these empty particles fell apart quite readily upon standing. Because these results were somewhat contradictory to Tenno et al. (1979), Alvarez and O'Brien (1982) reported further findings using NaOCl doses of less than 1 mg L^{-1} which concurred with the results of Tenno et al. (1979). Tenno and coworkers (1979) showed that the structural conversion of poliovirus to an empty particle was dependent upon chlorine concentration; and, the separation of viral components occurred with chlorine concentrations in excess of 0.8 mg L^{-1} NaOCl. They contend that these structural changes were not the primary cause of viral inactivation since they were unable to correlate percent viral inactivation with the percent conversion to the empty particle. O'Brien and his coworkers seem unable to accept the possibility of viral inactivation without the occurrence of some gross structural change.

Ozone treatment of the bacteriophage f2 by Kim and his coworkers (1980) caused a degradation of the capsid into protein subunits, with the subsequent release of RNA and inability to adsorb to host pili. These investigators suggested that the ozone treatment of water will cause RNA-containing enteric viruses to release their nucleic acid in an infectious form. In direct contrast, Roy and coworkers (1981) found that the RNA of ozone inactivated poliovirus remained within the protein capsid in a degraded state. The protein capsid was damaged but not to an extent that prevented adsorption to host cells. They concluded that inactivation was the result of a rate-limited diffusion process of the ozone across the protein barrier of the capsid, followed by degradation of the RNA. A study of the effect of ozone on poliovirus utilizing a deionized water system did not take into account the effects of pH and possible virus aggregation (Riesser et al., 1977). Even though the high concentrations of ozone completely degraded the poliovirus proteins into small fragments, the investigators speculated that the capsid was the site of inactivation.

Ward and Ashley (1976) determined that two structural modifications, possibly independent of one another, occurred when poliovirus was inactivated by an unidentified component found in the liquid fraction of anaerobically digested sludge. Two major capsid proteins, VP1 and VP2, were broken down, and the single-stranded RNA was irreparably nicked into fragments with an average sedimentation rate of 25S. Interestingly, the inactivated virion remained intact and did not

change in its sedimentation rate. In a comparative study of the effects of SDS and EDTA on rotaviruses, Ward and Ashley (1980) emphasized that the properties of the bonds between the two concentric protein shells of the virion capsid determine how the virus particle behaves to chemical treatment. Furthermore, the specific treatment and breaking of these bonds can aid in determining both the function and the properties of these intershell crosslinkages.

In a search for alternate disinfecting agents, Gowda and coworkers (1981) studied the kinetics of poliovirus inactivation by Chloramine-T (CAT). The similarity of the observed inactivation kinetics to those of the heat inactivation of tobacco mosaic virus, as reported by Ginoza (1958) and Ginoza et al. (1964), led Gowda et al. (1981) to propose the same mechanism of inactivation. That is, a denaturation of RNA was taking place by the formation of phosphotriester bonds with subsequent hydrolytic cleavage of the molecule.

Most recently, Alvarez and O'Brien (1982) investigated the inactivation of poliovirus by chlorine dioxide. At pH 10.0 the RNA was separated from the protein, converting the virion to an empty capsid, but at pH 6.0 there was no separation of viral components. Alkaline conditions causes chlorine dioxide to ionize into chlorite and chlorate ions which may be the cause of this phenomenon. These investigators also found inactivation affected poliovirus replication at some point after host cell penetration and virion uncoating. Olivieri et al (1982) studied the effect of chlorine dioxide on the bacteriophage f2 and found

that inactivation was not dependent upon damage to the viral RNA. The RNA extracted from inactivated phage remained infective.

The preceding has been a review of the analyses and theories associated with the most recent research into the mechanism by which various agents, both physical and chemical, inactivate viruses. Although the research has been primarily holistic in its approach, the results are somewhat superficial. The results, however, do give a basis from which further investigations can be employed, utilizing new, more sensitive techniques, in an attempt to determine what occurs on a molecular level. The criteria for choosing a physical or chemical disinfecting agent should include the ability of the agent to trigger an inactivation mechanism. The need for understanding inactivation mechanisms of viruses is essential for improving disinfection of our water supplies. This need can only be fulfilled by further investigations.

III. INACTIVATION KINETICS OF H-1 PARVOVIRUS

Introduction

The transmission of waterborne pathogens via drinking water supplies continues to occur, primarily in inadequately disinfected water. Investigators have determined that up to 50 percent of nonbacterial gastroenteritis cases derived from waterborne outbreaks are caused by viruses (Bitton, 1980). Recently, Craun (1981) reported that during 1971 to 1978, there were five outbreaks of waterborne gastroenteritis affecting almost 1500 persons, four of them due to inadequately disinfected water. The etiologic agent for all five outbreaks was identified as being parvovirus-like and similar to the Norwalk agent.

Little is known about the fate of parvovirus-like agents in water treatment systems. The inactivation of enteric viruses has been studied by many investigators using a variety of systems and inactivating agents. Several studies have been performed using chlorine to inactivate several of the enteric viruses including poliovirus, coxsackievirus, reovirus, rotavirus, and echovirus, all of which are RNA-containing enteric viruses. The only study utilizing a DNA-containing enteric virus was performed by Englebrecht and coworkers (1978 and 1980) in which Kilham rat virus (KRV) was subjected to chlorine inactivation. They found this parvovirus to be as resistant as picornaviruses to chlorine. However, they were comparing hemag-

glutination (HA) assays of KRV to plaque assays of the picornaviruses. If the antigenicity of the inactivated virus was unaffected then an HA assay would not differentiate between inactivated and infective virus.

This study used the parvovirus H-1, a putative human virus, (Toolan, 1968) as a model virus for chlorine inactivation experiments. Because outbreaks of waterborne gastroenteritis caused by parvovirus-like agents were associated with inadequately disinfected water supplies, chlorine levels used in the inactivation trials were equal to or less than the current minimum that EPA (1976) recommends, of a 0.2 mg L^{-1} . Control of chlorine species was provided by phosphate buffered saline, pH 7.0, that was chlorine-demand-free. The data obtained suggest that H-1 appears to be less resistant to chlorine than poliovirus.

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 cell line was grown to confluency in minimum essential media (MEM, Flow Labs, McLean, VA), to which was added $0.625 \text{ } \mu\text{g ml}^{-1}$ Fungizone (Flow Labs), 30 ml glutamine (Sigma, St. Louis), 10 percent (v/v) fetal bovine serum (FBS, Flow Labs), $100 \text{ units ml}^{-1}$ of penicillin (Sigma), and $100 \text{ } \mu\text{g ml}^{-1}$ of streptomycin (Sigma). The proper pH (7.2 to 7.6) was maintained by

adjustment with sterile 7.5 percent (w/v) sodium bicarbonate (NaHCO_3) and incubation in an atmosphere of one to two percent carbon dioxide.

Virus stocks were obtained by infecting barely confluent monolayers of NB cells in roller bottles. The cells were thoroughly rinsed with Dulbecco's solution, then infected with an approximate multiplicity of infection (MOI) of 0.1 (PFU/cell). The virus was allowed to adsorb for 30 to 60 minutes before MEM and serum (final FBS concentration of 10 percent) was added.

Virus Purification

The H-1 parvovirus was purified by a method similar to that previously described for bovine parvovirus (Patton, 1980). When 90 to 100 percent cytopathic effect (CPE) was observed (48 to 72 hours post-infection), the virus was harvested in the following manner. Cell debris and lysate were collected, centrifuged (27,000 rpm, two hours, 10°C, and SW27 rotor) and resuspended in a small volume (< 20 ml) of 50 mM Tris-HCl buffer at pH 8.0. The cell material was then digested with 50 $\mu\text{g L}^{-1}$ DNase I (Sigma) and 100 $\mu\text{g L}^{-1}$ RNase A (Sigma) in the presence of 5 mM MgCl_2 , at 37°C for 30 to 60 minutes. Then, 250 $\mu\text{g L}^{-1}$ trypsin (Sigma) and 25 $\mu\text{g L}^{-1}$ chymotrypsin (Sigma) was added and the samples were incubated an additional 15 minutes. A high speed (50,000 rpm, 60 minutes, 4°C, and Ti50 rotor) pellet was obtained from the supernatant of a low speed (10,000 rpm, 10 minutes, 20°C, and JA-17 rotor) centrifugation of the digested cell material. The high speed pellet

containing the virus was resuspended in a small volume (< 2 ml) of 50 mM Tris-HCl at pH 8.0. This sample was then loaded onto a preformed cesium chloride (CsCl, EM Reagents, Darmstadt, Ger.) gradient consisting, from bottom to top of the tube, of 2.0 ml of 40 percent CsCl, 4.0 ml of 35 percent CsCl, 2.0 ml of 30 percent CsCl, and 2.0 to 2.8 ml of 1 M sucrose (EM Reagents). All solutions were made with 50 mM Tris-HCl buffer at pH 8.0. The gradients were centrifuged at 34,000 rpm for 18 hours in an SW41 rotor at 15°C.

After isopycnic centrifugation of the virus in the preformed CsCl gradient, the opaque virus bands were located by light-scattering. The upper band containing light-full virus particles (banding at 1.39 to 1.42 g cm⁻³ in CsCl) was collected separately from a slightly lower band of heavy-full virus particles (banding at 1.46 to 1.48 g cm⁻³). These bands were collected by aspiration and dialyzed against 50 mM Tris-HCl buffer at pH 8.0, for a minimum of one to two hours. All dialysis tubing utilized had a molecular weight cut off of 12,000 to 14,000. The virus was then centrifuged into a 5 to 30 percent neutral sucrose gradient (50 mM Tris-HCl, 0.5 mM EDTA, pH 8.7) for two hours, at 41,000 rpm and 4°C in an SW41 rotor. The gradients were fractionated from the bottom and the fractions containing virus were located either directly by light-scattering, or indirectly from a concurrent gradient containing radioactively labeled virus which was detected by liquid scintillation spectrophotometry (Beckman, Model LS7500, Silver Spring, MD). The virus

was dialyzed overnight against chlorine-demand-free phosphate buffered saline at pH 7.0 and then collected and stored at 4°C.

Poliovirus type 1 (LSc) was purified by a Freon (1,1,2-trichloro-1,2,2-trifluoroethane, Fisher Scientific) extraction technique. This involved extracting cellular debris by adding to the cell lysate of infected cells an equal volume of Freon, then blending until emulsified. This mixture was centrifuged for 5 minutes at full speed in a table top clinical centrifuge. The supernatant was collected and put into dialysis bags with 12,000 to 14,000 molecular weight cut off. To concentrate the supernatant, the dialysis bags were laid in a tray and liberally covered with polyethylene glycol. When the volume of the supernatant was reduced sufficiently (approximately 150 ml), it was collected and placed on a step gradient of 10 ml of CsCl (Density = 1.34 g cm⁻³) and 5 ml of 25 percent (w/v) sucrose. The gradients were centrifuged for 30 hours at 25,000 rpm in an SW27 rotor. The virus band was located by light scattering and the appropriate 1 ml fractions were pooled. The virus was dialyzed against chlorine-demand-free phosphate buffered saline, then collected and stored at 4°C.

Preparation of Phosphate-Buffered Saline, pH 7

A phosphate-buffered saline (PBS) solution (0.01 M sodium phosphate, pH 7.0; 0.14 M sodium chloride) was prepared with reagent grade chemicals (Fisher Scientific Company). One liter stock solutions of both 1 M sodium monobasic phosphate (NaH₂PO₄) and 1 M sodium dibasic

phosphate (Na_2HPO_4) were prepared with either deionized, glass distilled water or water that had been treated by reverse osmosis, then deionized. Then, 58.5 ml of 1 M NaH_2PO_4 and 92.5 ml of 1 M Na_2HPO_4 were mixed with 14.85 L of 0.14 M sodium chloride (NaCl). The resulting solution was a 0.01 M phosphate buffer at pH 7.0.

In order to exhaust the demand of the buffer for chlorine, household bleach (5.25 percent sodium hypochlorite) was added to make a final concentration of 2.1 milligrams per liter (mg L^{-1}) and allowed to react for 24 hours. The solution was then irradiated with a submersible, quartz UV light (UV Products, Inc., San Gabriel, CA) until all remaining chlorine was quenched. This solution then was considered "chlorine-demand-free". The pH was determined with a standardized Fisher Accumet pH meter and was always found to be $\text{pH } 7.0 \pm 0.05$.

Preparation of Glassware and Plasticware

All glassware was routinely stored in a 10 percent (v/v) hydrochloric acid (HCl) bath. Just prior to use, it was dipped in a chlorine solution, then rinsed thoroughly, first with distilled water, then with chlorine-demand-free PBS.

Sodium Hypochlorite and Sodium Thiosulfate Solutions

A primary stock chlorine solution was freshly prepared for each experiment from household bleach (5.25 percent, w/v, sodium hypochlorite) by dilution with chlorine-demand-free PBS. The resulting stock

solution, 0.21 mg L^{-1} , was used to dose the three reaction vessels at either 0.05, 0.10, or 0.20 mg L^{-1} . Sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), either in crystalline form or as a one percent solution (w/v) made up in chlorine-demand-free PBS, was used to quench the residual chlorine in the samples. The $\text{Na}_2\text{S}_2\text{O}_3$ solution was made fresh for each experiment.

Inactivation Trials

Disinfection trials were performed using a jar test apparatus (Phipps and Bird, Richmond, VA) and a constant temperature water bath (Figure 1). In a typical trial, 700 ml of chlorine-demand-free PBS at pH 7 was transferred to a reactor that had been treated to remove chlorine demand. The reactor was placed in the water bath and allowed to equilibrate to the set temperature while being stirred with glass paddles at 65 rpm. Sodium hypochlorite was added to provide either 0.05, 0.10, or 0.20 mg L^{-1} free chlorine as determined by amperometric titration (Standard Methods, 1981). Once the chlorine level was determined in a particular reactor, viruses were added to achieve an infectivity level of 10^5 to 10^6 plaque-forming units per milliliter (PFU ml^{-1}). Samples withdrawn at 1, 2, 5, 10, 15, 20, and 30 minutes were immediately transferred to tubes containing sodium thiosulfate to neutralize residual chlorine, and then assayed for virus. These experiments were performed at 5, 10, 20, and 30°C for each chlorine level. A control reactor, containing PBS and virus, was present in all experiments. Samples were taken initially, midway, and at the end of

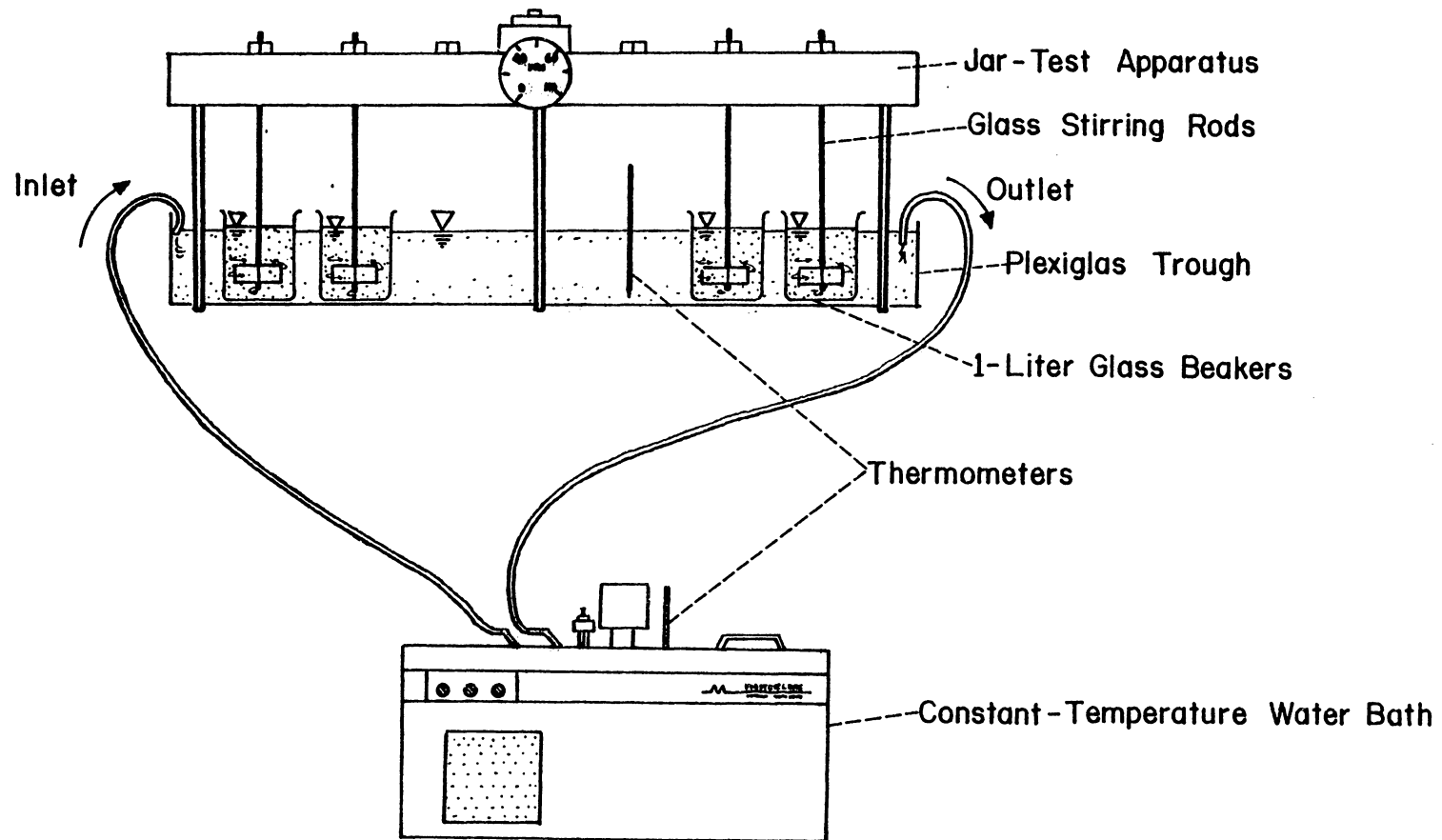


Figure 1. A schematic of the inactivation trial apparatus with the constant temperature water bath.

the experiment to determine if any die-off of the control virus occurred in the PBS. Chlorine levels of each reactor were determined at the end of each trial.

Plaque Assay

Parvovirus H-1 was enumerated by plaque assay as described by Ledinko (1967). Confluent monolayers of NB cells grown in plastic tissue culture dishes, 60 mm diameter, were rinsed with 2 ml of Dulbecco's solution and then infected with 0.25 ml of virus samples diluted in (MEM). After a 30 to 60 minute adsorption period, a 0.6 percent agarose (Bethesda Research Laboratories, Rockville, MD or FMC Corp., Marine Colloids Div., Rockland, ME) overlay solution, containing MEM and a final concentration of 10 percent FBS (Flow Labs), was added to the infected tissue cultures. The plates were incubated at 37°C in an atmosphere of 5 percent CO₂ for 5 to 6 days. At this time the agarose overlay was removed, and remaining viable cells were stained with a 0.1 percent (w/v) crystal violet stain. Visible plaques were counted and multiplied by the appropriate dilution factor. Results were expressed as plaque-forming-units per milliliter (PFU ml⁻¹).

The poliovirus type 1 (LSc) was also enumerated by plaque assay. Confluent monolayers of BGM cells were grown and treated in the same manner prior to infection as the NB cells. After infection with appropriate dilutions and a 30 to 60 minutes adsorption period, a 1.4 percent agar-overlay solution containing MEM and 10 percent FBS was

added to the tissue culture plates. The plates were incubated in the same atmosphere as used for the NB cells. After 48 hours, the plaques were visualized and counted as described for the parvovirus.

Results

Inactivation Trials

The reactors were adjusted to either 0.05, 0.10, 0.02 mg L⁻¹ free chlorine by addition of the proper dose from the primary stock solution (0.21 mg ml⁻¹) of NaOCl. The chlorine species was controlled by using PBS at pH 7.0, and quantification of surviving viruses at the various sampling times was determined by plaque assay. These assay results were used, along with the average virus titer from a control reactor, to calculate the surviving fraction of viruses for each sampling time. The surviving fraction is equal to N_t/N_0 where N_t is the PFU ml⁻¹ remaining at a specified time, t , and N_0 is the PFU ml⁻¹ at time equal zero (from the control reactor). The logarithm of the surviving fraction plotted versus time presents the inactivation data for parvovirus H-1 in Figures 2-5. Each figure illustrates the effect of a different temperature on the inactivation curve for the various chlorine doses. Reaction temperatures were 5, 10, 20, and 30°C. Each curve is representative of data obtained for two to four experiments with the exception of the 30°C data (Figure 5), which represents only one experiment.

