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Virion Conformational Forms and the Complex Inactivation Kinetics of Echovirus by Chlorine in Water

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Aberrant inactivation kinetics were observed when monodispersed echovirus type 1 (Farouk) was inactivated with chlorine. An initial 1- to 2-$\log_{10}$-unit decrease in titer was followed by a lag period, during which the titer stayed the same or increased, and this was followed by a final decline in titer. First-order kinetics were obtained with poliovirus type 1 under the same conditions. Isoelectric focusing studies of echovirus before chlorine treatment showed that the virus distributed into two pH-dependent and interconvertible isoelectric forms. After chlorine treatment all remaining virus infectivity was associated with a third pH-independent isoelectric form. The complex inactivation kinetics appeared to be due to shifts between these conformational forms during inactivation in certain ionic environments. Under certain conditions the conformational shifts resulted in substantial resistance of monodispersed echovirus to chlorine.

In a previous publication, we reported complex inactivation kinetics when echovirus type 1 (Farouk) was inactivated with 20 μM HOCl at pH 6.0 and 20°C (17). An initial 1- to 2-$\log_{10}$-unit decrease in titer in the first second of inactivation was followed by a lag period of 15 s before a final decline in titer. We demonstrated that this unusual departure from first-order kinetics was not due to virion aggregation, genetic heterogeneity of the virus suspension, or depletion of chlorine and suggested that conformational changes in the virion capsid might be involved. At the time these results were observed, the halogen inactivation experiments carried out in this laboratory with reovirus and poliovirus had shown first-order kinetics or only slight departures from first-order kinetics when virion aggregation was eliminated (2–4, 14–16). However, there is now evidence that suggests that the complex inactivation kinetics originally observed with echovirus are more common than was first expected. Kenyon and Schaub (9) have reported complex inactivation kinetics with poliovirus type 1 (LSc), and experiments in this laboratory have demonstrated complex kinetics for coxsackieviruses B3 and B5 (8). These findings suggest that (i) different enteric viruses may be inactivated by different mechanisms and (ii) some may become, in the course of inactivation, temporarily resistant to the effects of the inactivating agent.

The experiments reported here were performed to examine the effects of pH, salt concentration, temperature, and chlorine concentration on the unusual inactivation kinetics of echovirus. Isoelectric focusing studies were performed to determine virion conformational forms during disinfection. Evidence is presented in support of the hypothesis that the complex inactivation kinetics of echovirus are related to conformational changes in capsid structure.

MATERIALS AND METHODS

Virus preparation and assay. Echovirus type 1 (Farouk) was obtained from Mark Sobsey, School of Public Health, University of North Carolina. Details of its production in human epidermoid carcinoma (HEp-2) cells and purification of chlorine demand-free monodispersed suspensions were the same as described in an earlier work with poliovirus (2). ^{3}H-labeled monodispersed virus preparations were prepared by adding [^{3}H]uridine (Moravek Biochemicals) at a concentration of 10 to 20 μCi/ml to the media at 2 to 4 h postinfection. Physical assay of purified virus suspensions for aggregation analysis was made using the kinetic attachment method for electron microscopy (12).

Chlorine inactivation. Virus suspensions were exposed to chlorine solutions by using the Continuous Flow Apparatus as previously described (14). For the inactivation experiments at pH 6.0 and 7.0, the buffer solution (0.01 M phosphate and 0.1 M sodium chloride) used to maintain the desired pH was prepared from Fisher primary standard monobasic potassium phosphate, Mallinkrodt analytical reagent grade potassium phosphate, and Fisher biological grade sodium chloride. The phosphate-carbonate buffer solution used in the experiment at pH 10.0 was prepared with Fisher primary standard potassium phosphate, Fisher alkalimetric-grade sodium carbonate, and Fisher biological-grade sodium chloride as previously described (16). The sodium carbonate was heated at 250°C for 2 h to remove excessive chlorine-demanding impurities. The solutions for the experiments at pH 5.1 and 5.7 were prepared by adjusting chlorine demand-free water to the desired pH with HCl. All buffer solutions were prepared with deionized, glass-distilled water and were made chlorine demand-free as previously described (4). In all experiments the virus suspensions to be inactivated were diluted to the 5-ml injection volume with the chlorine demand-free buffer prepared for that particular experiment. In one experiment, virions that had been pretreated with chlorine were required. These were prepared by diluting purified echovirus fivefold with distilled water. Hypochlorous acid was diluted to a concentration that would reduce the plaque titer of the virus suspension about 1 $\log_{10}$ unit and this was added to the virus suspension. Treatment was allowed to continue until no more chlorine was present (ca. 2 min). In this way, highly concentrated chlorine-treated virions which had not been exposed to sodium thiosulfate were obtained, and these were further inactivated by using the Continuous Flow Apparatus. Free-