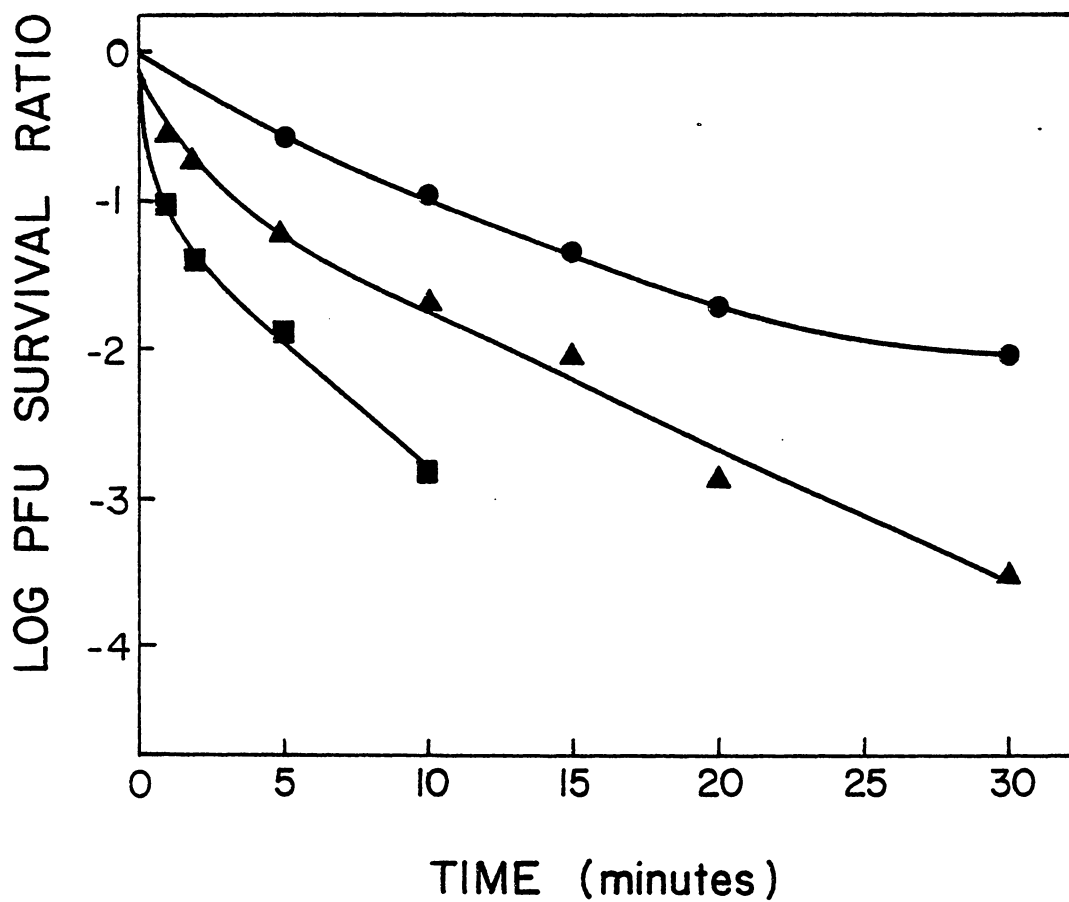


Figure 2. The response of H-1 light-full (LF) parvoviruses at 5°C and pH 7.0 to various levels of free chlorine. (● 0.05 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

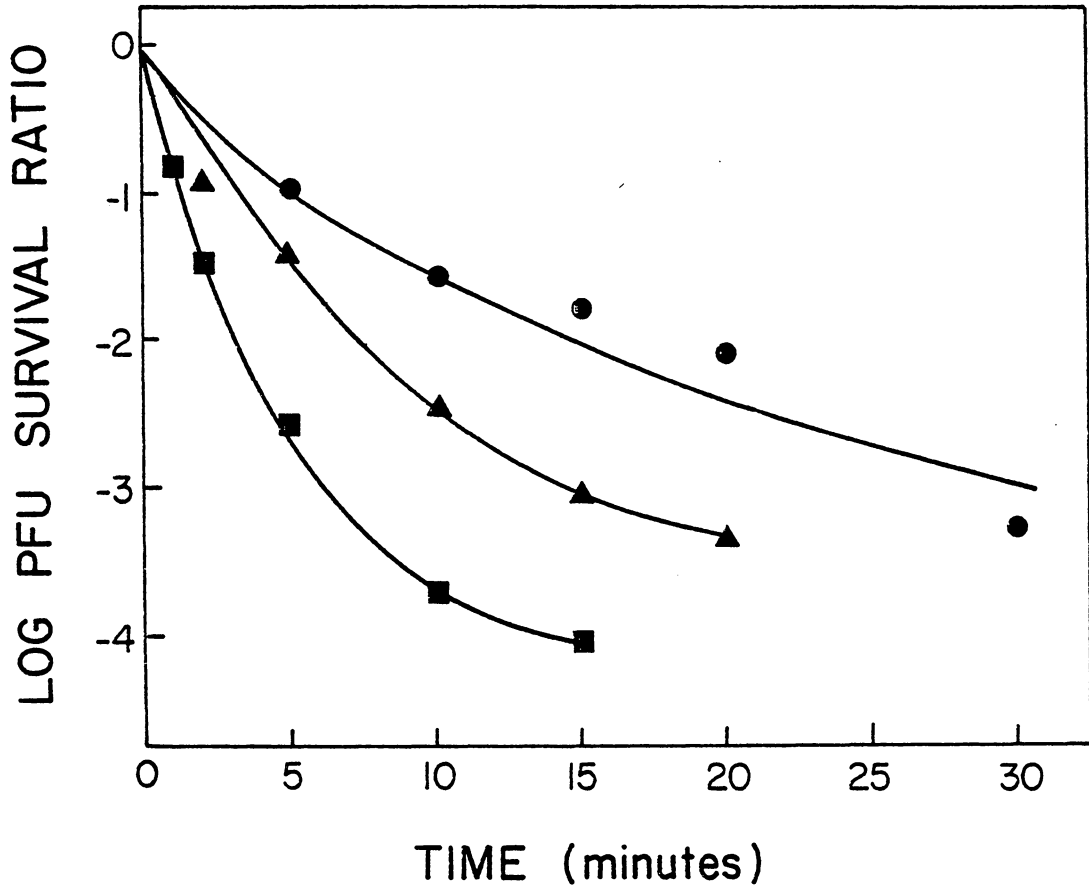


Figure 3. The response of H-1 light-full (LF) parvoviruses at 10°C and pH 7.0 to various levels of free chlorine. (● 0.5 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

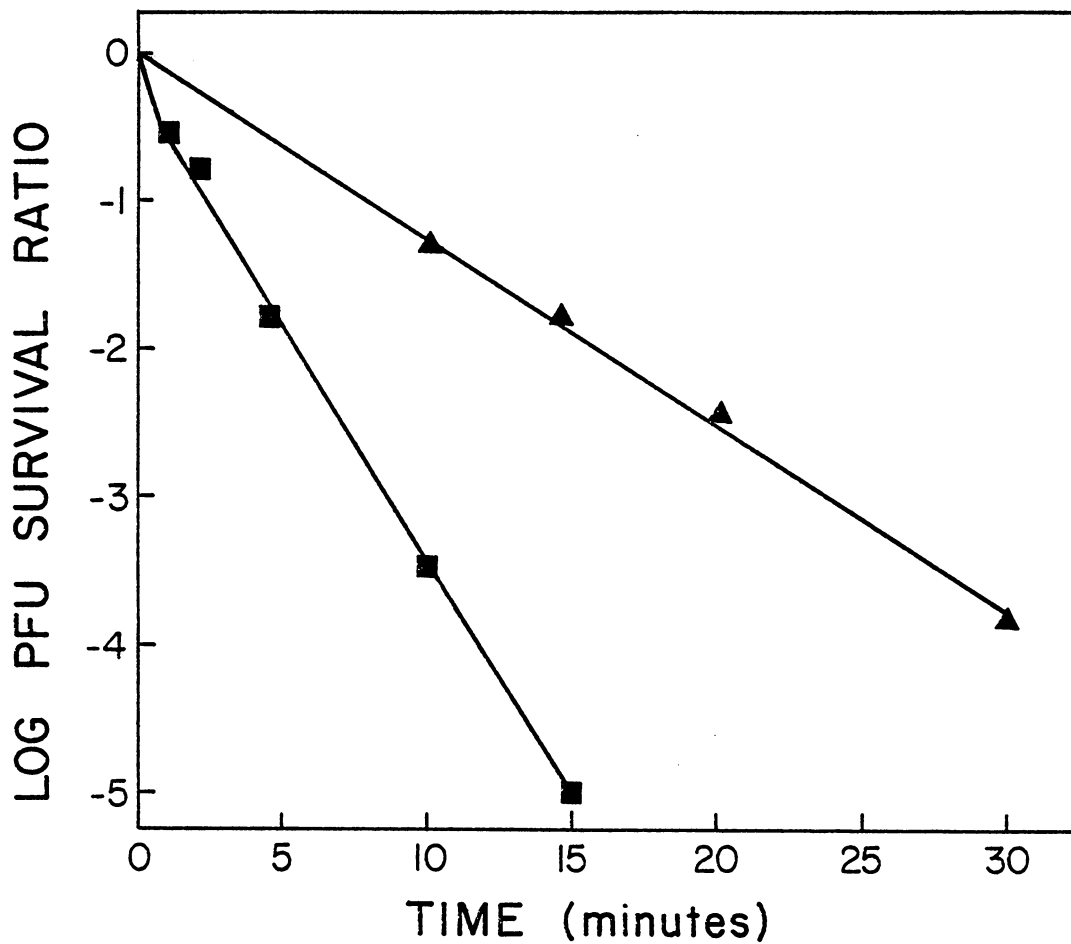


Figure 4. The response of H-1 light-full (LF) parvoviruses at 20°C and pH 7.0 to various levels of free chlorine. (● 0.05 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

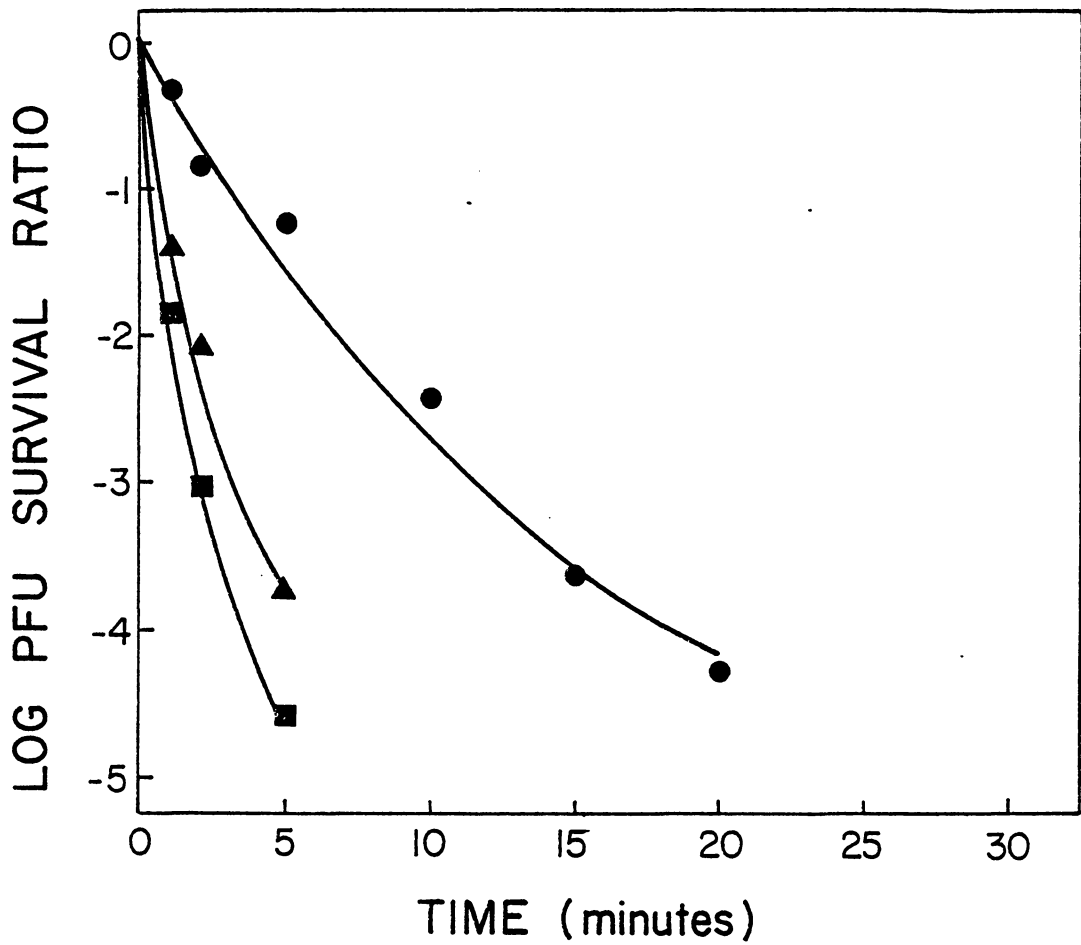


Figure 5. The response of H-1 light-full (LF) parvoviruses at 30°C and pH 7.0 to various levels of free chlorine. (● 0.05 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

The general trend demonstrated by all these graphs is that as the chlorine dose increases, the rate of inactivation increases. Also, increasing contact time effected increased levels of inactivation, but not linearly. The majority of the curves "tail-away" from the slope of a line created by the first few sampling times.

Figure 6 presents inactivation data at 20°C for the more dense or heavy-full particle type of parvovirus H-1, in contrast to Figure 2-5 which present inactivation data for the less dense or light-full particle type. For comparative purposes, the inactivation of poliovirus type 1 (LSc) at 20°C using this system is presented in Figure 7. The same general trends exhibited by the data in Figures 2-5 are evident in Figures 6 and 7.

Effectiveness of Chlorine Concentration

The contact time required for a two-log reduction in virus titer can be determined from the inactivation trial curves (Figures 2-5). These data can be used to denote the effect of chlorine concentration on inactivation and can be expressed by the van't Hoff equation (Kimball, 1953; Gurian, 1956; described in Fair et al., 1971; and Metcalf and Eddy, Inc., 1979):

$$C^n t_{99} = k$$

Where:

C = concentration

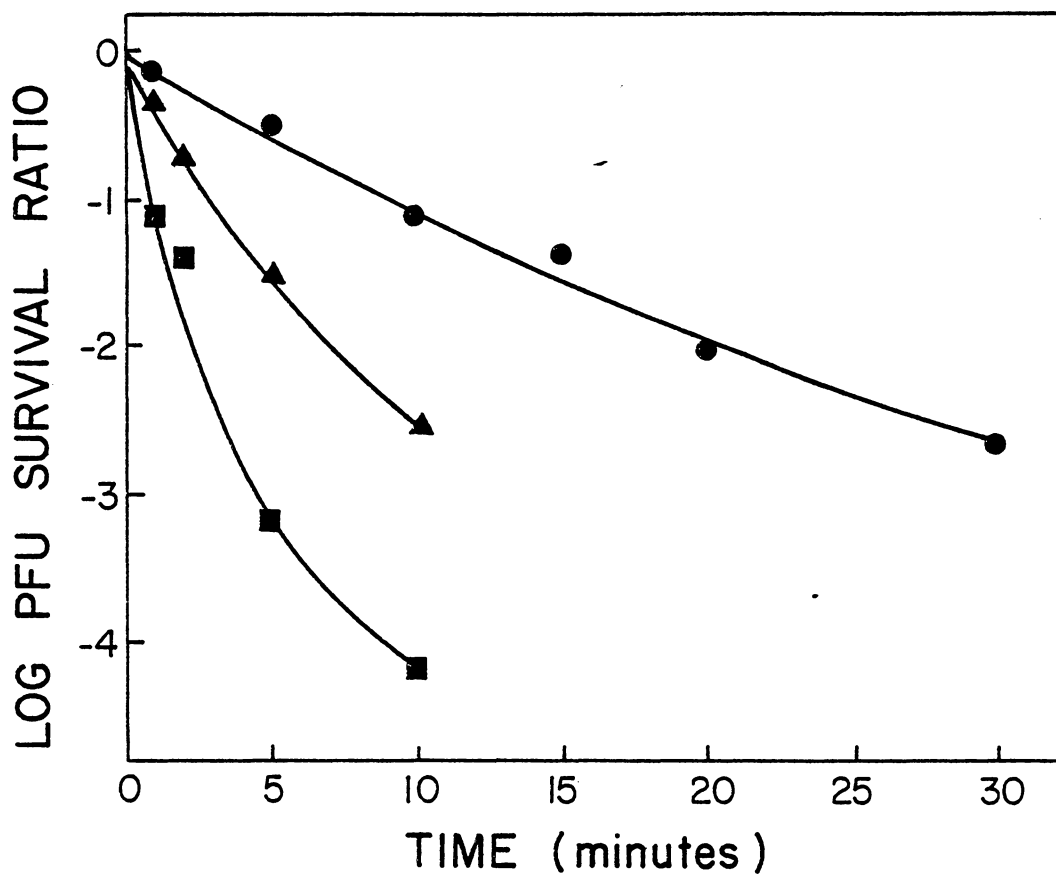


Figure 6. The response of H-1 heavy-full (HF) parvoviruses at 20°C and pH 7.0 to various levels of free chlorine. (● 0.05 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

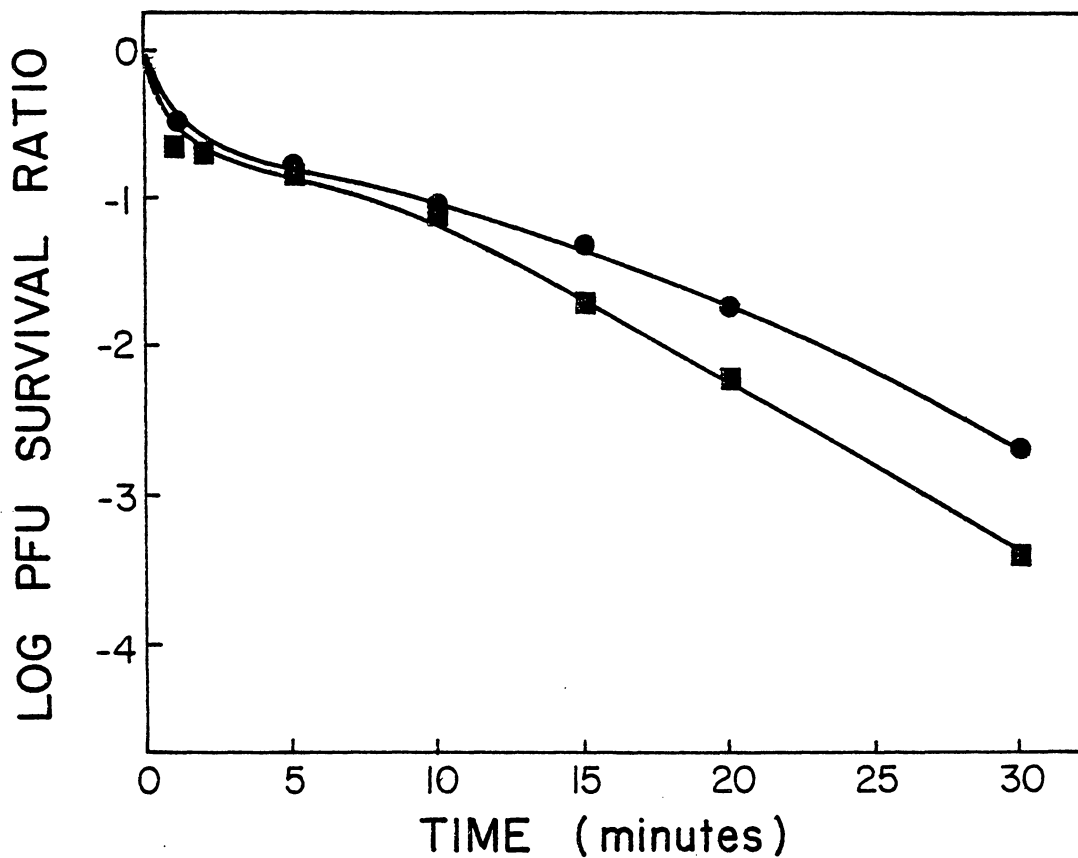


Figure 7. The response of poliovirus type 1 (LSc) at 20°C and pH 7.0 to various levels of free chlorine. (● 0.05 mg L⁻¹, ▲ 0.10 mg L⁻¹, ■ 0.2 mg L⁻¹).

n = coefficient of dilution

k = empirical constant

t_{99} = time required for 99 percent inactivation

Rearranging, a straight line relationship can be developed

$$\log C = 1/n \log k - 1/n \log t_{99}$$

A log-log plot of concentration versus time provides both the value for n , as the slope equals $-1/n$ and the reaction rate constant (or k value) from the y -coordinate when $t_{99} = 1$. The line of best fit was obtained for each temperature by least squares linear regression analyses.

Figure 8 represents a van't Hoff plot for 99 percent inactivation of the virus at 5, 10, and 20°C. An apparent anomaly noted in these graphs is the relationship of the 5°C line to both the 10 and 20°C lines. Its position relative to the others indicates that disinfection was better at the coldest temperature. Also illustrated in the previous inactivation trial graphs, (Figs. 2-5) the rate of inactivation at 5°C was faster than at 10 or 20°C.

Both the 30°C inactivation data and the 20°C inactivation data for heavy-full virions were also subjected to van't Hoff graphic analysis (Figure 9). Although the 20°C line for heavy-fulls is in the same vicinity as the 20°C line for light-fulls, it has a slightly different slope, making the empirical constant, k , in the van't Hoff equation less than those describing the inactivation of the light-fulls.

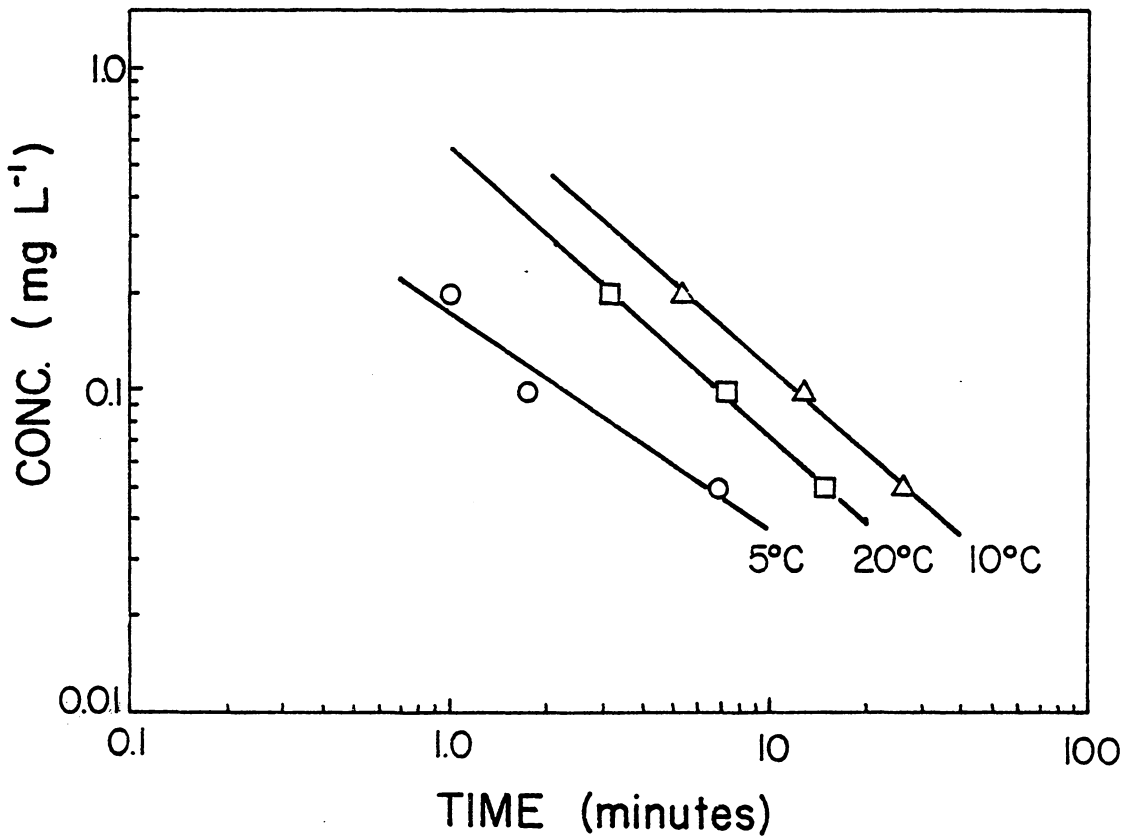


Figure 8. Relationship of time and chlorine concentration for 99 percent inactivation of H-1 light-full (LF) parvoviruses at 5, 10, and 20°C.

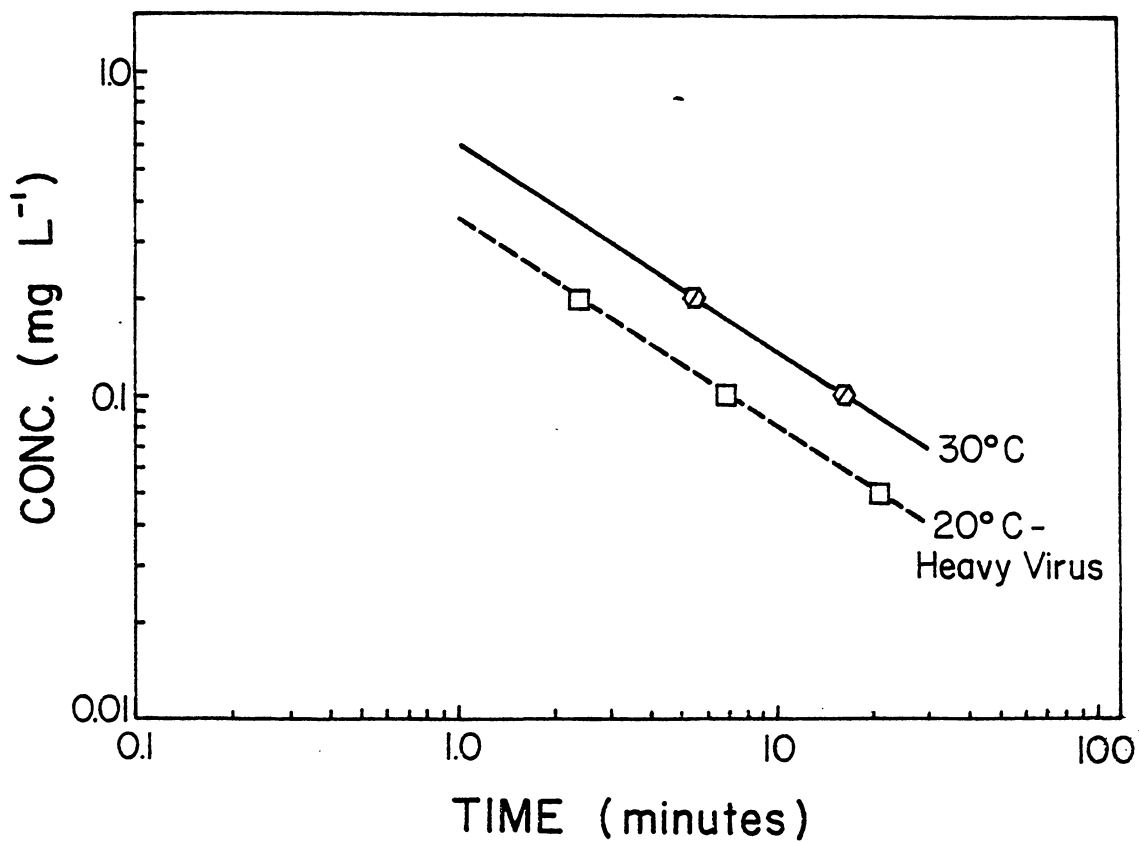


Figure 9. Relationship of time and chlorine concentration for 99 percent inactivation of H-1 light-full (LF) parvoviruses at 30°C, and of H-1 heavy-full (HF) parvoviruses at 20°C.

Table 2 presents the n and k values computed from the inactivation data presented in Figures 8 and 9. These values can be used to predict the time required for 99 percent inactivation at a particular temperature if the concentration of chlorine is known. For example, at 20°C, pH 7, the following formula would apply:

$$C^{1.12} t_{99} = 0.53$$

Although there is some incoherence present in the graphic representations, Table 2 shows an orderly progression of increasing k values for the light-full inactivation data at 10, 20 and 30°C.

Conformation to Chick's Law

The inactivation data of Figures 2-5 were analyzed to determine the degree of conformation to Chick's Law. Chick (1908) determined that the longer the contact time with a disinfectant the greater the kill. The original Chick's Law has since been modified by the constant, m, to the empirical formula (Fair et al., 1971; Metcalf and Eddy, Inc., 1979):

$$\ln \frac{N_t}{N_0} = e^{-kt^m}$$

Where: N_t = number of organisms at time t

N_0 = initial number of organisms at time equal zero

k = constant, time⁻¹

t = time

Table 2. Van't Hoff coefficients of dilution, n , and empirical constants, k , for various temperatures.

Temp, °C	n	Observed k
5	1.49	0.075
10	1.15	0.85
20	1.12	0.53
30	1.54	0.46
20-Heavy-Full	1.55	0.20

$$m = \text{constant}$$

Rearranging, a straight line equation can be developed:

$$\log (-\ln N_t/N_0) = \log k + m \log t$$

where m is the slope of the line and k is the y -coordinate when t is unity. The values of $-\ln N_t/N_0$ versus t were plotted on log-log paper and the straight line of best fit was determined for each chlorine dose by a least squares linear regression analysis. These graphs are presented in Figures 10 to 13, and illustrate the curves generated for the observed inactivations at 5, 10, 20, and 30°C. The slopes of all the lines, regardless of temperature and chlorine concentration, are less than 1.0. The observed k rates are listed in Table 3 as k_{OBS} .

The observed k values in Table 3 (k_{OBS}) were plotted versus chlorine concentration on log-log paper for each temperature. These graphs are presented in Figures 14 and 15. Straight lines of best fit were determined by least squares linear regression analyses, and the resulting lines for 10 and 20°C were found to be approximately parallel (Fig. 14), as were the 5 and 30°C, and 20°C heavy-full lines (Fig. 15). From the straight lines of best fit, new k values were obtained so that any incoherence in the reaction rate data for individual temperatures is minimized. These values are listed in Table 3 under the heading k_{CALC} . It should be noted, however, that the data for 5 and 10°C exhibit a significant amount of deviation from the straight line of best fit.

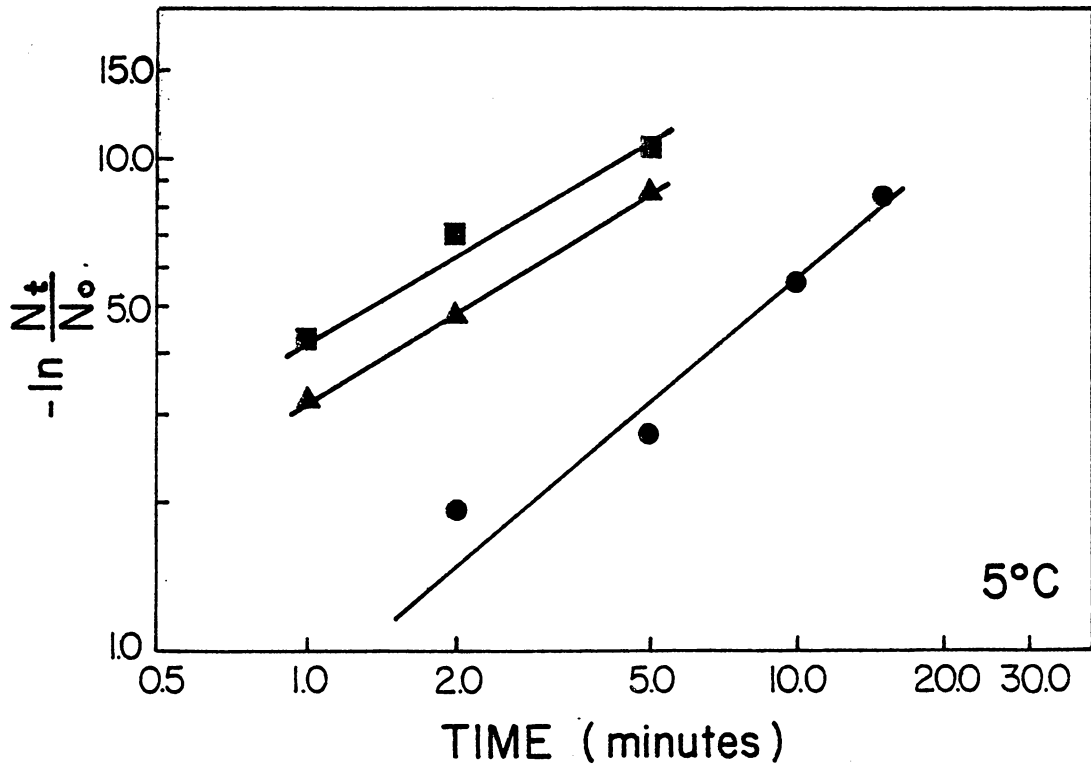


Figure 10. The relationship of the surviving fraction of parvovirus H-1 during chlorine inactivation studies at 5°C with time. (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

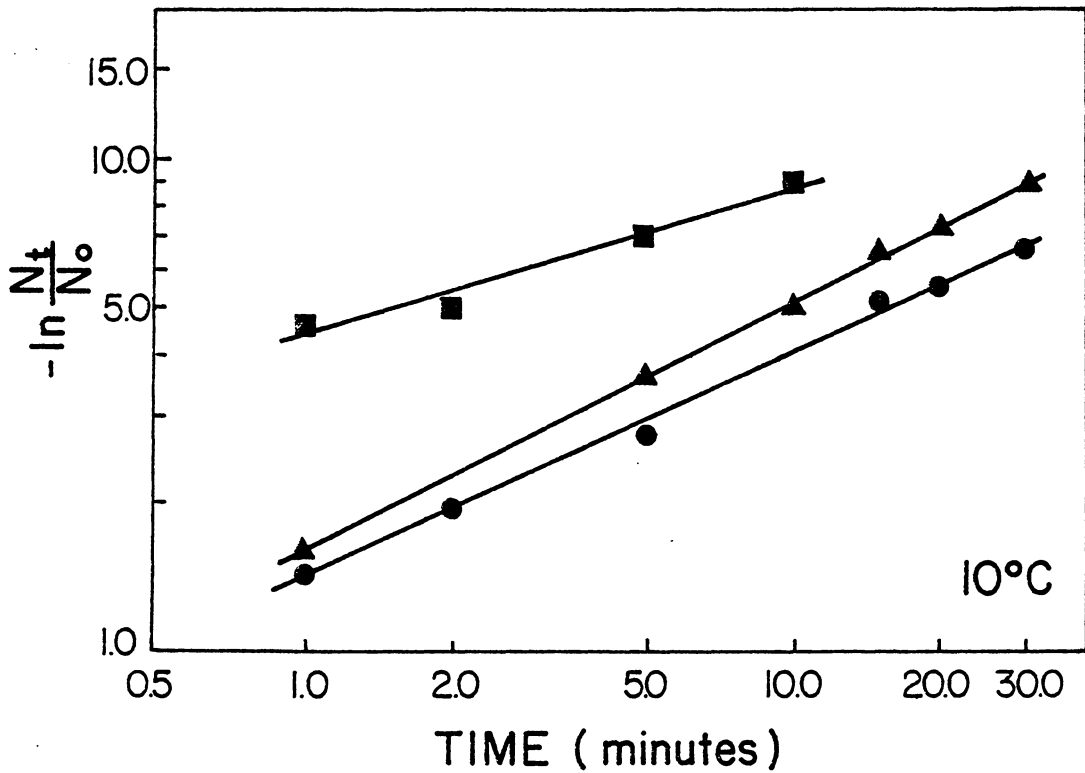


Figure 11. The relationship of the surviving fraction of parvovirus H-1 during chlorine inactivation studies at 10°C with time. (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

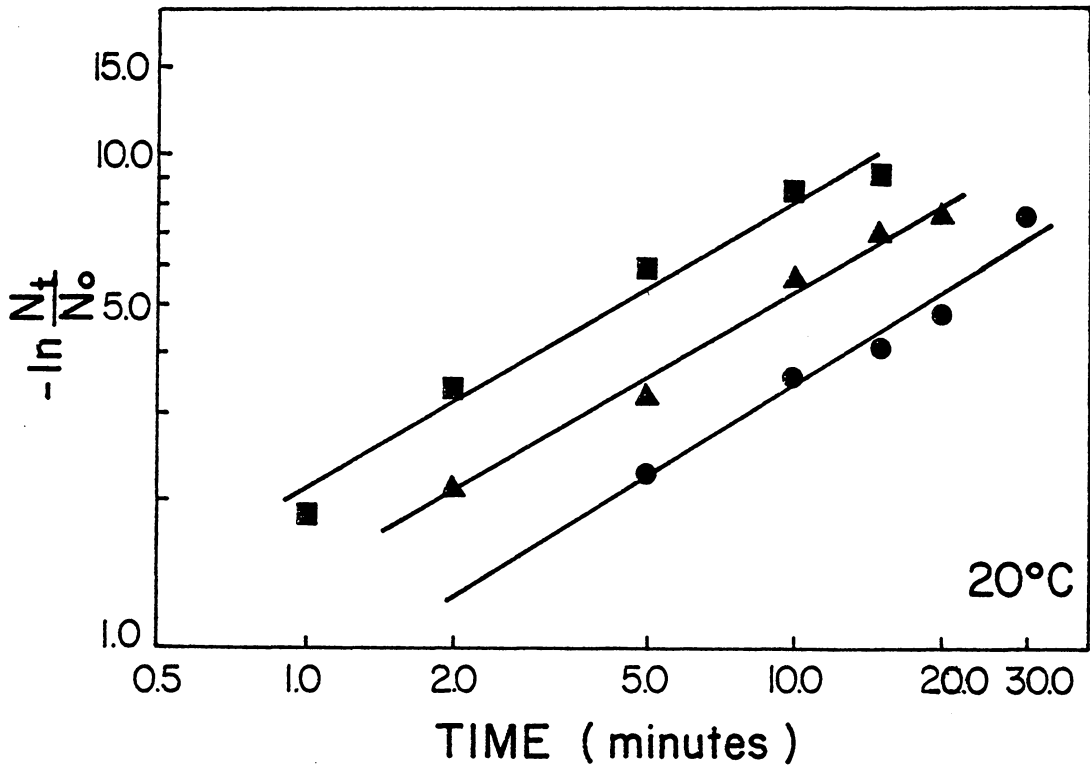


Figure 12. The relationship of the surviving fraction of parvovirus H-1 during chlorine inactivation studies at 20°C with time. (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

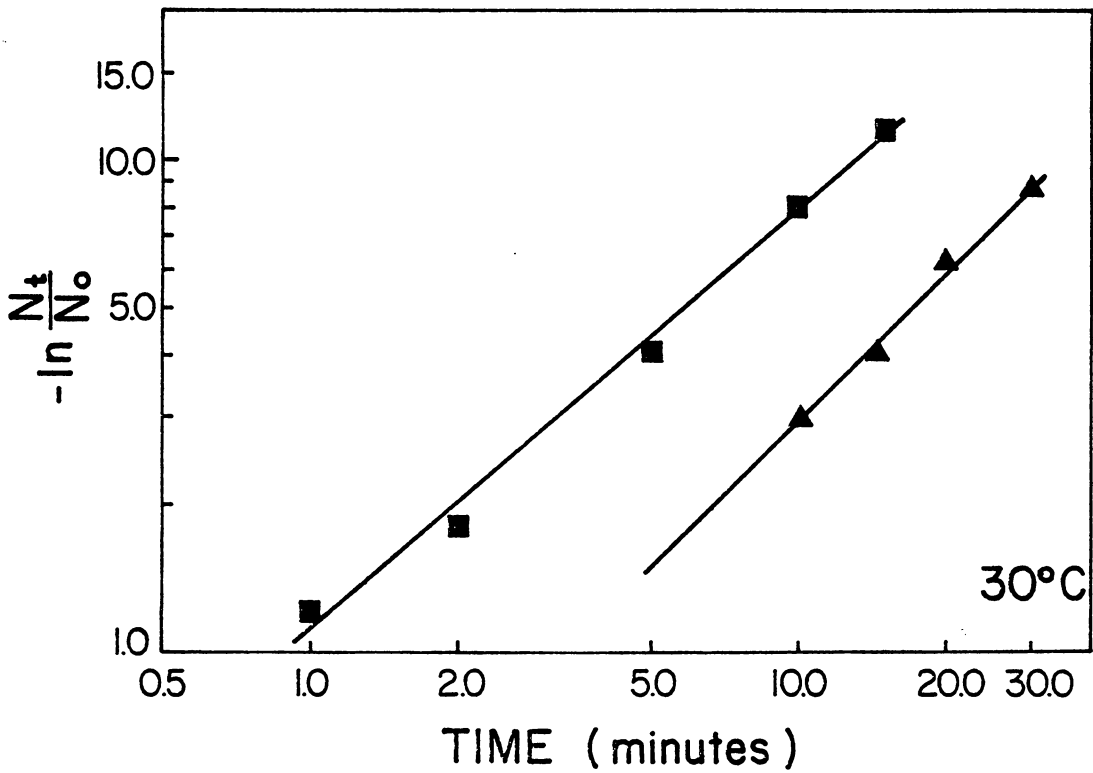


Figure 13. The relationship of the surviving fraction of parvovirus H-1 during chlorine inactivation studies at 30°C with time (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

Table 3. Survival reaction rate constants as described by a modified Chick's Law for data observed and calculated from the line of best fit.