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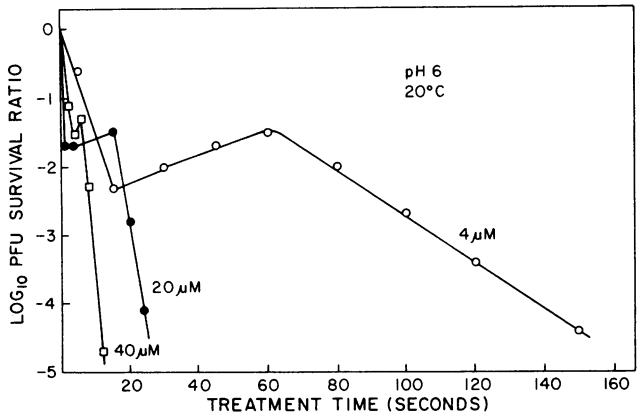

FIG. 1. Inactivation of echovirus with 4, 20, and 40 μM HOCl at pH 6.0 and 20°C.

chlorine concentrations were determined by amperometric titration with phenylarsine oxide (1). Surviving virus infectivity was determined at each point by duplicate plaque titrations.

Isoelectric focusing. Isoelectric focusing of radiolabeled echovirus was performed as previously described for poliovirus and reovirus (7) in an LKB 8101 Ampholine column of 110 ml with 1% Ampholines covering the pH range of 3 to 10. Depending on the experiment, the virus sample was mixed throughout or added to the alkaline or acid region only of the column of 5 to 40% sucrose. For those experiments in which the concentration or specific activity of the virus suspension was low, a modified isoelectric focusing procedure was used (10). The virus was added to a 10-ml column of 5 to 40% sucrose containing 1% Ampholines in a glass tube (13 by 1 cm) stoppered with a small piece of dialysis tubing. The column was placed in a standard tube gel apparatus. For electrophoresis, the bottom reservoir contained 1% sulfuric acid (vol/vol) in 40% sucrose, and the top reservoir contained 2% ethanolamine. The bottom reservoir was filled to cover the column and continually stirred throughout the experiment to prevent overheating of the column. After electrophoresis at 400 V at 4°C for 6 h, 3- to 4-drop fractions were collected and diluted with 0.5 ml of distilled water. The pH of each fraction was determined, a sample was removed for plaque titration, and the fractions were adjusted to 7% trichloroacetic acid. The trichloroacetic acid-precipitable material was collected on membrane filters and counted by using a Beckman LS 7500 liquid scintillation counter.

RESULTS

Inactivation of echovirus at pH 6.0 and 20°C. Previously reported experiments with monodispersed preparations of poliovirus (4) were performed with solutions containing 0.01 M phosphate buffer at pH 6.0 to insure that all of the reacting chlorine was in the form of HOCl and in the presence of 0.1 M NaCl to prevent the highly concentrated suspensions of poliovirus from aggregating. Poliovirus suspensions will aggregate at pH 6.0 in buffers of low ionic strength (5, 6). Although concentrated echovirus suspensions do not aggregate in low-ionic-strength buffers unless the pH is below 5 (17), 0.1 M NaCl was added to the echovirus experiments to provide uniformity. The inactivation of echovirus under these conditions at 20°C with 4, 20, and 40 μM HOCl is shown in Fig. 1. At all three concentrations, there was an initial 1- to 2-$\log_{10}$-unit decrease in titer, a lag period during which the titer remained about the same or gradually increased, and a final steady decline in titer. The length of the lag period varied indirectly with the chlorine concentration and lasted nearly 60 s with 4 μM HOCl. Reducing the temperature to 2°C had little effect on the general shape of the inactivation curve (data not shown). However, the reaction was slower at 2°C than at 20°C at the same concentration of HOCl (20 μM), as evidenced by a longer lag period (60 s compared with 15 s) and a slower rate for the final decline in titer (0.65 $\log_{10}$ units/10 s compared with 2.9 $\log_{10}$ units/10 s).