Chlorine Dosage mg L ⁻¹	Temp, °C	k _{OBS} , min ⁻¹	k _{CALC} , min ⁻¹
0.05	5	0.83	0.89
	10	1.42	1.09
	20	0.81	0.82
	30	N.D. ^a	0.08
	20°-Heavy-Full	0.26	0.27
0.10	5	3.21	2.28
	10	1.59	2.14
	20	1.38	1.32
	30	0.30	0.30
	20°-Heavy-Full	0.98	0.84
0.20	5	4.45	5.86
	10	4.35	4.22
	20	2.07	2.12
	30	1.10	1.10
	20°-Heavy-Full	2.40	2.62

^aN.D. = not determined

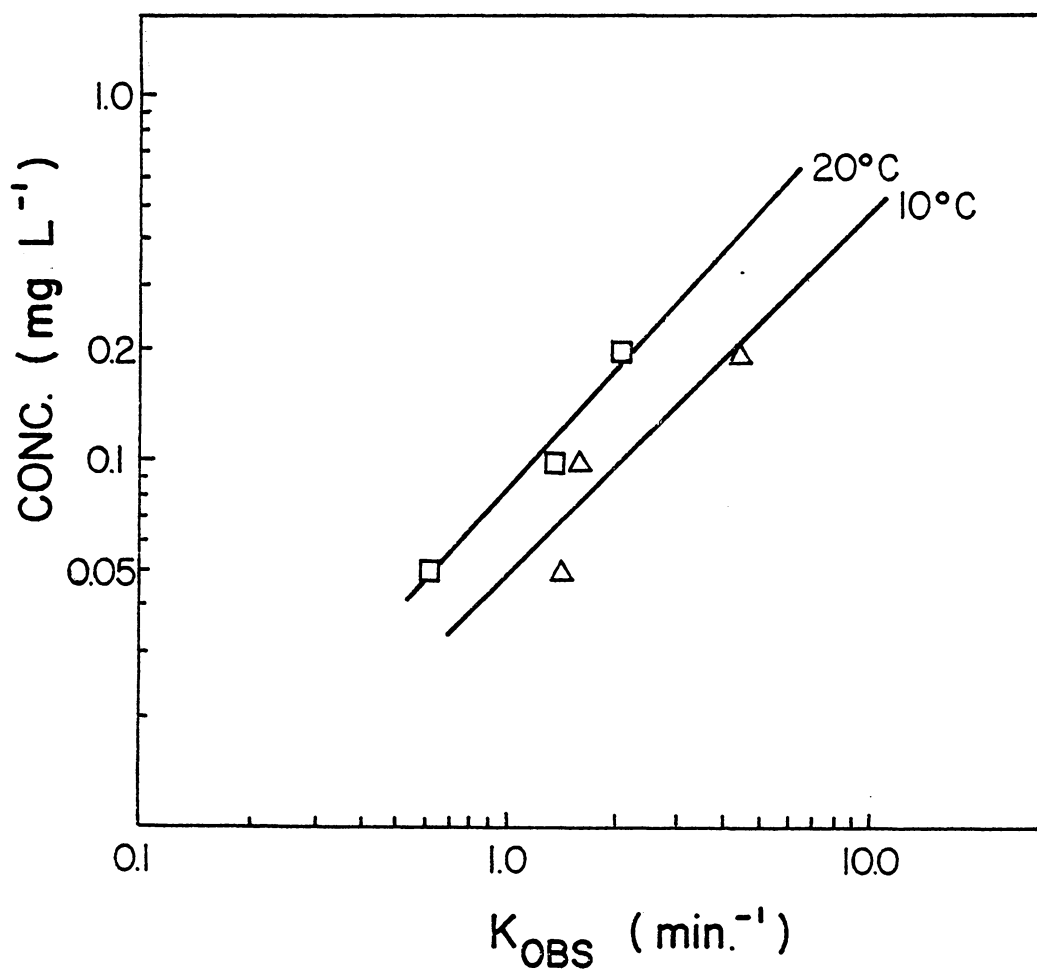


Figure 14. The relationship of the survival rates observed during chlorine inactivation studies of parvovirus H-1 at 10°C (Δ) and 20°C (□) with chlorine concentration.

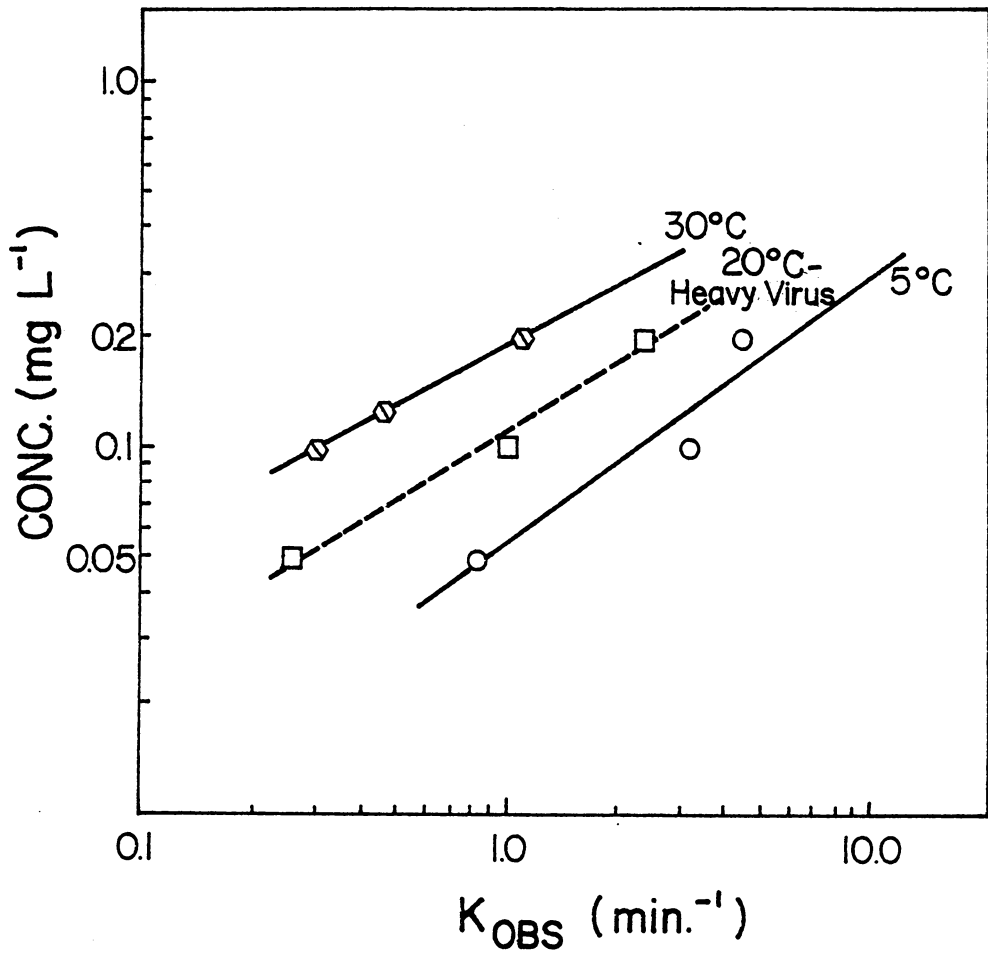


Figure 15. The relationship of the survival rates observed during chlorine inactivation studies of parvovirus H-1 with chlorine concentration. Light-falls at 5°C (○) and 30°C (◊), and heavy-falls at 20°C (◻).

Such deviation could be due either to experimental error or it could be real.

Temperature Effects on Reaction Rate

The classical Arrhenius equation (Hiatt, 1964):

$$k = Ae^{-E_a/RT}$$

Where: k = constant

A = collision factor

E_a = activation energy

R = universal gas constant

T = temperature

can be converted to a straight line formula by taking the natural logarithm of both sides:

$$\ln k = \ln A - E_a/R (1/T)$$

A graph of the $\ln k$ versus the inverse of the absolute temperature should be a straight line with slope equal to $-E_a/R$. Such a graph is presented in Figure 16 using k_{CALC} values in units of minutes^{-1} . Figure 16 presents the effect of temperature on the survival rate of the virus. For example, at 5°C and 0.2 mg L^{-1} chlorine, the survival rate was less than at 10° and 0.2 mg L^{-1} chlorine. This creates the positive slope for the 0.2 mg L^{-1} chlorine curve.

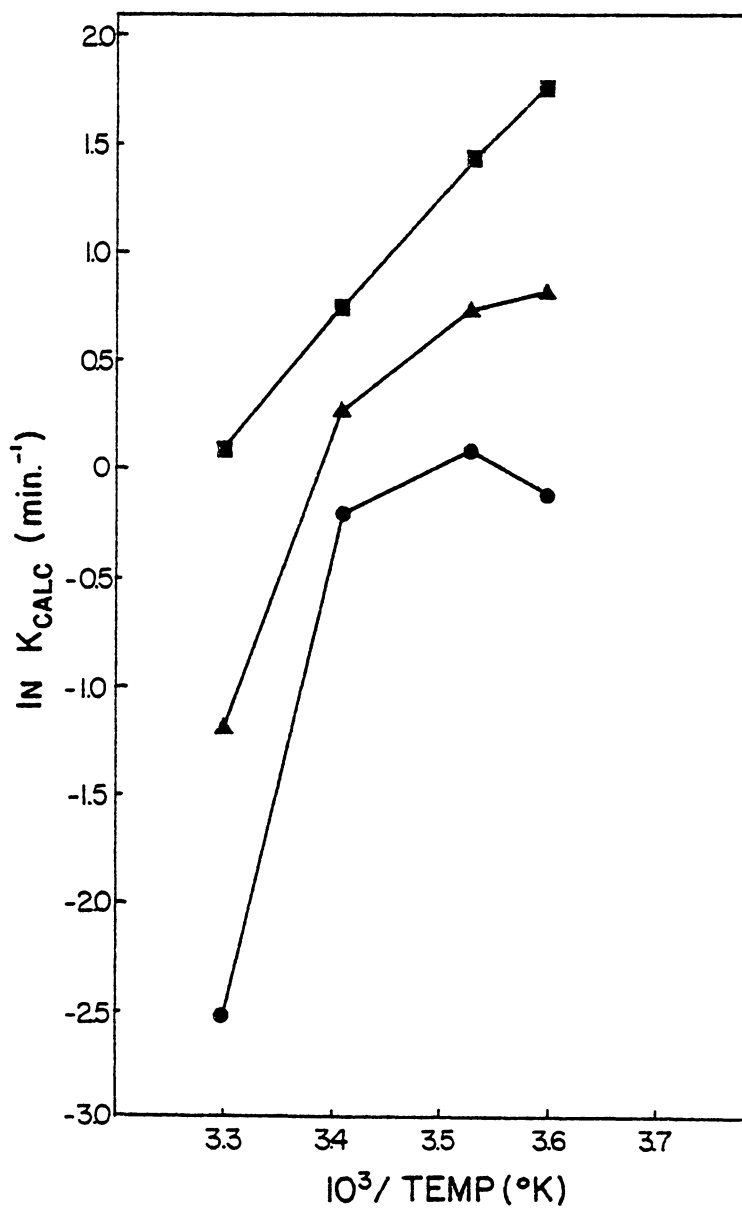


Figure 16. An Arrhenius plot of survival rates versus temperature for each chlorine concentration (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

The k' values for the rate of formation of inactivated virus were calculated in a manner similar to the method used for calculation of survival-rate k values. These rates are presented in Table 4. The effect of temperature on the inactivation rate is illustrated in Figure 17. The curve for 0.05 mg L^{-1} chlorine best demonstrates an effect which is just opposite that of the survival rates. The slope of this curve is $-E_a/R$ which in this case is equal to an energy of activation of 2421 cal/mole. (2.4 Kcal/mole). The apparent anomalies of the other two dose curves presents the calculation of activation energies.

A more rigorous determination of activation energy can be obtained by solving simultaneous Eyring's equations for the 5 and 30°C k' values. Eyring's equation (Hiatt, 1964) states that:

$$\ln K = \ln (K_0 T_i/h) + S/R - E_a/RT_i$$

Where; K = rate constant, min^{-1}
 K_0 = Boltzmann's constant
 T_i = absolute temperature
 h = Planck's constant
 S = entropy
 R = universal gas constant
 E_a = activation energy

In good agreement with the graphical determination of $E_a = 2.4 \text{ Kcal mole}^{-1}$, Eyring's equation yields an E_a of $2.0 \text{ Kcal mole}^{-1}$ for the k'

Table 4. Inactivation reaction rate constants as described by a modified Chick's Law for data observed and calculated from the line of best fit.

Chlorine Dosage mg L ⁻¹	Temp, °C	k' _{OBS} , min ⁻¹	k' _{CALC} , min ⁻¹
0.05	5	0.62	0.63
	10	0.69	0.68
	20	0.83 ^a	0.79
	30	N.D. ^a	0.90
0.10	5	0.97	0.84
	10	0.79	0.80
	20	0.81	0.84
	30	0.93	0.93
0.20	5	0.99	1.13
	10	0.96	0.95
	20	0.89	0.90
	30	0.96	0.96

^aN.D. = not determined

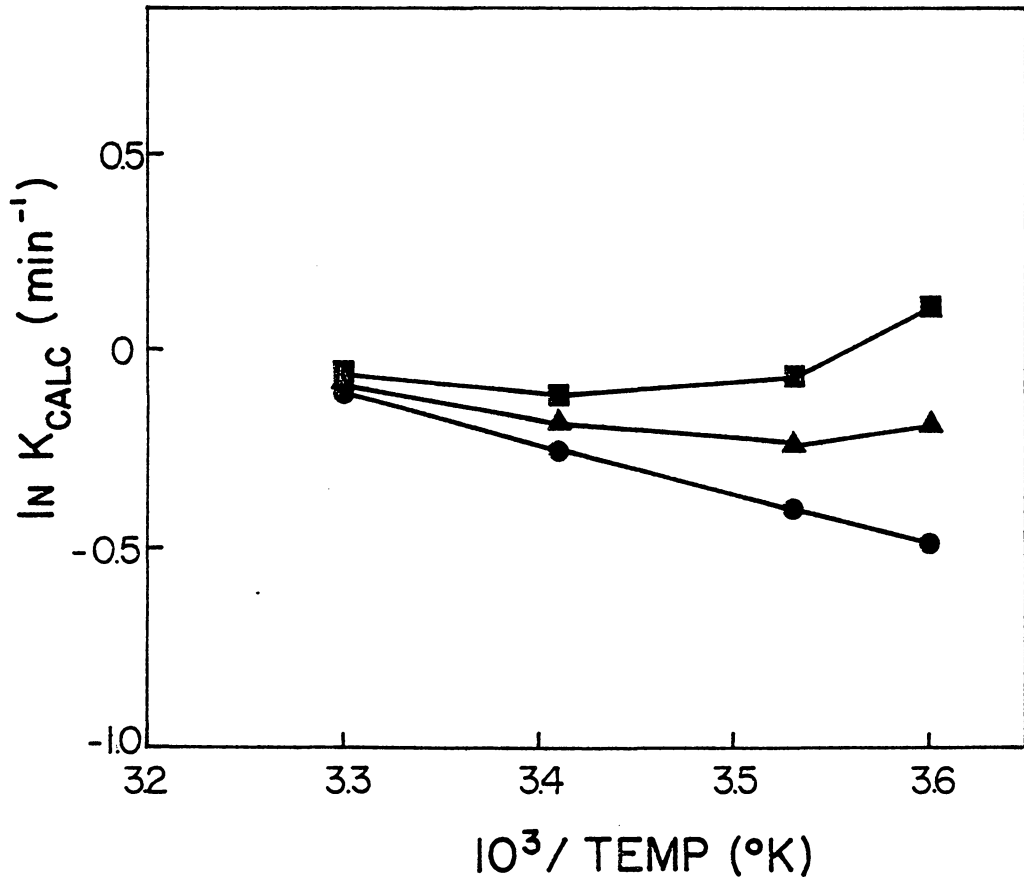


Figure 17. An Arrhenius plot of inactivation rates versus temperature for each chlorine concentration (● 0.05 mg L⁻¹ NaOCl, ▲ 0.1 mg L⁻¹, ■ 0.2 mg L⁻¹).

values of 0.05 mg L^{-1} over the temperature range of 5 to 30°C . The change in entropy was also determined and found to be -52.34 entropy units.

Discussion

The inactivation curves presented in Figures 2-5 clearly illustrate the usual dose-response relationship of an enteric virus, in this case, parvovirus H-1 (light-fulls), to treatment with NaOCl. For example, the greater the chlorine dose the faster the inactivation rate at any specific temperature. Also, for any particular chlorine dose, as contact time increases, the rate of inactivation increases. Many of the inactivation curves, however, exhibit a progressive "tail-away" effect in relation to the time of contact. This is characteristic of varying reaction rates, possibly due to either the presence of resistant virus particles, certain interferences, or complex reaction mechanisms (Fair, *et al.*, 1971). The protection of surviving viruses by aggregation is also a possibility.

The effect of chlorine concentration on inactivation rates at each temperature was examined by fitting the data to the van't Hoff's equation. The slopes of the resulting lines of best fit were equal to $-1/n$, where n , a dilution factor, is indicative of the order of reaction. The similar slopes existing for 10 and 20°C (1.15 and 1.12, respectively) support the premise that the chlorine concentration and time of contact were of equal importance at these two temperatures, and

that the reaction rate was first-order. However, the curves for 5 and 30°C indicate that, at these temperatures, the lower chlorine concentrations had less inactivation potential. This also holds true for the heavy-full particle type of H-1 (Figures 6 and 9). The heavy-full particle type differs from the light-full particle type by the configuration of viral protein 2 (VP2). The conversion of heavy to light particles occurs when certain bonds of VP2' are cleaved to form a slightly smaller protein, VP2 (Paradiso, 1981). Both particles are equally infectious, even though they differ in protein content and density. The lines representing the 5 and 30°C data (Figure 15) for the light-full virions are parallel to the 20°C data for the heavy-full virions. This could be interpreted to mean that the capsid protein of the light-full virions are affected by temperature to an extent that they react to chlorine at 5 and 30°C in a manner similar to the heavy-full virions at 20°C.

There are two possible explanations for this apparent thermodynamic anomaly which is best presented in Figure 8. It is possible that an added sucrose-gradient step in the purification of later virus preparations could have removed some chlorine-inhibiting or chlorine-demanding material found in earlier preparations. Because there was never a detectable chlorine demand with any of the virus preparations, it was felt that this was not the case. A more feasible explanation to this investigator would be related to the effect of temperature on the protein configuration of the viral capsid. It is possible that the

colder temperature might have affected the relationship of the capsid proteins with the surrounding medium. This effect could have possibly altered the spatial arrangement of the capsid proteins in a manner so that inactivation sites were either more exposed or more reactive. Admittedly, however, this latter explanation would need experimental verification moreso than the former.

If the virus has only one inactivation site and requires just a single hit by chlorine to be inactivated, then the reaction between virus and chlorine, as defined by Chick's Law (1908), is of first-order. Because the number of surviving viruses is proportional to the number of inactivated viruses, the graphic presentation of the inactivation data based on Chick's Law normally utilizes this surviving-fraction term. If the inactivation reaction was first-order, it would have a slope of one, but, as presented in Figures 10-13, the slopes of the lines of best fit are all less than one. Again, this is indicative of declining reaction rates with time. Not surprisingly, the inactivation data does not fit Chick's Law. It does imply, however, that either there are a number of inactivation sites on an individual virion, all of which must be hit to cause inactivation, or multiple hits of a single inactivating site are required for inactivation.

A correlation between the inactivation kinetics and the mechanism of inactivation is illustrated more clearly upon examination of the reaction rate dependence on temperature (Figure 15). A typical Arrhenius plot of the natural logarithms of k_{CALC} values in Table 3

versus the inverse of temperature shows an unexpected amount of data scattering at the lower chlorine doses of 0.05 and 0.10 mg L⁻¹. The gross variations in the slope of the line connecting these points would be indicative of more than one reaction mechanism if viewed in terms of textbook chemical kinetics. Although experimental error was possibly greater at the low concentrations of chlorine compared to that at the higher concentrations, the orderly progression in the increasing linearity of the data from 0.05 mg L⁻¹ to 0.2 mg L⁻¹ is believed to be significant. This indirectly suggests that minor events causing inactivation at the lower dose of chlorine are preempted by a major event that is created by an increase in the amount of chlorine. Such a major event may merely be the summation of all minor events due to the abundance of chlorine in contact with the virion.

The summation effect can be graphically demonstrated by an Arrhenius plot of the k'_{CALC} rates of inactivation from Table 4 (Figure 16) rather than survival k_{CALC} rates, as in Figure 15. The curve for 0.05 mg L⁻¹ of chlorine was rather straight indicating only one reaction mechanism. The slope of this line is equal to $-E_a/R$; and so the energy of activation was calculated to be approximately 2.4 Kcal mole⁻¹. As the chlorine dose was increased, the variations in slope of lines drawn between the data points indicated a diversity of reaction mechanisms, which were denoted earlier as a summation of reactions into one major event. For this reason, no attempt was made to determine a single activation energy for the higher doses.

By solving simultaneous Eyring's equations for the 5 and 30°C k'_{CALC} values, both the inactivation energy and change in entropy could be determined. The E_a of 2.0 Kcal mole⁻¹ obtained was in good agreement with the graphical result of 2.4 kcal mole⁻¹. The negative entropy value indicates that a more orderly state of the virus has been created.

IV. MECHANISM OF CHLORINE INACTIVATION OF H-1 PARVOVIRUS

Introduction

In 1968, a waterborne pathogen, later determined to be a parvovirus-like agent, caused an outbreak of gastroenteritis in Norwalk, Ohio (CDC, 1977). A recent epidemiological report on waterborne outbreaks of disease since then (Craun, 1981) has implicated a similar agent as being responsible for five outbreaks of waterborne gastroenteritis during the period between 1971 and 1978. Four out 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. Elucidation of inactivation mechanisms perhaps will aid in the optimization of chlorine disinfection of drinking water supplies. They have dealt, however, with only the RNA-containing poliovirus (Tenno, et al., 1979; O'Brien and Newman, 1979; Gowda, et al., 1981; Alvarez and O'Brien, 1982).

The relative simplicity of enteric viruses accomodates them to one of three possible mechanisms of inactivation: 1) an alteration of the protein capsid, 2) an alteration of the nucleic acid, or 3) a combination of both modes. Tenno and coworkers (1979), and more recently Alvarez and O'Brien (1982), have determined that the mode of action of chlorine on poliovirus was 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 the DNA-containing ones, remains to be ascertained.

The experimentation described here utilized a small DNA-containing enteric virus. The parvovirus H-1 is a putative human virus (Toolan, 1968) 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 (Chapter III) and Kilham rat virus (Englebrecht, 1981) have been determined. This investigation determined the effects of chlorine on the whole virion, the viral protein, and the extracted viral nucleic acid.

Materials and Methods

Common Procedures

The propagation of parvovirus H-1 was performed as described in Chapter III with one exception. At 10 to 12 hours post-infection (PI), the virus was radioactively labeled in either the protein capsid or the nucleic acid by adding 0.25 millicurie (mCi) per roller bottle of tritiated methione ($^3\text{H-Met}$) or tritiated thymidine ($^3\text{H-TdR}$, New England Nuclear, Boston, MA), respectively. Both the purification and enumeration procedures also described earlier were followed in the experiments performed in this chapter.

The PBS, sodium hypochlorite, and sodium thiosulfate solutions were made in the same manner. A secondary stock solution was prepared from

the NaOCl primary stock solution by further dilution with chlorine demand-free PBS. These secondary stock solutions were designed to provide desired NaOCl residuals when mixed with either 50 or 100 microliters (μl) of virus stock. A final volume of 300 μl was always utilized in these "micro" experiments and the NaOCl dosages ranged from 1 to 5 mg L^{-1} for various experiments. Plastic "micro" tubes (Brinkmann Instruments, Westbury, Ny) were soaked in 10 percent (v/v) HCl, filled with household bleach, and rinsed with distilled water.

Sucrose Gradient Analysis

Sedimentation-rate analysis of control and chlorine-treated whole virions was performed by rate zonal centrifugation. Samples were layered onto 5 to 30 percent neutral sucrose gradients (50 mM TRIS-HCl, 0.5 mM Na_2 EDTA, pH 8.7). These were centrifuged for two hours at 41,000 rpm and 4°C. Gradients were fractionated from the bottom by needle puncture, and approximately 0.5 ml (12 drops) fractions were collected. The radioactivity associated with each fraction was determined by liquid scintillation spectrophotometry (Beckman Model LS7500). In some instances, when the fraction was to undergo further analysis, only 2.5 to 10 percent of the fraction volume was counted.

Rate zonal centrifugation of the DNA from both control and chlorine-treated whole virions was performed on either 5 to 20 percent or 5 to 30 percent alkaline sucrose gradients (0.3 M NaOH, 0.7 M NaCl,

1 mM Na₂ EDTA, 0.15 percent sarkosyl, pH 12.1). Samples to be analyzed were made 0.25 M NaOH and incubated at 37°C for 10 minutes for protein digestion prior to centrifugation. The 5 to 30 percent sucrose gradients were centrifuged in an SW41 rotor for 15 hours, at 30,000 rpm and 20°C; and the 5 to 20 percent sucrose gradients were centrifuged in an SW50.1 rotor for four hours, at 48,000 rpm, and 5°C. Gradients were fractionated into either 0.4 ml (22 drops) or 0.175 ml (8 drops) fractions, respectively. Prior to measuring the radioactivity associated with each fraction either the entire fraction or an aliquot of 10 percent 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 N₂ and resuspended in 20 to 40 µl Laemmli application buffer (Laemmli, 1970).

Polyacrylamide gels were run by the discontinuous gel system of Laemmli (1970). Electrophoresis was performed using a constant current of 10 milliamperes (mA) for the stacking gel and 25 mA for the resolving gel. Acrylamide (7.5 percent acrylamide, 0.193 percent bis-acrylamide) gels were prepared for fluorography by the method of Bonner and Laskey (1974). Gels were dried onto Whatman filter paper and exposed to Kodak

X-omat R film with the aid of a DuPont Cronex Lightning-Plus intensifying screen at -80°C .

Molecular weights of unknowns were determined by the method of Shapiro et al., (1967). Marker proteins included phosphorylase A (92,500d), human transferrin (80,000d), a bovine serum albumin (67,000d), catalase (55,000d), ovalbumin (45,000d), and carbonic anhydrase (30,000d)(Sigma).

Analysis of nucleic acids was performed by electrophoresis in 1.4 percent agarose (BRL or Sea-Kem LE) using a tris-sodium acetate buffer (40 mM TRIS-HOAc, 20 mM sodium acetate, and 2 mM EDTA, pH 8.3). The pelleted DNA samples were dried under N_2 , and resuspended in 20 μl distilled H_2O , 4 μl Ficoll, 1.25 μl bromphenol blue, and 1.0 μl of 20 percent (w/v) sodium dodecyl sulfate (SDS).

DNA Isolation, Replication, and Electrophoresis

Tritiated thymidine labeled H-1 whole virions were subjected to high pH by making samples 0.3 M NaOH (pH 12.1) and incubated at 37°C for 10 minutes. The labeled DNA was banded in 5 to 20 percent alkaline sucrose gradients as described previously, and found by determination of radioactivity. Peak fractions were pooled and dialyzed overnight against TE (50 mM TRIS-HCl, 0.5 mM EDTA, pH 8.0). The DNA in each sample was precipitated at -80°C by adding 2.5 volumes of cold ethanol after making the sample 0.3 M sodium acetate, pH 5.2, and adding transfer RNA to a final concentration of $50 \mu\text{g ml}^{-1}$. Samples were

centrifuged for 30 minutes in a microcentrifuge (Brinkmann Instruments, Eppendorf Div., Westbury, NY, Model #5412).

The resulting pellets were then resuspended in 100 μ l of a reaction mixture for replication. This mixture consisted of 50 mM TRIS-HCl, pH 8.0; 50 mM NaCl; 10 mM MgCl₂; 80 μ M dGTP, dATP, and dTTP; 20 μ M dCTP; 25 μ g BSA; [-³²P] dCTP; and distilled water to volume. Then one unit of Klenow DNA polymerase I was added and the samples incubated for one hour at 37°C. Replication was terminated 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 by Neal and Florini (1973). The samples were made 0.3 M sodium acetate, pH 5.2, and the DNA ethanol precipitated as before.

Pellets were dried under N₂ and resuspended in electrophoresis application buffer [20 μ l distilled H₂O, 4 μ l Ficol, 1.25 μ l bromphenol blue, and 0.1 μ l of 20 percent (w/v) SDS]. Samples were loaded onto a 1.4 percent agarose (Bethesda Research Laboratories) gel and subjected to electrophoresis at 75 to 90 mV for 2.75 hours. The gel was blotted dry and exposed to Kodak X-omat R film (Kodak, Rochester, NY) at -80°C with the aid of a DuPont Cronex Lightning-Plus intensifying screen. Both single- and double-stranded DNA from bovine parvovirus were used as markers.

Restriction Endonuclease Treatment

The restriction endonucleases (Bethesda Research Laboratories) Eco RI, Pst I, and Bcl I were used to cut the double-stranded, replicated DNA. Conditions for each reaction were those recommended by the manufacturer. The assay buffer and assay temperature for Eco RI was 100 mM TRIS-HCl (pH 7.2), 5 mM MgCl₂, 2 mM 2-mercaptoethanol, and 50 mM NaCl at 37°C; for Pst I it was 20 mM TRIS-HCl (pH 7.5), 10 mM MgCl₂, 50 mM (NH₄)₂SO₄, and 100 µg ml⁻¹ BSA at 37°C; and for Bcl I it was 12 mM TRIS-HCl (pH 7.4), 12 mM MgCl₂, 12 mM NaCl, and 0.05 mM dithiothreitol at 50°C. Samples were incubated for 1 to 1.5 hours at the required temperature. Then, the fragmented DNA was ethanol precipitated as previously described. Analysis of the resulting fragments was performed by electrophoresis on 1.4 percent agarose gels following the same procedure as outlined for double-stranded DNA analysis. Autoradiography was also performed as previously described.

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 washed twice with Dulbecco's solution. Radioactively labeled virus [³H]-Met) samples, both chlorine-treated and untreated, were diluted with PBS to a final volume of 0.5 ml. The entire sample was placed onto the washed monolayers and allowed to absorb for 60 minutes with agitation at 15 minute 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 solutions. The filters were dried under a heat lamp, then placed in a toluene cocktail for radioactive assay by liquid scintillation spectrophotometry (Beckman, Model LS7500).

Results

Sedimentation Rate Analysis

The effect of chlorine on H-1 was investigated by exposing [³H]-methionine labeled virions to doses of NaOCl ranging from 1 to 5 mg L⁻¹ for various exposure times. The change in sedimentation rate of the treated virus was then determined by rate zonal centrifugation using 5 to 30 percent neutral sucrose gradients. Treatment of the capsid labeled virus with 1 mg L⁻¹ NaOCl for 60 minutes revealed the formation of a new particle type located in fraction 18 (Figure 18, Panel B). By increasing the NaOCl dosage to 5 mg L⁻¹, the enhancement of the formation of the new particle type by increasing exposure times is readily apparent (Figure 18, Panels C and D). Total conversion of the full virion to this new particle could be achieved by chlorinating at 5 mg L⁻¹ NaOCl for 60 minutes (Figure 19). The sedimentation coefficients for full virions, empty particles, and the new particle type were estimated to be approximately 116S, 73S, and 43S by the method of Griffith (1976). The sedimentation rate coefficients for the full

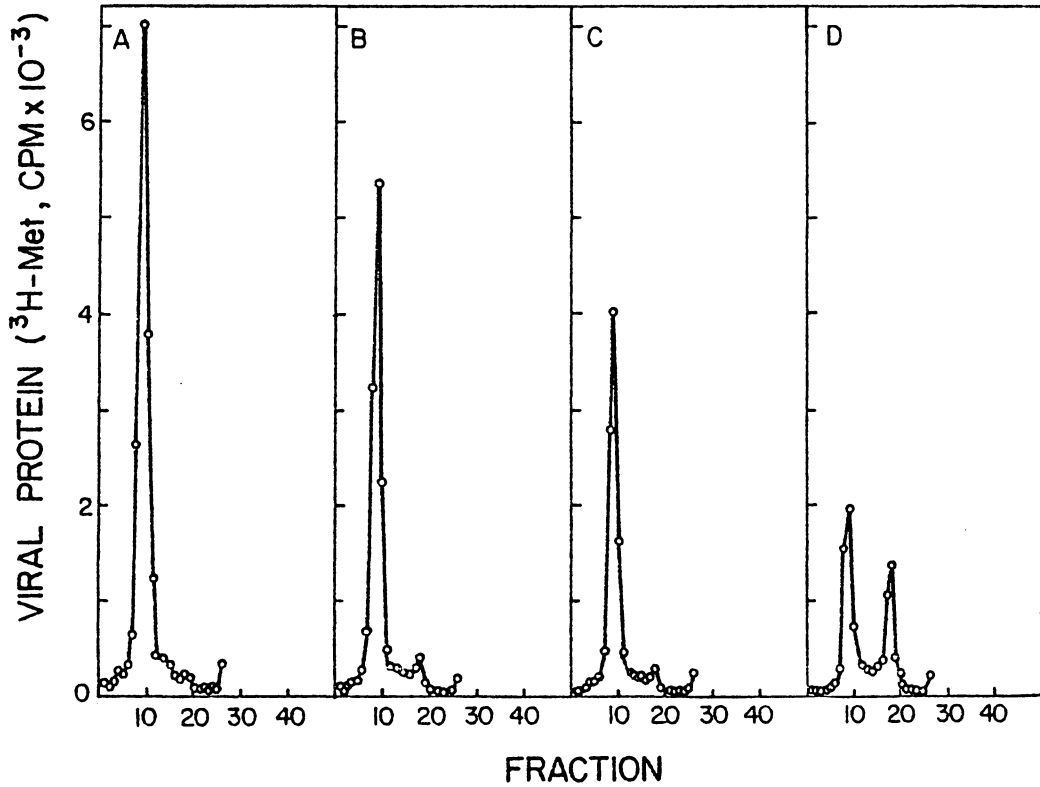


Figure 18. Sedimentation rate analysis of chlorine-treated, [³H]-Met labeled, whole virus by a 5 to 30 percent neutral sucrose gradient. Panel A: Control; Panel B: 1 mg L⁻¹ NaOCl, 60 min. exposure; Panel C: 5 mg L⁻¹ NaOCl, 5 min exposure; Panel D: 5 mg L⁻¹ NaOCl, 60 min exposure.

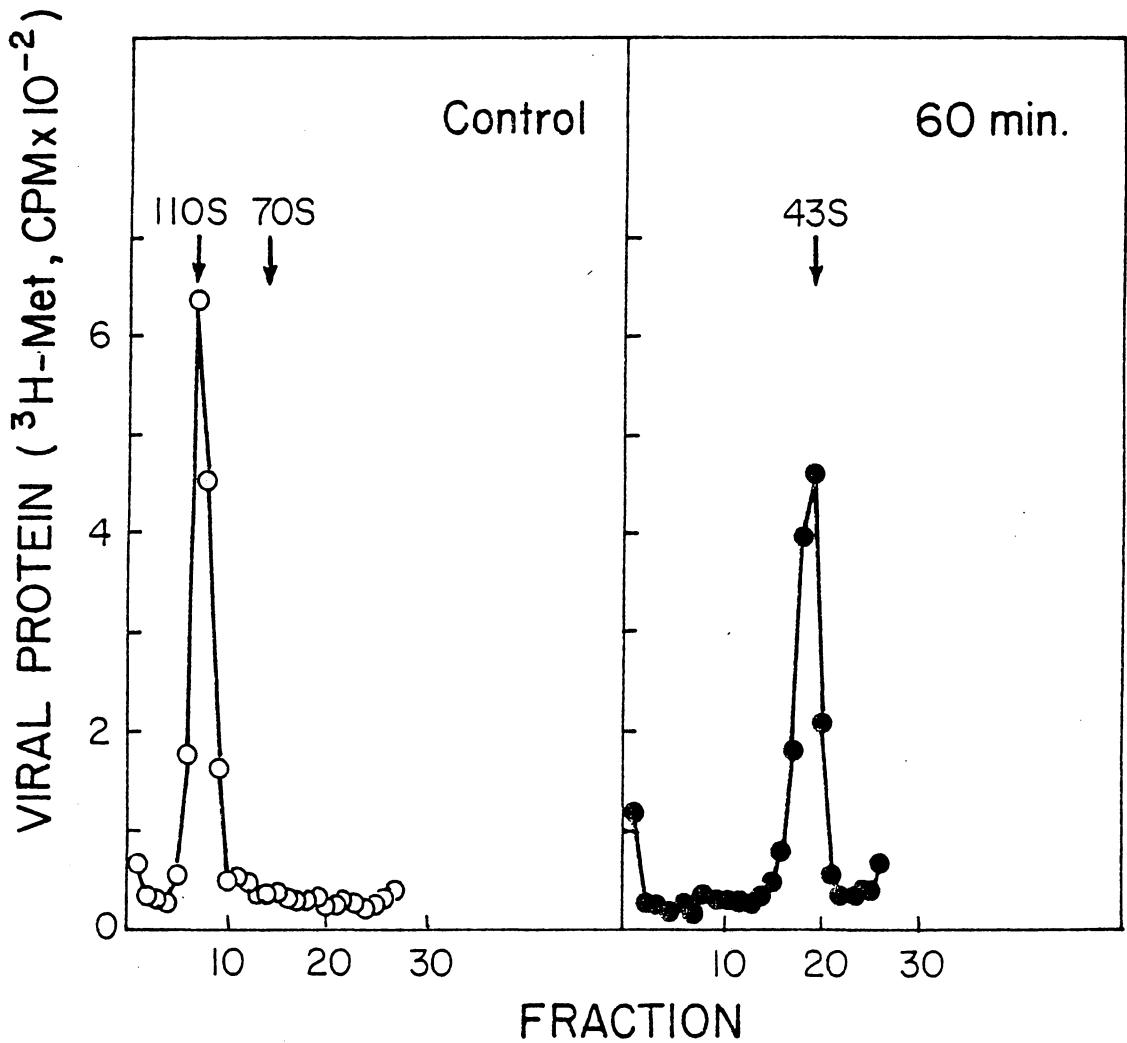


Figure 19. Sedimentation rate analysis of chlorine-treated, $[^3\text{H}]$ -Met labeled, whole virus by a 5 to 30 percent neutral sucrose gradient. Left Panel: Control showing location of 110S and 70S (empty capsid) particles; Right Panel: 5 mg L^{-1} NaOCl , 60 min exposure.

virions agree well Salzman (1978). Chlorine, at the levels used in experiments with full virions, caused no alteration in the sedimentation rate of empty H-1 capsids. The graphic presentation of the radioactivity detected in the gradient fractions of the control and the chlorine-treated empty capsids were similar to one another, with the peak of radioactivity associated with fraction 14 in both.

Because these experiments reveal only what occurs with the protein component of the virus, the same protocol was used to determine what would happen to [³H]-thymidine labeled virus. Figure 20 shows that the same results were obtained indicating that the 43S peak contains not only protein but also nucleic acid.

Infectivity Assay of Peak Fractions

The peak fractions in Panels A and B, Figure 18, and in Figure 19, were then plaque assayed for infectivity, the results of which are shown in Table 5. Treatment of the virus with 1 mg L⁻¹ NaOCl for 60 minutes caused a 3-log decrease in titer, and there was no infective particles in fraction 18 of the new peak. The infective virus titer of the individual fractions 7, 8, and 9 of the control in Figure 20 ranged from 10⁶ to 10⁷ PFU mL⁻¹. There was no infectivity in the corresponding fractions of the virus that had been treated with 5 mg L⁻¹ NaOCl for 60 minutes. Also, there were no infective particles associated with the 43S peak, even though both the capsid protein and nucleic acid were present in this peak.