Inactivation of echovirus at pH 7.0 and 20°C. At pH 7.0 an initial 2-$\log_{10}$-unit decrease in titer was followed by a very dramatic rise in titer of 1.5 $\log_{10}$ units before a final decline in titer (Fig. 2). Although there was a change in the distribution of HOCl and OCl^- when the buffer was adjusted from pH 6.0 to 7.0, 75% of the chlorine remained in the form of HOCl, with 25% in the form of OCl^-. Several experiments were performed to demonstrate clearly the early transition points of this complex inactivation curve.

Conformational states of the echovirus capsid. Mandel (11) has reported three conformational states for poliovirus type 1 (Brunhilde). He observed that poliovirus is distributed during isoelectric focusing into two pH-dependent and interconvertible forms with isoelectric points (pIs) of 7.0 (A form) and 4.5 (B form). The A form predominates at pH values greater than 7.0, and the B form predominates at pH values less than 4.5. At pH values in between, both A and B forms are present. Heat and UV irradiation stabilize the virion irreversibly in the B form. In this paper, this form will be referred to as the B* form, since it is no longer interconvertible with the A form.

We have observed three corresponding isoelectric forms for echovirus. Radioactively labeled echovirus was distrib-

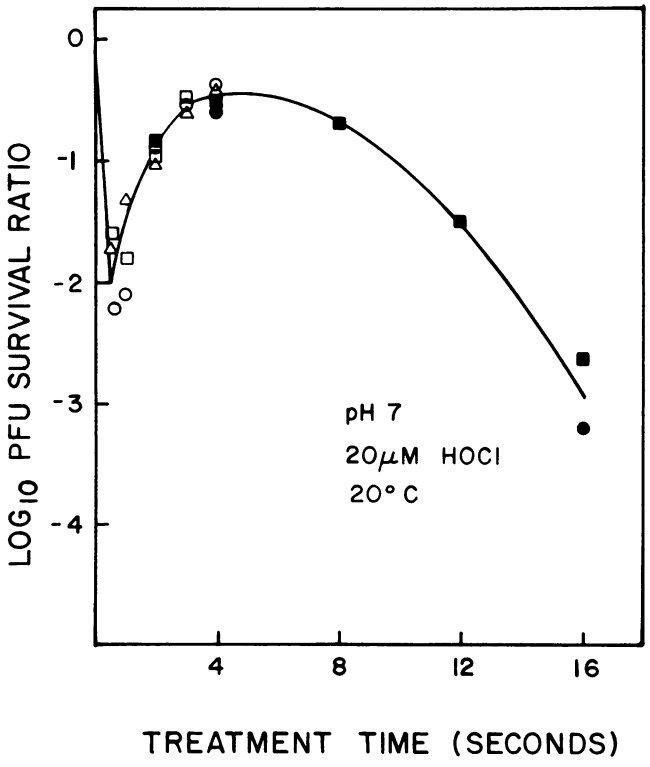

FIG. 2. Inactivation of echovirus with 20 μM HOCl at pH 7.0 and 20°C (different symbols indicate five repeat experiments).

uted into two isoelectric forms with pIs of 5.7 (A form) and 5.1 (B form) (Fig. 3) when the virus was distributed throughout the column before the start of the experiment. Under these conditions, the virus was exposed to both alkaline and acid conditions, since the pH gradient was established before the virus finished migrating. When the virus was added to the acid or alkaline region of the column, only one peak, at pH 5.1 or 5.7, respectively, was observed (Fig. 4), indicating that the forms were pH dependent. The forms were also interconvertible. When virus taken from the peak at pH 5.7 was introduced into another column in the acid region, the virus migrated to pH 5.1 (data not shown). Virus that focused at pH 5.1 similarly migrated to pH 5.7 when introduced in the alkaline region of another gradient. The B form appeared to have a specific infectivity of at least one to two orders of magnitude less than the A form, since, in this experiment, as well as in a previously reported experiment (17), all of the infectivity was associated with the peak at pH 5.7.

Isoelectric focusing of several of the samples along the complex inactivation curve at pH 7.0 (Fig. 2) showed a sequential stabilization of the virus infectivity at a pI of 5.1 (Fig. 5). This appeared to represent a third form for the virus. Unlike the B form, this form (B*) was no longer in reversible equilibrium with the A form, since no infectivity was observed at a pI of 5.7 after 4 s, despite the fact that the virus was mixed throughout the entire column at the beginning of the experiment. In addition, the B* form appeared to have a specific infectivity comparable to that of the A form, since most of the lost titer was regained after 4 s of treatment.

Inactivation of echovirus in the absence of 0.1 M NaCl and after stabilization in the B* conformational state. As previously mentioned, 0.1 M NaCl is not required to prevent echovirus aggregation since this virus does not tend to aggregate unless the pH is lowered below 5. The elimination of sodium chloride resulted in first-order inactivation kinetics (Fig. 6, circles), indicating that sodium chloride was involved in the unusual kinetics of echovirus at this pH. The rate of inactivation was 2.3 times slower than that for the final decline in titer of the three-phase inactivation curve in the presence of 0.1 M NaCl (triangles). Virus which had been pretreated with chlorine to stabilize it in the B* conformational form gave first-order kinetics even in the presence of 0.1 M NaCl, and the inactivation rate was the same as that for the final decline in titer of the three-phase curve (squares).

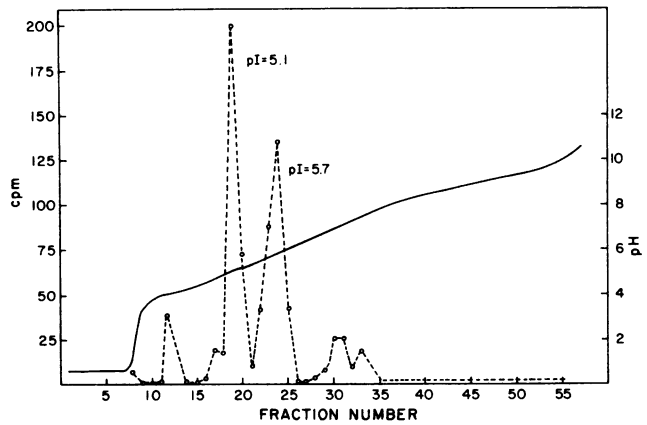

FIG. 3. Isoelectric focusing of echovirus radiolabeled with [^{3}H]uridine. The virus was mixed throughout the gradient at the start of the run. The infectivity of the virus was associated with the isoelectric peak at pH 5.7 (17). -----, cpm; ———, pH.

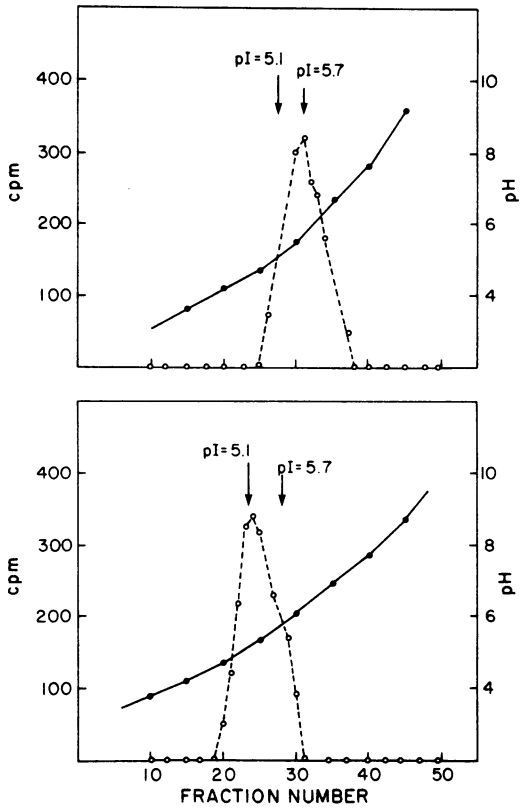

FIG. 4. The effect of the initial position of echovirus in the column on the final distribution after isoelectric focusing. Echovirus, radiolabeled with [^{3}H]uridine, was added to the alkaline region (top panel) or acid region (bottom panel) of the column at the start of the run. -----, cpm; ———, pH.