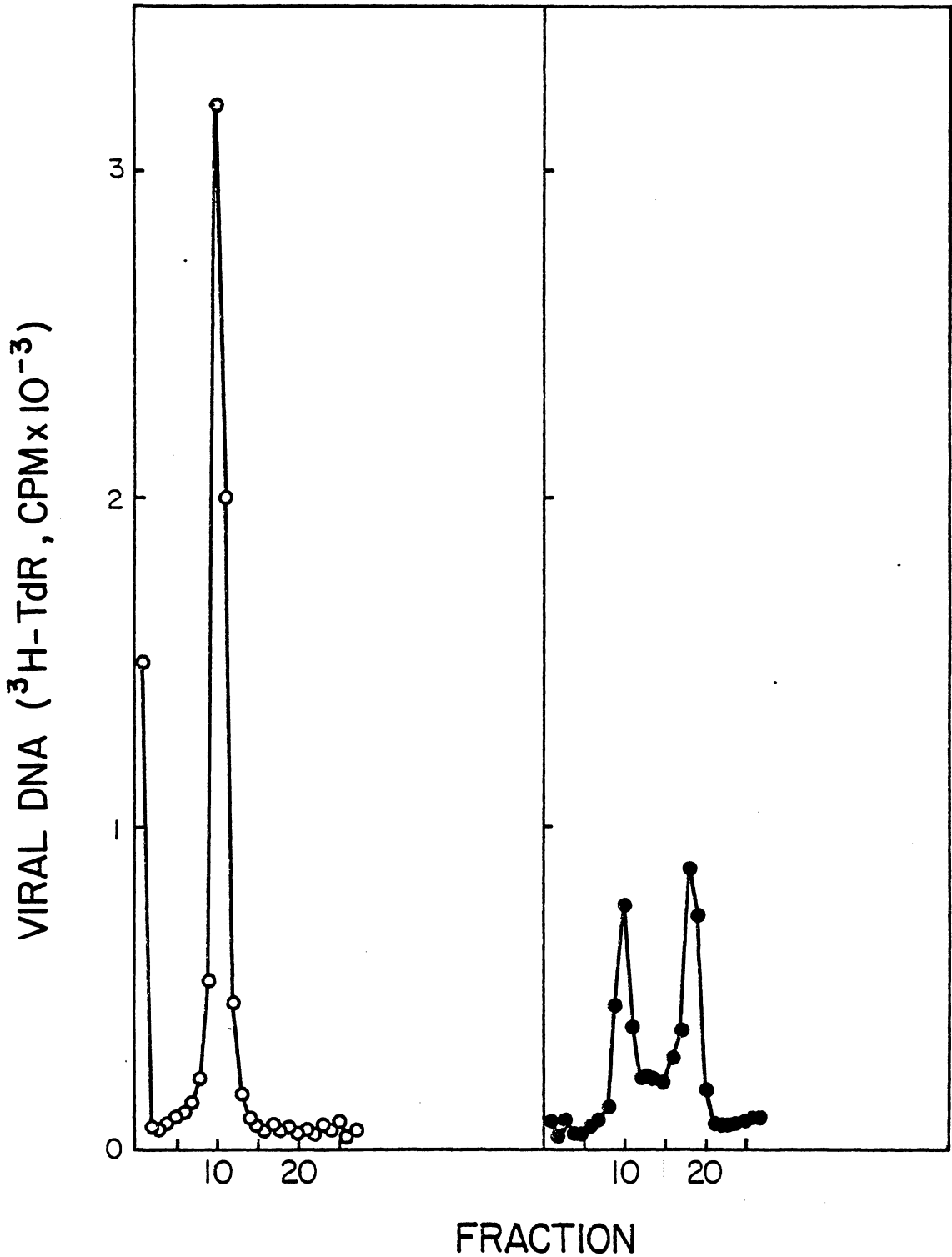


Figure 20. Sedimentation rate analysis of chlorine-treated, [³H]-TdR labeled, whole virus by a 5 to 30 percent neutral sucrose gradient. Left Panel: Control; Right Panel: 5 mg L⁻¹ NaOCl, 60 min exposure.

Table 5. Infectivity Assay of Peak Fractions

Sample	Fraction	PFU ml ⁻¹
Control	8	1.8 x 10 ⁸
1 mg L ⁻¹ , 60 min	9	8.0 x 10 ⁴
	18	0
Control	7	1.6 x 10 ⁷
	8	4.4 x 10 ⁶
	9	2.8 x 10 ⁶
5 mg L ⁻¹ , 60 min	7	0
	8	0
	9	0
	18	0
	19	0
	20	0

Electron Microscopy

To determine what occurred during 60-minute exposure to 5 mg L^{-1} of NaOCl, the virus was observed at various time intervals by electron microscopy. Treated virions were allowed to electrostatically attach to grids and were negatively stained with one percent uranyl acetate. Figure 21 shows electron micrographs taken of the control (Panel A), the virus exposed for 10 minutes (Panel B), and the virus exposed for 60 minutes (Panel C). The micrograph of the virus exposed for 10 minutes revealed that the protein capsid has been altered to such an extent that the uranyl acetate stain had entered the particle and made it appear dark. Conspicuous, however, are the tail-like extrusions from the ruptured particles (indicated by the arrow). Although there was some aggregation after 10 minutes exposure, after 60 minutes aggregation was so extensive that the tail-like extrusions were no longer discernible. It was thought that this extrusion was a strand of DNA which had been released from the viral particle.

Protein Analysis by SDS-Polyacrylamide Electrophoresis

The effect of chlorine on the protein component was investigated further. Tritiated methionine-labeled H-1 was treated with varying doses of NaOCl ranging in dose from 1 to 5 mg L^{-1} for exposure times of less than 5 minutes. The chlorine was neutralized by addition of sodium thiosulfate. The samples were adjusted to 0.3 M sodium acetate, pH 5.2,

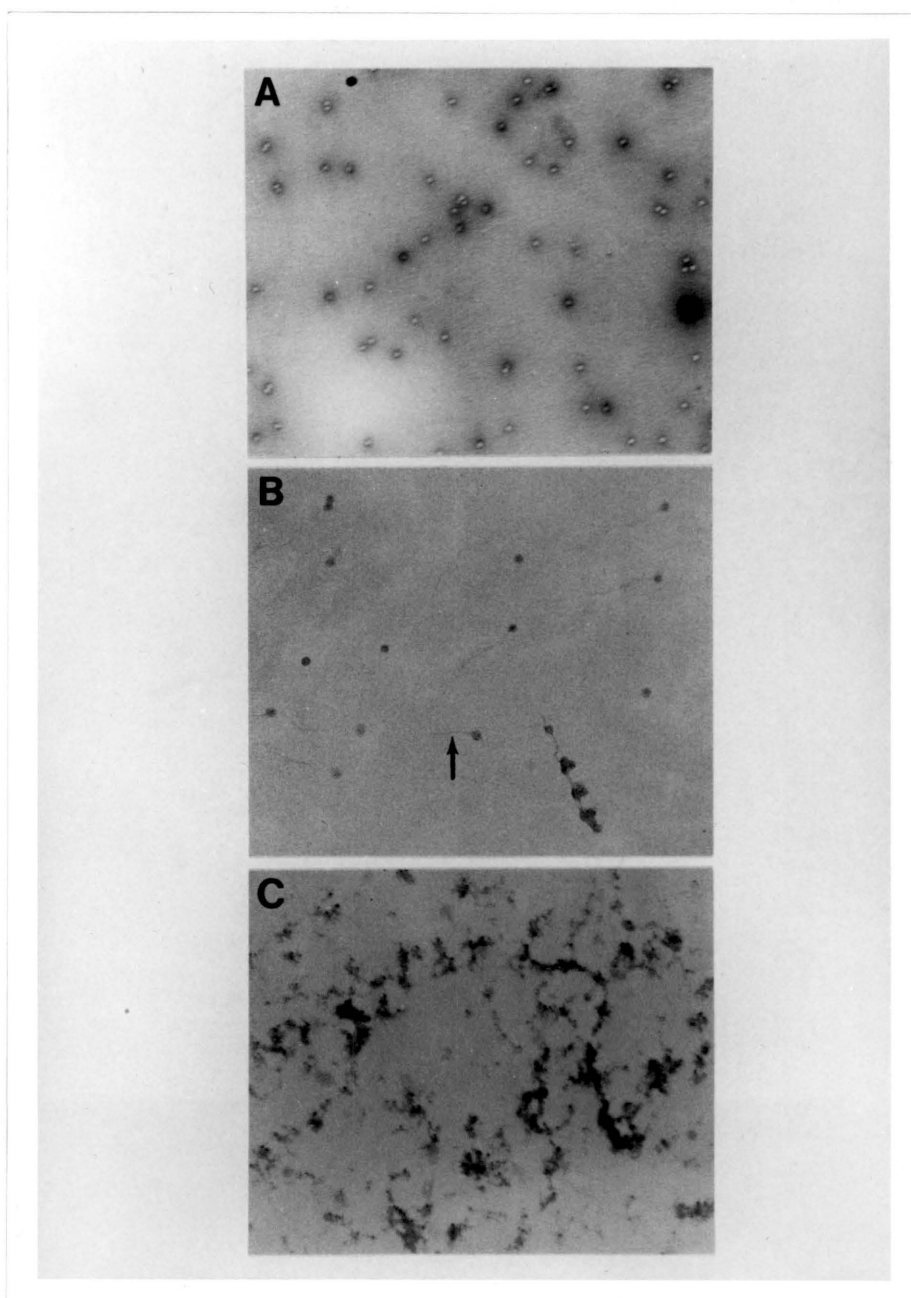


Figure 21. Electron micrographs of negatively stained untreated parvovirus H-1 and virus treated with 5 mg L^{-1} NaOCl for: 10 min (B); and 60 min (C). The virus particles in (A) are 18 to 22 nm in diameter. Of particular interest are the tail-like structures (arrow) extruded from the virus particles (B). This is thought to be released ssDNA.

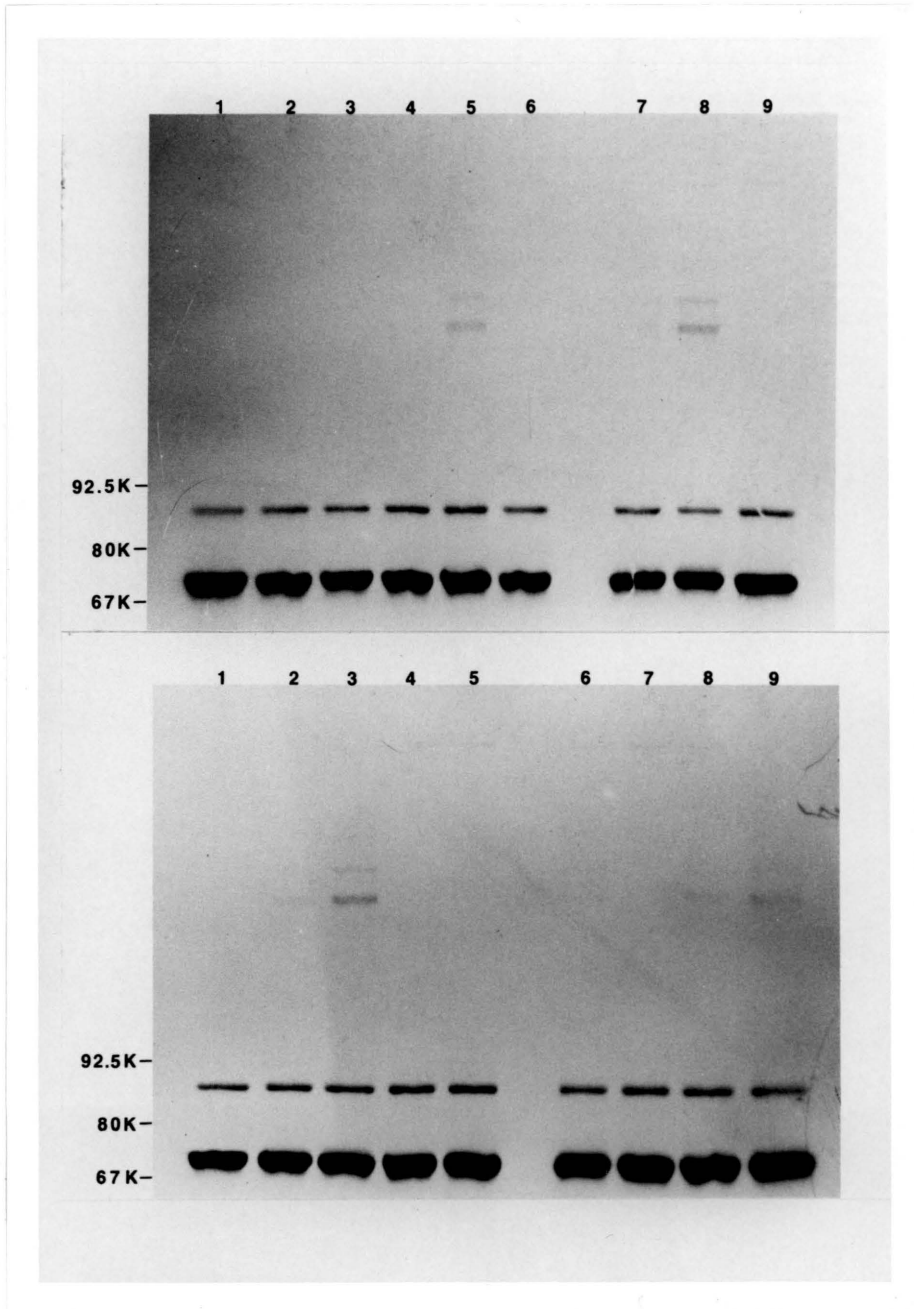
and the protein was precipitated at -80°C by adding ethanol (2.5 times the volume). Protein pellets were resuspended in Laemmli application buffer and subjected to electrophoresis on SDS-polyacrylamide gels. Since the proteins were radioactively labeled the gels were prepared for fluorography and the resulting films are shown in Figure 22. All sample lanes show clearly the two polypeptides, VP1 and VP2, that comprise the virus capsid. However, as the chlorine dose increased or the exposure time increased, there is an indication that several new, higher molecular weight polypeptides were being formed. Again enhancement of this effect is achieved by 5 mg L^{-1} NaOCl at exposure times of up to 60 minutes. The chlorine was neutralized by addition of either one percent (w/v) sodium thiosulfate or one percent (w/v) sodium sulfite. The resulting fluorograph is presented in Figure 23. Lane 1 is the control, and the remaining lanes represent increasing exposure times and show 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 different aggregate sizes. By 60 minutes 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 (SDS) only covalently cross-linked polypeptides remained intact.

Pairs of replicate samples were exposed to 5 mg L^{-1} NaOCl for 10, 30, and 60 minutes to determine if there was any protein-DNA

Figure 22. SDS-polyacrylamide (7.5 percent) gel electrophoresis and fluorography of the capsid proteins extracted from whole parvovirus H-1, both untreated and chlorine treated. Exposure times varied for each chlorine dosage. Panel A, Lane 1: Control showing the two major polypeptides comprising the H-1 capsid; Lane 2: Control; Lane 3: 1 mg L^{-1} NaOCl, 30s exposure; Lane 4: 1 mg L^{-1} , 2 min; Lane 5: 1 mg L^{-1} NaOCl, 5 min; Lane 6: 2 mg L^{-1} NaOCl, 30s exposure; Lane 7: Standard markers; Lane 8: 2 mg L^{-1} , 2 min; Lane 9: 2 mg L^{-1} , 5 min; Lane 10: 3 mg L^{-1} NaOCl, 30s exposure.

Panel B, Lane 1: Control; Lane 2: 3 mg L^{-1} , 2 min; Lane 3: 3 mg L^{-1} , 4 min; Lane 4: 4 mg L^{-1} NaOCl, 30s exposure; Lane 5: 4 mg L^{-1} , 1 min; Lane 6: Standard markers; Lane 7: 4 mg L^{-1} , 2 min; Lane 8: 5 mg L^{-1} NaOCl, 30s exposure; Lane 9: 5 mg L^{-1} , 1 min; Lane 10: 5 mg L^{-1} , 2 min.

Panel A, Lanes 5, 8, and 9, as well as, Panel B, Lanes 2, 3, 7, 9, and 10, show the formation of high molecular aggregates with increasing exposure time, or increasing chlorine dosage. The relative positions and molecular weights of the standard markers are indicated in the left margin ($K = \times 10^3$ daltons)



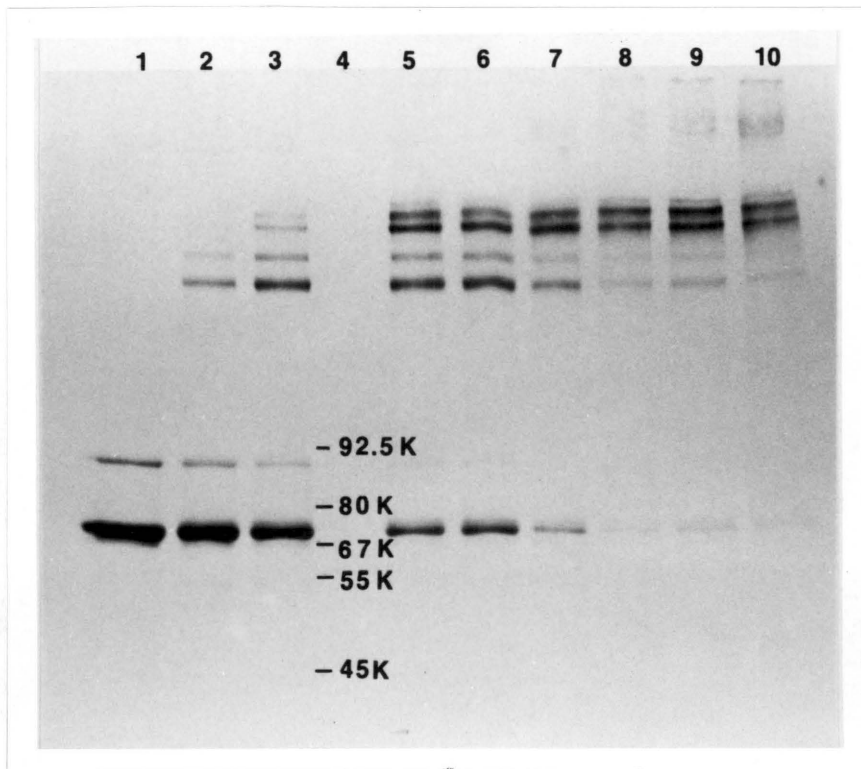


Figure 23. SDS-polyacrylamide (7.5 percent) gel electrophoresis and fluorography of the capsid proteins extracted from whole parvovirus H-1, both untreated and chlorine treated. All treated samples were exposed to 5 mg L^{-1} NaOCl for various exposure times. Lane 1: Control; Lane 2: 3 min; Lane 3: 5 min; Lane 4: Standard markers, relative positions on the stained gel and the individual molecular weight values are indicated; Lane 5: 10 min; Lane 6: 10 min, sodium sulfite used to quench the reaction rather than sodium thiosulfate; Lane 7: 15 min; Lane 8: 30 min; Lane 9: 30 min, sodium sulfite; and Lane 10: 60 min.