Inactivation of echovirus at pH 5.1 and 5.7. Since the conformational forms of the virus were pH dependent, it was possible to perform experiments under conditions where the virus was known to be in a certain conformational form at the start of the experiment. Therefore, chlorine demand-free water was adjusted with HCl to either pH 5.7, where the virus was in the A form, or to pH 5.1, where the virus was in the B form. First-order kinetics were observed at pH 5.7 (Fig. 7, squares) and complex kinetics at pH 5.1 (circles).

Inactivation of echovirus at pH 10.0. The most dramatic effect of the unusual inactivation kinetics for echovirus was observed at pH 10.0, where the chlorine was in the form of OCl^-. In Fig. 8, the inactivation of echovirus at pH 10.0 is compared with previously reported inactivation kinetics for monodispersed poliovirus, type 1 (Mahoney [16] and Brunhilde [15]) and for heavily aggregated coxsackievirus B5 (8). Complex kinetics were observed with monodispersed echovirus, whereas first-order kinetics were observed with monodispersed poliovirus. The kinetics of inactivation for coxsackievirus were typical of those for a heavily aggregated suspension of virus. Significantly, monodispersed echovirus was resistant for 8 min to a concentration of chlorine that inactivated monodispersed poliovirus 4 $\log_{10}$ units in less than 30 s and inactivated aggregated coxsackievirus 4 $\log_{10}$ units in less than 2 min.

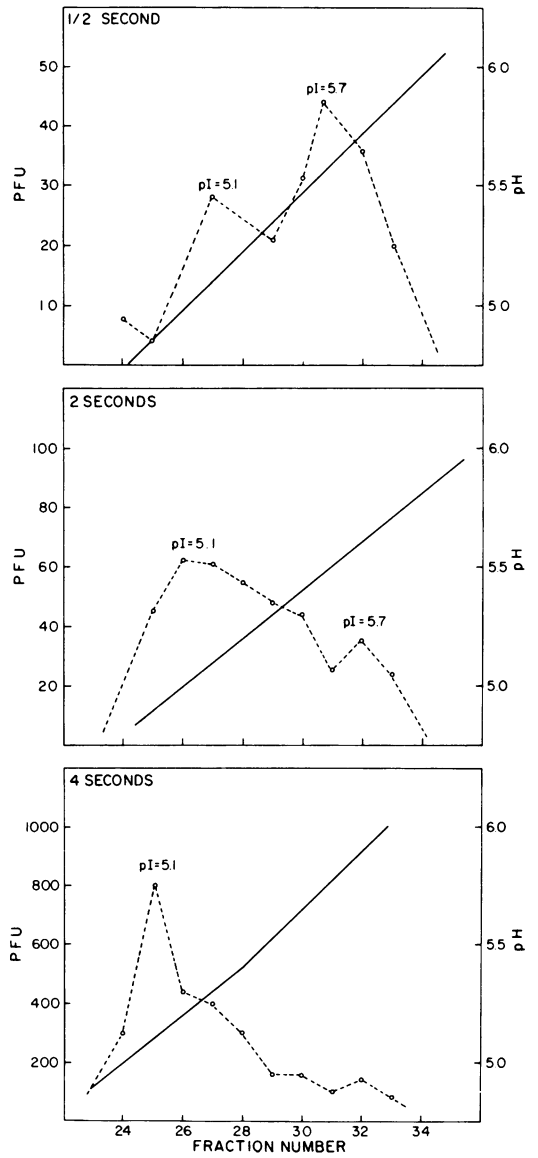

FIG. 5. Virus samples taken from the inactivation experiment at pH 7.0 (Fig. 2, open circles) after 0.5, 2, and 4 s of treatment were electrofocused and assayed for infectivity. -----, PFU; ———, pH.

DISCUSSION

Departures from linearity in virus inactivation kinetics are usually attributed to aggregation among the virions, with the assumption being made that if no aggregation were present, the reaction would be first order. The complex kinetics of inactivation of echovirus by chlorine in water is an exception, and it is becoming apparent that such exceptions are more common than was first thought. Such complexities in disinfection kinetics indicate that the picornaviruses can become, in certain ionic environments, resistant for a period of time to the effects of chlorine. The success achieved in any effort to destroy virus in water with chlorine must necessarily depend on an understanding of this mechanism of virus survival.

The experiments reported here suggest that the complex inactivation kinetics may be related to conformational shifts in the picornavirus capsid. As previously mentioned, Mandel (11) reported three conformational states for poliovirus based on isoelectric focusing measurements. In this study, we observed three corresponding isoelectric forms for echovirus (Fig. 3–5). There are two differences between our results and those of Mandel. First, the two pH-dependent and interconvertible isoelectric forms which Mandel observed for poliovirus at pH 4.5 (B form) and pH 7.0 (A form) were both infectious (11). With echovirus, the specific infectivity of the B form appeared to be at least 1 to 2 $\log_{10}$ units lower than that of the A form, since only one infectious peak was observed. Second, when poliovirus was irreversibly stabilized in the B* form at pH 4.5 by heat or UV treatment, it was no longer infectious. In fact, all remaining infectivity after heat or UV treatment was associated with the A-form virus. In contrast, echovirus was irreversibly stabilized in the B* form after exposure to chlorine, but before inactivation.

Isoelectric focusing of samples along the complex inactivation curve at pH 7.0 showed a sequential stabilization of remaining virus infectivity in the B* isoelectric form (Fig. 5). After 0.5 s of treatment, the titer had dropped almost 2 $\log_{10}$ units, and the virus infectivity was distributed between the two isoelectric peaks. As the titer began to increase, more of the virus infectivity focused at pH 5.1. At the peak of the increase in titer, all of the remaining infectivity focused at pH 5.1. The low titer after 0.5 s of treatment may be due to the majority of the virion particles being in the less infectious B form. This would be reflected by some infectivity at both isoelectric points. The increase in titer appears to be related to stabilization of the virus in the B* form. Thus, these results suggest that the virus may go through a transition from the A form to the B form to the B* form during the course of inactivation.

In addition to having a lower specific infectivity, the B form may be more resistant to the effects of chlorine. This is suggested by the lag periods which lasted as long as 60 s at pH 6.0, both at low temperature (2°C, 20 μM HOCl) and at low HOCl concentration (4 μM, 20°C). The most remarkable effect was observed at pH 10.0, where monodispersed echovirus was resistant for 8 min to a concentration of OCl^-

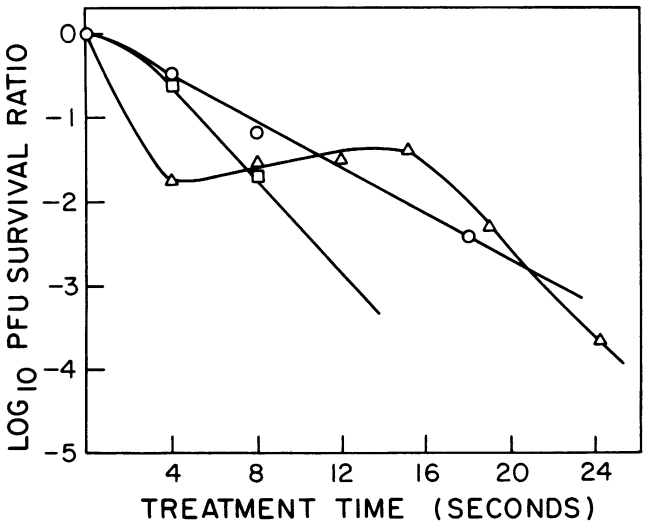

FIG. 6. Inactivation of echovirus with 20 μM HOCl at pH 6.0 and 20°C in the presence (triangles) and absence (circles) of 0.1 M NaCl and of irreversibly stabilized (B*) virus in the presence of 0.1 M NaCl (squares).

that inactivated heavily aggregated coxsackievirus 4 $\log_{10}$ units in 2 min.