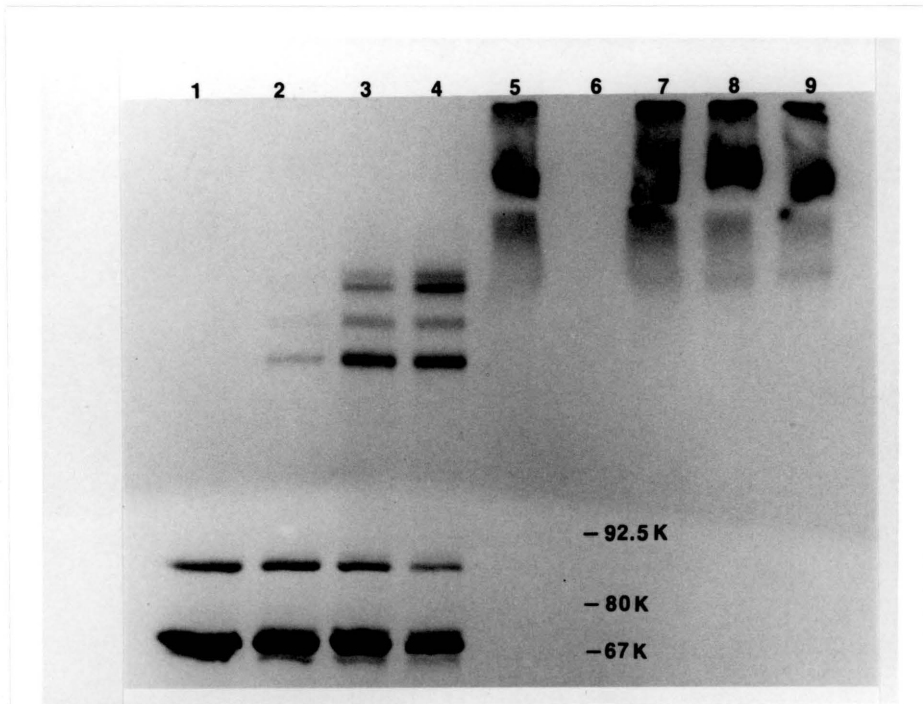
crosslinking caused by chlorination. One sample of the pair was radioactively labeled with [^3H]-Met, the other with [^3H]-TdR. The protocol just described for precipitating the virus was used for both samples. After electrophoresis, staining with Coomassie blue showed similar protein patterns for both samples in the pair. A fluorograph of the gel, (Figure 24) however, revealed the DNA was not associated with the protein and had remained in the stacking gel.

Analysis of the Integrity of the Nucleic Acid

Treatment with 5 mg L^{-1} NaOCl of H-1 virions containing a full complement of DNA caused a complete loss of infectivity. The integrity of the remaining DNA was questionable considering capsid proteins had been altered significantly. To determine if the loss of infectivity was associated with capsid alteration or damage to the nucleic acid, the intactness of the DNA was investigated.

This question was initially addressed by digesting any associated protein in the sample under highly alkaline conditions. The alkaline state was maintained on 5 to 20 percent sucrose gradients during analysis of the intactness of the DNA. The graphs (Figures 25 and 26) of these fractionated gradients illustrate that regardless of exposure time (up to a maximum of 60 minutes) the DNA of chlorinated virions sedimented to the same position in the gradient as the control DNA (Fraction 18). Also, none of the gradients showed any extra peaks which would be indicative of fragmentation. Although this gradient analysis

Figure 24. SDS-polyacrylamide (7.5 percent) gel electrophoresis and fluorography of a dual experiment in which whole H-1 virions radioactively labeled in either the protein or nucleic acid were exposed to 5 mg L^{-1} NaOCl. The viral protein was extracted from all samples and electrophoresed. Staining by Coomassie blue produced identical protein patterns between Lanes 1 and 5, 2 and 7, 3 and 8, and 4 and 9. The fluorograph presented above shows the protein pattern in Lanes 1-4. Lanes 5, and 7-9 indicate that the viral DNA is not associated with the high molecular weight aggregates produced by chlorination. Lane 1: Control, [^3H]-Met; Lane 2: 10 min of exposure; Lane 3: 30 min; Lane 4: 60 min; Lane 5: Control, [^3H]-TdR; Lane 6: Standard markers, relative positions and molecular weight values are indicated; Lane 7: 10 min; Lane 8: 30 min; and Lane 9: 60 min.



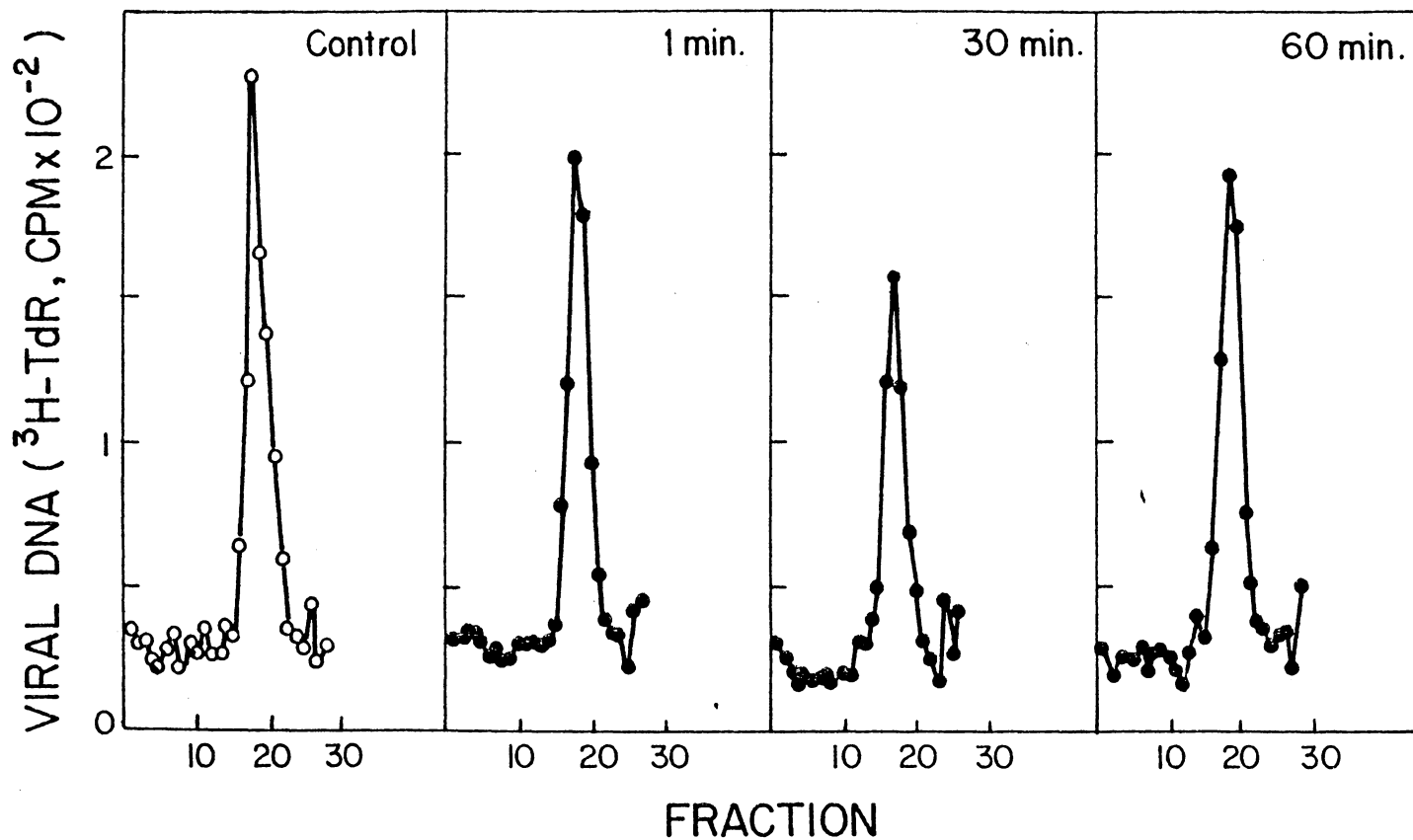


Figure 25. Sedimentation rate analysis of [³H]-TdR labeled DNA extracted from chlorine-treated whole virus. Parvovirus H-1 was treated with 5 mg L⁻¹ NaOCl for the indicated time of exposure and the DNA analyzed by 5 to 20 percent alkaline sucrose gradients.

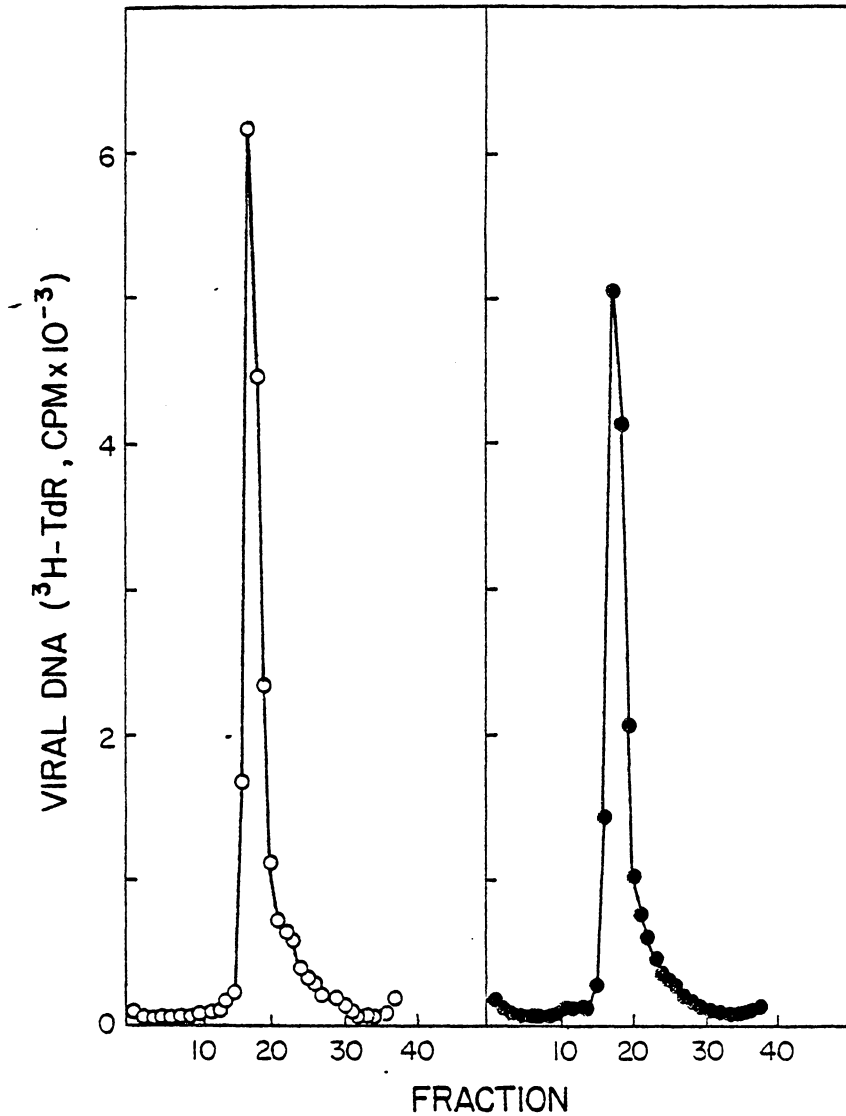


Figure 26. Sedimentation rate analysis of [³H]-TdR labeled DNA extracted from chlorine-treated whole virus. Parvovirus H-1 was treated with 5 mg L⁻¹ NaOCl for 60 min and the DNA analyzed by a 5 to 30 percent alkaline sucrose gradient (Right Panel); Left Panel: Control.

showed the intactness of the nucleic acid, it was unable to indicate whether any subtle changes may have occurred in the DNA. For example, a chlorine-induced lesion may be slight but still prevent 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 chlorine-treated DNA was implemented. Again, the whole virion was treated with 5 mg L^{-1} of NaOCl for various exposure times. However, this time the DNA was extracted and resuspended in a Tris buffer. A polymerase was added to the single stranded DNA after a mixture of nucleotides had been added (one of which was ^{32}P labeled) in order to initiate in vitro replication. If initiation was possible, the DNA would replicate either completely or until a chlorine-induced lesion prevented the polymerase from reading the entire strand. In either case, the ^{32}P -labeled nucleotide would be incorporated. The DNA was then precipitated and resuspended in an appropriate buffer for analysis of the degree of replication by electrophoresis. An autoradiogram of the resulting agarose gel (Figure 27) contains both single and double-stranded (lighter band) DNA markers in the middle lane. The other lanes, representing controls and various chlorine exposure times, revealed that the single-stranded DNA was capable of in vitro replication to double-stranded DNA even after a 60 minute exposure of the whole virion to chlorine.

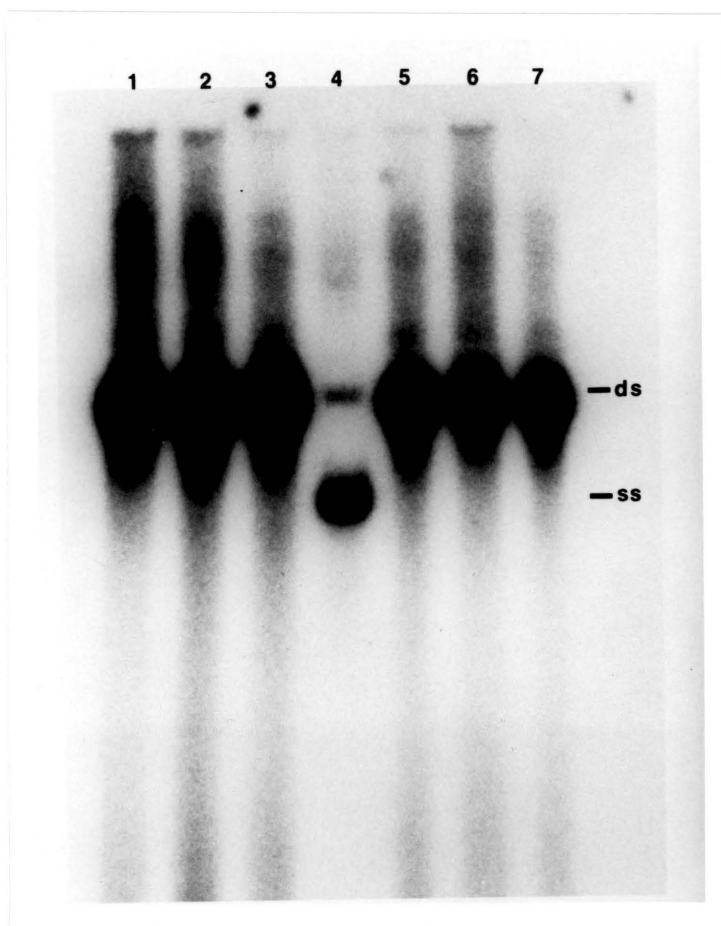


Figure 27. Agarose (1.4 percent) gel electrophoresis and autoradiograph of *in vitro* replicated DNA which had been extracted from $^{-1}$ whole H-1 parvovirus exposed for various times to 5 mg L^{-1} NaOCl. Lane 1: Control without sodium thiosulfate; Lane 2: Control; Lane 3: 1 min exposure; Lane 4: bovine parvovirus single-stranded (ss) and double-stranded (ds) DNA markers; Lane 5: 10 min exposure; Lane 6: 30 min; Lane 7: 60 min.

Endonuclease Experiments

The previous experiments indicated that the ssDNA extracted from chlorine treated H-1 whole virions was not fragmented and was capable of in vitro replication. To ensure that the replicated DNA was an intact double-strand, it was subjected to three endonucleases, which were known to cleave dsDNA into certain size fragments. The size of these fragments resulting from treatment by each endonuclease is shown in a physical map of H-1 (Figure 28). The three fragments created by Eco RI are seen in all lanes of the fluorograph in Figure 29. The two other endonucleases, Bcl I and Pst I, were harder to work with, and Figure 30 shows some incomplete cuts in a few of the lanes in the autoradiogram. The fact that the lanes representing the 60-minute exposure have all the proper fragments negates any anomalies present in the other lanes.

Adsorption Studies

The ability of the chlorine treated virus to adsorb to host cells was investigated. The chlorine treated [³H] methionine labeled virus was allowed to adsorb to host cells for 45 to 60 minutes at 37°C. This time period should have been adequate to allow for adsorption, but not penetration. Each virus-cell mixture was collected and filtered, then subjected to washing by PBS or PBS containing EDTA. The purpose of the EDTA was to eliminate any nonspecific binding of the damaged virions (Linser et al., 1979). Table 6 shows the results of this assay. Based

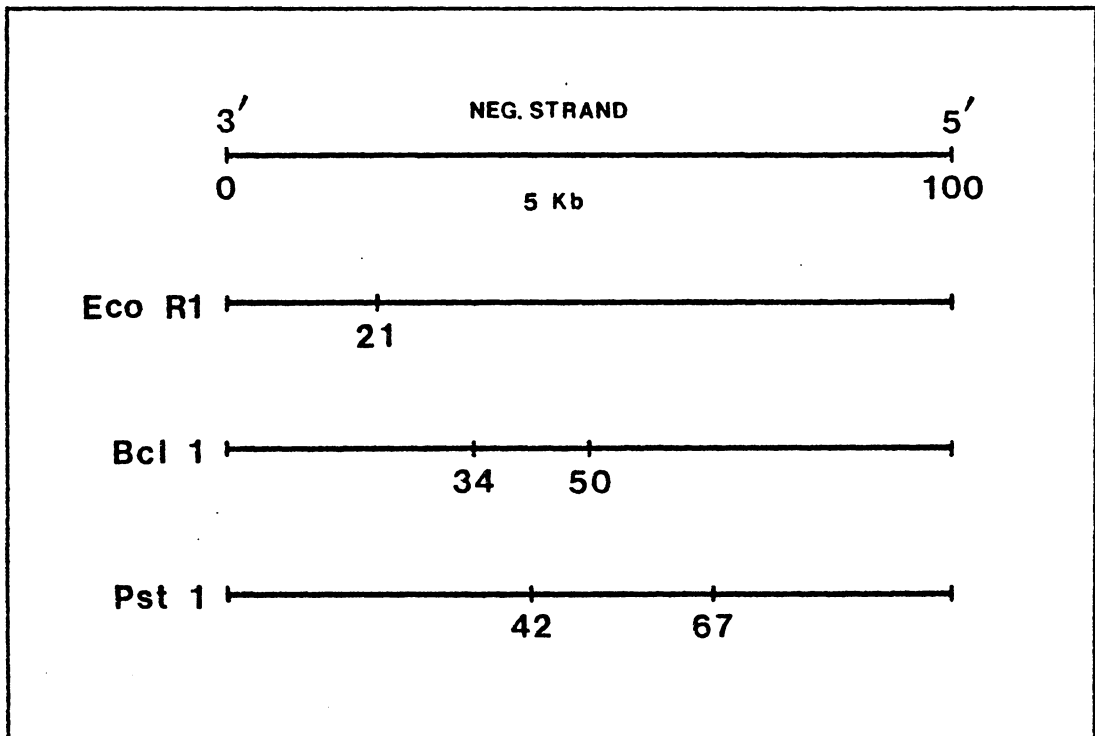


Figure 28. Physical map of parvovirus H-1 (Rhode, 1982). The relative locations of endonuclease cleavage sites are indicated for the restriction enzymes Eco RI, Bcl I, and Pst I. The location of the cleavage sites are represented by numbers representing the percentage of the original length of five kilobases (Kb).

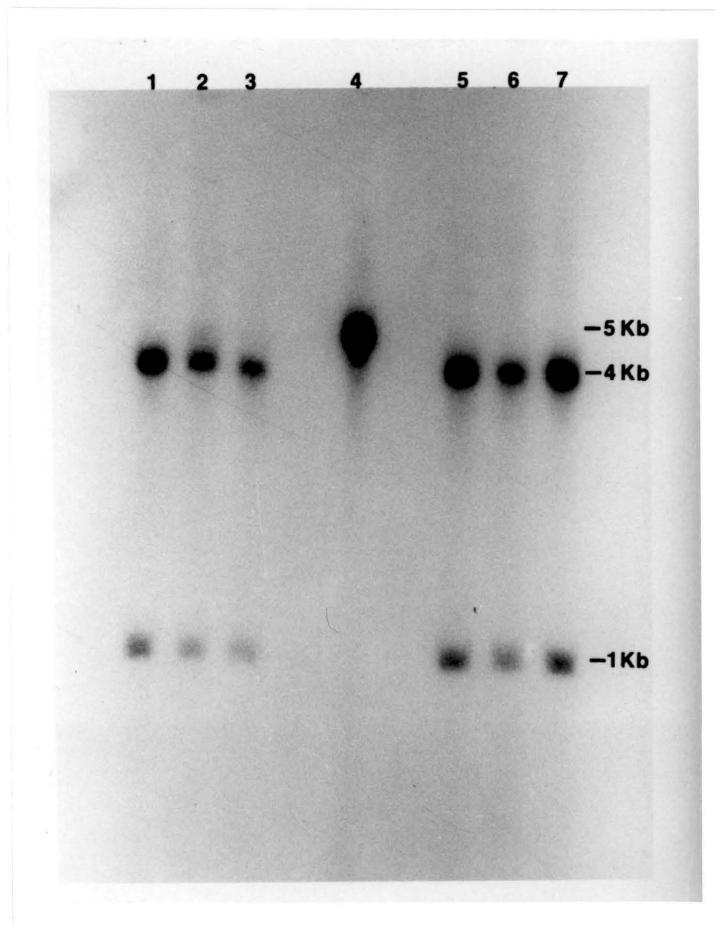


Figure 29. Autoradiogram of Eco RI restriction endonuclease analysis of in vitro replicated DNA. Whole virus was treated with 5 mg L^{-1} NaOCl for various exposure times, and the ssDNA isolated. After in vitro replication of the DNA, aliquots of these samples were treated with endonuclease Eco RI and analyzed by electrophoresis through a 1.4 percent agarose gel. Lane 1: Control without sodium thiosulfate (5Kb = 5000 base pairs); Lane 2: Control; Lane 3: 1 min exposure; Lane 4: Replicated control (dsDNA) which was not treated with Eco RI; Lane 5: 10 min exposure; Lane 6: 30 min; and Lane 7: 60 min.