Three different inactivation curves were obtained for echovirus with 20 μM HOCl at pH 6.0 and 20°C (Fig. 6). In the presence of sodium chloride, complex kinetics were observed. In the absence of sodium chloride, first-order kinetics were observed. Virus irreversibly stabilized in the B* form no longer showed complex kinetics even in the presence of sodium chloride. The experiments at pH 5.1 and 5.7 in chlorine demand-free water with no buffer and no salt showed complex kinetics at pH 5.1 and first-order kinetics at pH 5.7. Since the conformational forms of the virus were pH dependent, the virus at pH 5.1 was presumably in the B form and at pH 5.7 in the A form. These results suggest that when conditions were such that the virus was in the A form (pH ≥ 5.7, no NaCl) or B* form (pretreatment with HOCl) throughout the experiment, first-order kinetics were obtained. When the virus was initially in the B form (pH 5.1) or conditions favored a transition to the B form after exposure to HOCl (pH 6.0, 0.1 M NaCl), complex kinetics were observed.

Complex inactivation kinetics have also been observed for coxsackieviruses B3 and B5 (8) and poliovirus type 1 (LSc) (9). Like echovirus, coxsackievirus B5 gave complex inactivation kinetics at pH 6.0 in the presence of sodium chloride but not in its absence (8). However, complex kinetics were not seen with coxsackievirus B5 at pH 10.0. With coxsackievirus B3, complex kinetics were observed at pH 6.0 in the presence or absence of sodium chloride, and first-order kinetics were observed at pH 10.0. We have not observed complex kinetics for either of two strains of poliovirus type 1 (Mahoney and Brunhilde) under any of the above conditions (4, 15, 16). However, Kenyon and Schaub reported complex kinetics for poliovirus type 1 (LSc) in 0.005 M acetate buffer at pH 5.0 and 6°C (9). Thus, it appears that different ionic environments may favor conformational transitions for different picornaviruses.

Picornaviruses are released from the infected cell in the form of single particles and large and small aggregates (18). We have previously demonstrated two mechanisms which can account for the increased survival of aggregates in water. These are (i) protection—where halogen molecules are prevented from reaching hidden particles in large aggregates (13) and (ii) multiplicity reactivation—where an aggregate of inactivated virions can produce a plaque, although no individual virion is infectious alone (19). The results presented here demonstrate a third mechanism of virus survival through which dispersed virus can resist inactivation and suggest a new avenue of investigation of the complex problems involved in the inactivation of viruses in water by halogen compounds.

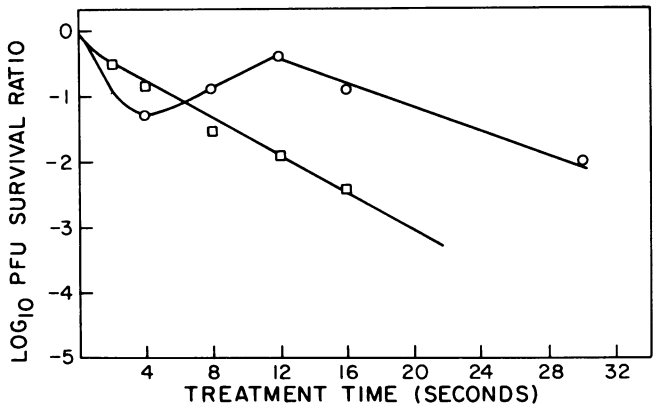

FIG. 7. Inactivation of echovirus with 20 μM HOCl at pH 5.1 (circles) and pH 5.7 (squares) and 20°C in the absence of buffer salts or sodium chloride.

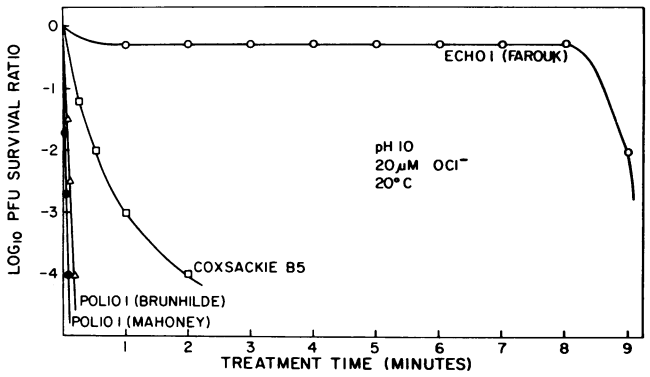

FIG. 8. Inactivation of monodispersed echovirus, two strains of monodispersed poliovirus (15, 16), and heavily aggregated coxsackievirus (8) with 20 μM OCl^- at pH 10.0 and 20°C in the presence of 0.1 M NaCl.

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