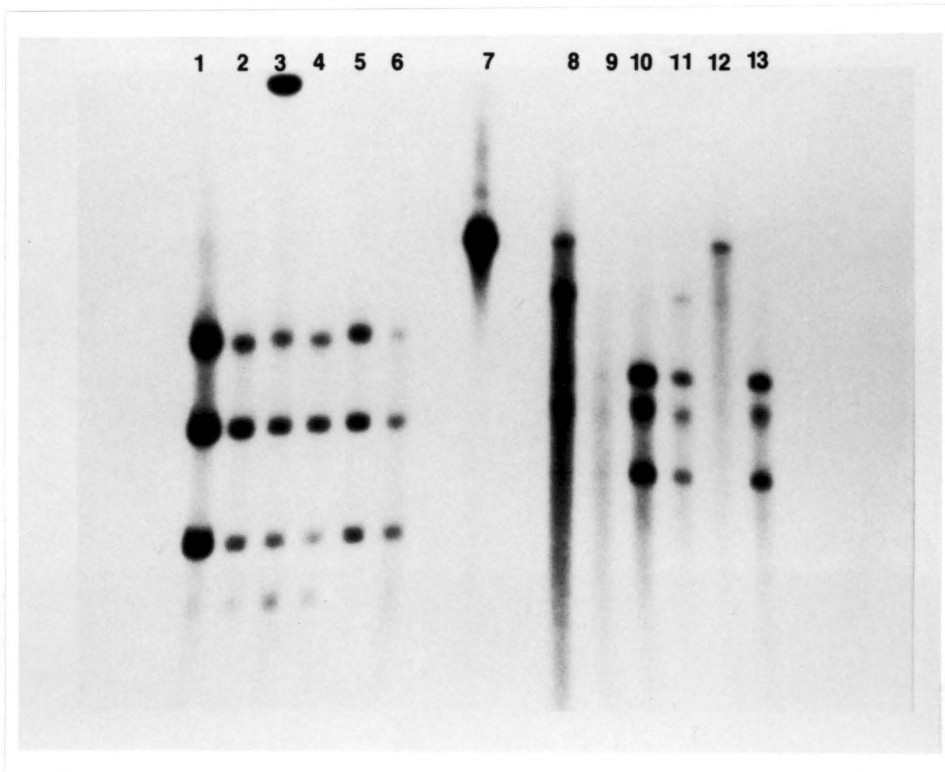


Figure 30. Autoradiogram of Bcl I and Pst I restriction endonuclease analysis of in vitro replicated DNA. Whole virus was treated with 5 mg L^{-1} NaOCl for various exposure times, and the ssDNA isolated. After in vitro replication of the ssDNA, aliquots of these samples were treated with either endonuclease Bcl I (Lanes 1-6) or Pst I (Lanes 8-13) and analyzed by electrophoresis through a 1.4 percent agarose gel. Lanes 1 and 8: Control without sodium thiosulfate; Lanes 2 and 9: Control; Lanes 3 and 10: 1 min exposure; Lanes 4 and 11: 10 min; Lanes 5 and 12: 30 min; Lanes 6 and 13: 60 min; and Lane 7: Replicated control (dsDNA) which was not treated with either endonuclease.

Table 6. The Adsorptive Ability of Chlorine-Treated Empty and Full H-1 Parvovirus.

Sample Treatment	Input cpm	Adsorbed cpm	% Input	% Control
<u>Full virus</u>	70700			
PBS Wash				
Control		21360	30.0	--
5 mg L ⁻¹ 60 min		20349	29.1	95.2
Blank Filter		68	--	--
PBS + EDTA Wash				
Control		18308	25.7	--
5 mg L ⁻¹ 60 min		8594	12.1	46.9
Blank Filter		164	--	--
<u>Empty Virus</u>	67025			
PBS + EDTA Wash				
Control		24557	36.6	--
5 mg L ⁻¹ 60 min		10552	15.6	42.9

on radioactivity, the levels of chlorine-treated virus remaining bound to host cells after washing with PBS were similar to the control. The PBS-EDTA wash, however, revealed that approximately 50 percent of the treated virus had non-specifically adsorbed to the host cell.

Discussion

The previous section on the study of inactivation kinetics indicated that there may be more than one inactivation site on the H-1 parvovirus. To determine the location of these sites on the virion, the whole virion 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.

The effect of chlorine on the whole virion was first denoted in the sedimentation rate analysis data presented in Figures 18 - 20. When the radioactively labeled virus was exposed to 1.0 mg L^{-1} NaOCl for 60 minutes, the sedimentation rate of only a small number of virions was changed, even though there was a three-log decrease in titer. This change was indicated when some of the virion-associated radioactivity, ($[^3\text{H}]\text{-Met}$) was detected in fraction 18 of the sucrose gradient. Using the calculation methodology of Griffith (1970), the sedimentation rate coefficient for the whole virion control was determined to be 116S which is in good agreement with the literature value of 110S (Salzman, 1978). Also in good agreement with the literature value of 70S for empty

virions (Salzman, 1978), was the calculated value of 73S. The radioactive peak in fraction 18 was determined to be 43S. Figures 18 and 19 also show that an increase in the chlorine dosage to 5 mg L^{-1} NaOCl enhanced the development of the 43S peak with a simultaneous loss of radioactivity in the 110S peak of the whole virion. 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 even though both viral components were present.

After a typical inactivation experiment, the treated virus was examined by electron microscopy to determine what visible morphological changes had taken place. A 10-minute exposure to 5 mg L^{-1} NaOCl had created a "tail-like" extrusion extending from the virus particles. It is felt that this extrusion was the viral DNA that was expelled from the altered virions. This single-stranded molecule was apparently visible by simple negative-staining because some protein was associated with it. It also appeared to remain attached to the virus particle. The electron micrograph of virus exposed for 60 minutes to chlorine indicated that there was a massive aggregation of these nucleoprotein complexes, perhaps due to the continued reaction of the viral particle with chlorine, or itself, or both.

To further study the effect of chlorine on the viral protein, a relatively new technique was utilized. By chlorinating [^3H]-Met labeled virus, not only could alterations in the proteins be determined by gel

electrophoresis, but the sensitivity of tritium detection could be increased by impregnating the gel with a fluorescent chemical. Initially, [³H]-Met whole virions were exposed to several concentrations of NaOCl for short periods of time (< 5 minutes). The resulting fluorograph (Figure 21) indicated that the two capsid proteins were not broken into smaller peptides but had covalently linked into higher molecular weight aggregates. Figure 22 is a fluorograph that clearly illustrates the sequence of events leading to the state of aggregation found at 60 minutes exposure. Some of the combinations must still have had reactive sites, as they are intermediary to the large polypeptide aggregates that eventually formed.

It is presumed that the reactive sites were located on exposed R-groups of certain amino acids comprising the protein. This implies that the architecture of the entire capsid, as well as the spatial relationship of the two major polypeptides, may be important because they direct which capsid protein and/or R-group is most exposed to chlorine. Because there are only 12 molecules of VP1 per virion these are likely to be found at the twelve apexes of the capsid (Kongsvik, 1974) and possibly have the greater exposure of the two viral proteins. The disappearance of VP1 before VP2 on the fluorograph in Figure 24 suggests this may have been the case; however, there was five times as much VP2 as VP1, so the reactivity of VP2 may be masked.

Further investigation revealed that the treatment of full H-1 virions with chlorine (5 mg L⁻¹ NaOCl) 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. (1979) and O'Brien and Newman (1979) who reported that 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. and Alvarez and O'Brien (1982) who demonstrated that inactivation of poliovirus by low concentrations of chlorine occurred before the nucleic acid lost its biological activity.

If inactivation of H-1 parvovirus is caused by an alteration of the protein capsid, then any steps of the infectious process that require the protein capsid be in its original state might be blocked. The adsorption of the virus to its host cell is the first step involving the protein capsid which is the reason that the investigation to determine if chlorine-treated whole virions had lost any ability to adsorb to host cells was undertaken. Table 2 indicates that there was no difference in the adsorptive capabilities of chlorine-treated virions versus the control when the cell material was washed with PBS. However, to eliminate any non-specific binding of the virus to the host cell, a PBS wash containing 0.001 M EDTA (Linser et al., 1979) was used for replicate samples. The apparent chelation of metallic ions by the EDTA caused approximately 50 percent of the adsorbed chlorine-treated viruses to be eluted from the cells. The interaction of chlorine-treated viruses and nontreated viruses with host cells is significantly

different. Further investigation is needed, however, to determine if the non-specifically bound chlorine-treated virus is able to penetrate the cell.

V. DISCUSSION AND CONCLUDING REMARKS

The previous two sections of this dissertation have discussed the results of an investigation into the kinetics and mechanism of inactivation of H-1 parvovirus by chlorine. It is the first mechanism of inactivation study utilizing a DNA-containing enteric virus. The results of this research hopefully will provide a basis for further investigations into disinfection kinetics and mechanism studies. Ideas for further investigations suggested from the results presented in both this study and in the literature will now be introduced.

Some investigators have proposed that inactivation of viruses takes place by the loss of a pertinent portion of the viral capsid. In the case of the enteroviruses that have four major polypeptides, the action of chlorine caused the loss of viral protein four (VP4) from the capsid (Tenno et al., 1979). The virus was then considered inactivated because it was no longer able to adsorb to the host cell. Based on this evidence, Tenno et al. (1979) believed VP4 to be the protein primarily responsible for adsorption. Increasing the exposure time of an enterovirus to chlorine leads to significant damage to VP1 and VP2 on the capsid. Detailed research by Lonberg-Holm (1981) demonstrated that VP4 is buried well within the protein capsid of poliovirus and is not exposed on the surface. (Furthermore, Lonberg-Holm believed it is not responsible for adsorption to cell receptor sites.) If one accepts that VP4 is not exposed on the capsid surface, then it appears that the release of VP4 could occur only with a major alteration of the entire

capsid. This alteration may not be the result of degradation of any of the other polypeptides, merely a rearrangement of them. In the process of this rearrangement, the concomitant release of the nucleic acid might also occur. The simultaneous occurrence of these two events possibly happened in the research of O'Brien and Newman (1979) when they were studying the effects of chlorine on poliovirus. When Tenno et al. (1979) used lower doses of chlorine, the nucleic acid was not released; however, the results did reveal VP4 was missing.

The structural simplicity of parvovirions relative to enteroviruses aids in demonstrating that either inactivation mechanisms vary with the virus under study or the loss of a polypeptide is not required for the loss of adsorptive capabilities. In the case of H-1 parvovirus the use of low dosages of chlorine caused inactivation by a structural alteration of the capsid integrity. The nucleic acid was exposed, but there was no indication that one of the two major polypeptides was lost from the capsid. The ability of the virus to specifically adsorb to the host cell, however, decreased significantly. 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. It seems more reasonable that the ability of the virus to adsorb to a host cell is dependent upon a particular spatial arrangement of all the capsid protein, rather than just one polypeptide. Initiation of the infectious process, therefore, may be prevented by alteration in any special spatial arrangement of proteins required for adsorption.

If one were to separate the capsid proteins of H-1 parvovirus by gel electrophoresis, it would be possible to extract these polypeptides individually from the gel. The ability of each polypeptide to adsorb to host cells could then be tested. This investigator believes that neither of the two polypeptides will specifically adsorb to the host cell on an individual basis. Adsorption most likely will take place when the two polypeptides are associated in a special arrangement to one another in the form of a viral capsid. Interestingly, empty capsids of H-1 parvovirus appeared to be able to adsorb to host cells just as readily as the full virions.

Another important aspect of inactivation mentioned earlier was the observation that chlorine causes both enteroviruses and parvoviruses to release their nucleic acids. In the case of the parvovirus the DNA remains attached to the protein component, whereas, the RNA of poliovirus is released and separates from the capsid. Whether the nucleic acid remains associated with the capsid or not, the release occurs as if induced by a definite mechanism. It resembles the triggered mechanism of nucleic acid release from a bacteriophage into a host cell. The release of the RNA from chlorine treated poliovirus capsids is also complete. Perhaps chlorine induces the same mechanisms that are operative in the normal infective cycle of viruses. Parvoviruses, on the other hand, replicate in the cell nucleus and the DNA must be protected during the virion's journey through the cytoplasm into the nucleus. Upon reaching the nucleus the DNA is thought to be

released. This mechanism of DNA release may be similar to or the same as that induced by chlorine during inactivation. The agent responsible in the cell may be a protease acting on the capsid in a manner similar to chlorine. The same can be said for the chlorine treatment of poliovirus. Extracellular enzymes may trigger the release of the RNA into the cell in the same manner that chlorine inactivates the virus. The details of the infection process for parvoviruses are unknown at this time, but again, the relative simplicity of this virus may prove useful in determining if the release of the DNA by chlorination occurs in the same fashion as the release of the DNA prior to the replication of the virus. Also, the fact that even high doses of chlorine are unable to strip the nucleic acid completely away from the capsid may be indicative of how important the protein shell is for the protection of the DNA from intracellular enzymes. It would be interesting to compare experimentally the uncoating mechanism of enteric viruses during the infectious process to the mechanism of chlorine inactivation.

The apparent anomalies associated with the inactivation rates of parvovirus H-1 at various temperatures needs further study. These results indicated that perhaps more than one reaction mechanism was responsible for inactivation, and the reaction mechanism was a function of temperature. If this is so, then "micro-scale" inactivation trials could be performed at various temperatures ranging from 5 to 30°C, and the protein analyzed by gel electrophoresis. It is known that inactivation trials at room temperature (22 to 25°C) caused high

molecular weight aggregates to form. The reaction mechanism at a different temperature may not cause protein aggregates of the same size.

Lastly, from the literature review the difficulties of comparing the results of the many inactivation studies became readily evident. An initial question to be answered in any inactivation study is what type of media should be used for the virus-disinfectant reaction. Some investigators, for obvious reasons, prefer a well-defined buffered system that is chlorine-demand-free. Others, however, feel that inactivation studies should employ finished waters, ideally from any and all sources under investigation, so that the characteristics of the actual water are automatically included in the analysis. Dependent upon the goals of the project, either approach may be appropriate. Basic research with proper control of the variables is required for defining the complex aspects of inactivation. On the other hand, it is important to recognize the effect of a variety of characteristics inherent in different water supplies.

Prudent consideration should be given to adopting a standard procedure for virucide testing in environmental systems. Thus far, research indicates that there appears to be no one virus suitable as an indicator of viral disinfection. Differences in rates of inactivation are known to exist even between the various strains of subgroups of enteroviruses, thereby refuting the case for using just poliovirus as a model. Furthermore, there is a possibility that even subtle differences between particle types of a single strain, (i.e., particle density) can

affect inactivation rates. Also, environmental factors, such as pH, temperature, and salt concentrations can affect not only the concentrations of various species of a virucide but also, and more importantly the relative resistance of a particular virus. This phenomenon was evident for poliovirus in the studies by Englebrecht et al. (1980), and Jensen et al. (1980).

Although this research was separated into two distinct efforts, there is a definite complementation of the kinetic data with the mechanism of inaction. Based on the research in its entirety, the following statements can be made.

- 1) The expected dose-response relationship of the H-1 parvovirus to chlorine was exhibited in the inactivation trials.
- 2) Analysis by the van't Hoff equation indicated that chlorine concentration and contact time of the virus with chlorine were of equal importance.
- 3) The kinetic data does not fully conform to Chick's Law because of the "tail-away" effect.
- 4) Analysis by the Arrhenius equation indicated that there are several reaction mechanisms capable of causing inactivation.
- 5) The energy of activation for 0.05 mg L⁻¹ chlorine at 5 to 30°C was graphically determined to be 2.4 Kcal mole⁻¹ and was calculated to be 2.0 Kcal mole⁻¹ by Eyring's equation.

- 6) It was demonstrated using Eyring's equation that a negative change in entropy occurred (-52.34 relative entropy units) indicating a more orderly state had been created.
- 7) Chlorine significantly altered the protein capsid and the ability of a significant fraction of the viruses to specifically adsorb to host cells.
- 8) The exposure of H-1 to 5 mg L^{-1} chlorine for times ranging from 3 to 60 minutes caused a progressive and eventually, massive covalent cross-linking to occur between the polypeptides of the viral capsid that resulted in the release of the DNA and a change in the sedimentation rate of the viral particle.
- 9) The DNA of the H-1 parvovirus retained its integrity and the ability to undergo in vitro replication, even after being exposed to 5 mg L^{-1} of NaOCl for 60 minutes.

In summary the parvovirus H-1, a putative human virus containing ssDNA, was used as a model virus for chlorine inactivation experiments. Inactivation occurred in the usual dose-response relationship, that is, increasing the chlorine dosage caused an increase in the rate of inactivation. The results indicated that perhaps more than one reaction mechanism was responsible for inactivation, and the reaction mechanism was a function of temperature.

From the mechanism study it was concluded that the initial action of chlorine on parvovirus H-1 was on the capsid. Alterations in the two

major capsid proteins caused the virion to rupture and, as evidenced by electron microscopy the ssDNA was exposed. Also, the adsorption ability of the chlorine-treated virions to host cells was significantly inhibited. This was presumably due to the effect on the spatial arrangement of the capsid proteins in their entirety rather than a loss of, or change, in only one polypeptide. The sedimentation rate of the chlorine-treated whole virus decreased from 116S to 43S. The chlorine caused certain sites on the capsid proteins to become highly reactive which facilitated the formation of higher molecular weight aggregates as detected by fluorographs of electrophoretic protein patterns in polyacrylamide gels. Most significant was the discovery that the ssDNA remained undamaged and was still capable of in vitro replication even after 60 minutes of exposure to 5 mg L^{-1} of sodium hypochlorite at pH 7.

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A STUDY OF THE KINETICS AND MECHANISM OF INACTIVATION
OF A DNA-CONTAINING ENTERIC VIRUS BY CHLORINE

by

C. Calvert Churn, III

(ABSTRACT)

A newly discovered enteric virus has recently been associated with large outbreaks of waterborne gastroenteritis. Most commonly referred to as the Norwalk agent, this virus appears to be morphologically and biophysically similar to the parvoviruses. Presently there is very little known about the fate of parvoviruses in environmental systems.

In this study the parvovirus H-1, a putative human virus containing single-stranded DNA (ssDNA), was used as a model virus for chlorine inactivation experiments. The purpose of this research was two-fold: first, to investigate the kinetics of inactivation of parvovirus H-1 by low levels of free chlorine (0.05 - 0.20 mg L⁻¹) at pH 7 and at 5, 10, 20, and 30°C; and secondly, to determine the mechanism by which chlorine inactivates this virus.

Inactivation occurred in the usual dose-response relationship, that is, increasing the chlorine dose caused an increase in the rate of inactivation. The results indicated that perhaps more than one reaction mechanism was responsible for inactivation, and the reaction mechanism was a function of temperature. The energy required for the inactivation reaction using 0.05 mg L⁻¹ free chlorine from 5 to 30°C was graphically

determined to be $2.4 \text{ Kcal mole}^{-1}$. The change in entropy was calculated to be -52.34 entropy units.

From the mechanism study it was concluded that the initial action of chlorine on parvovirus H-1 was on the capsid. Alterations in the two major capsid proteins caused the virion to rupture, and, as evidenced by electron microscopy the ssDNA was exposed. Also, the adsorption ability of the chlorine-treated virions to host cells was significantly inhibited. This was presumably due to the effect on the spatial arrangement of the capsid proteins in their entirety rather than a loss of, or change, in only one polypeptide. The sedimentation rate of the chlorine-treated whole virus decreased from 116S to 43S. The chlorine caused certain sites on the capsid proteins to become highly reactive which facilitated the formation of higher molecular weight aggregates as detected by fluorographs of electrophoretic protein patterns in polyacrylamide gels. Most significant was the discovery that the ssDNA remained undamaged and was still capable of in vitro replication even after 60 minutes of exposure to 5 mg L^{-1} of sodium hypochlorite at pH 7.