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The Complex Reaction Kinetics of ECHO-1 Virus with Chlorine in Water (39965)

DOROTHY C. YOUNG, J. D. JOHNSON, AND D. G. SHARP*

* *Department of Bacteriology and Immunology, School of Medicine, and Department of Environmental Science and Engineering, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514*

Introduction. Those who would demonstrate the power of an inactivating agent on viruses in water have often presented their data in terms of log infectivity vs time of action. Departures from linearity in such a graph have frequently been attributed to aggregation among the virions, the assumption being that were no aggregation present, the line would be straight, and the reaction first order (1). In some cases, experiment has shown this to be true (2–5). But the present case of the inactivation of ECHO-1 virus by HOCl in water is a notable exception. Some other perturbation of the straight line, first-order reaction kinetics, must occur and it appears that conformational changes in the capsid of the virion may be involved. What follows is an account of the kinetic aspects of the inactivation of monodispersed ECHO-1 virus in water containing HOCl. Particular attention has been directed to the initial phases of the reaction which take place so quickly that they could be overlooked.

Materials and methods. ECHO-1 virus (Farouk Strain) was provided by Dr. Mark Sobsey of this University. It was produced in quantity in human epidermoid carcinoma cells (HEp-2), and neutralization tests with Farouk antisera resulted in plaque reduction of 99 to 99.9%. Details of virus production, purification, plaque assay, and physical assay of single virions and clumps by quantitative electron microscopy were all the same as described in a publication of earlier work with poliovirus (6).

The degree of aggregation among virions was further tested in some cases by differential centrifugation in a step-density gradient of sucrose. This is the red, white, and blue (RWB) technique (5), so called because of the dye contained in the different density levels. After an appropriate amount of centrifugation, only single virions remain in the top (white) region which contained all of the original virus suspension. If aggregates are present, the pairs and other small groups will be found just below, in the blue region, while large aggregates will be in the lowest (red) region of the centrifuge tube. Plaque titration of these three regions together with a similar set of fractions from a control tube of monodispersed virus provides a very sensitive means for detecting aggregation at concentration levels too low for electron microscopy, as well as a rough measure of the size and frequency of the aggregates present.

A stock solution of sodium hypochlorite was prepared by diluting Fisher 5% reagent grade hypochlorite solution 10:1. An appropriate amount of this stock was then added to 21 liters of buffered chlorine demand-free water at pH 6.0 to obtain the desired HOCl concentration. This concentration was determined immediately prior to a run by amperometric titration with phenylarsine oxide (PAO). The buffer (0.01 *M* phosphate and 0.1 *M* sodium chloride) was prepared from Fisher primary standard monobasic potassium phosphate, Mallinckrodt analytical reagent grade dibasic potassium phosphate, Fisher biological grade sodium chloride, and deionized glass-distilled water containing 2.5 mg/liter of chlorine. If the buffer still contained 1.5–2.0 mg/liter of chlorine after 2 days, it was dechlorinated with ultraviolet light and considered to be chlorine demand free.

The virus diluted in 0.14 *M* NaCl was injected into a turbulent flowing stream of the prepared chlorine solution and samples were withdrawn at appropriate time intervals downstream for rapid mixing with 2 mM sodium thiosulfate solution to reduce any unreacted HOCl and for plaque titration. The apparatus (4) operates satisfactorily with exposure times as short as 0.5 sec.

The isoelectric point of ECHO virus was determined in the electrofocusing appara-

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tus, Model 8100 (LKB Produkter AB, Stockholm, Sweden), using 1% ampholine carrier ampholytes covering the range of pH 3–10.

Experiments and results. The inactivation of ECHO virus by 20 μM HOCl at pH 6 and 20° is shown as a function of time in Fig. 1. Several experiments were required to demonstrate clearly the two transition points of this complex curve. During the first second of exposure 98% of the PFU were inactivated. Then no further change was observed until 15 sec, after which rapid inactivation was resumed, reaching the 10^{-4} level of survival in about 24 sec. Suspecting virion aggregation at this point, sodium chloride up to 0.3 M was added to the 0.01 M phosphate buffer in the chlorinated water (6) and the experiments were repeated. The inactivation curve remained the same.

Virus was picked from plaques surviving 15 sec of HOCl exposure (plateau region of Fig. 1), and with this the inactivation experiment was repeated. These progeny of the surviving plaque gave the same response as the parent virus preparation. There was no initial lag, which would be expected from a more resistant mutant, and the time when inactivation was resumed was the same also.

For inactivation of ECHO virus by HOCl, pH 6.0 was chosen in order that the results might not be complicated by the presence of chlorine in any other active form, Cl_2 or OCl. The virus proved to be isoelectric at pH 5.7 (Fig. 2), but there was no tendency for aggregation to occur in this region. Single particle approximation (SPA) tests (7) carried out in several 0.05 M buffers covering the range of pH from 3 to 11 showed virion aggregation in the acid range below pH 5 but none above this point (Fig. 2). Electron microscopy also showed that the virus preparations at pH 6 (with and without added NaCl) contained very few aggregates. Virions attached to aluminum-coated collodion films by Brownian bombardment [KA method, Ref. (8)] revealed about 30 pairs per thousand, three triplets per thousand, and larger groups in progressively smaller numbers. No clumps as large as 10 could be found. Furthermore, RWB tests of suspensions of PFU that had survived 15 sec of HOCl treatment showed that they sedimented at the same rate as single virions. They were not aggregates, not even pairs.

Further evidence that virion aggregation does not influence the shape of the inactivation curve is supplied by repeating the experiment at a lower virion concentration. Most of the experiments were done with suspensions of 10^8 virions/ml. When this was reduced to 10^7 virions/ml, the inactivation curve remained essentially the same. The persistent level was 99% and inactivation resumed between 15 and 19 sec.

Discussion. When infectivity declines rap-

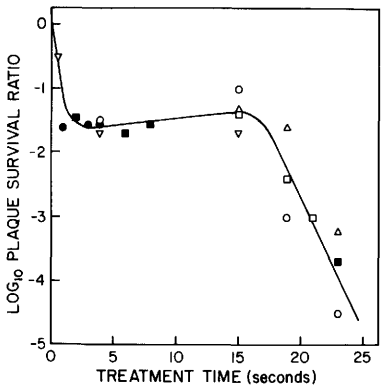

FIG. 1. Inactivation of ECHO-1 with 20 μM hypochlorous acid (HOCl) at pH 6 and 20°. Ninety-eight percent of the virus is inactivated in 1 sec, followed by a 15-sec delay before final decline in titer (different symbols indicate six repeat experiments). Squares show results obtained with progeny of a single plaque selected from survivors of 15-sec HOCl treatment.

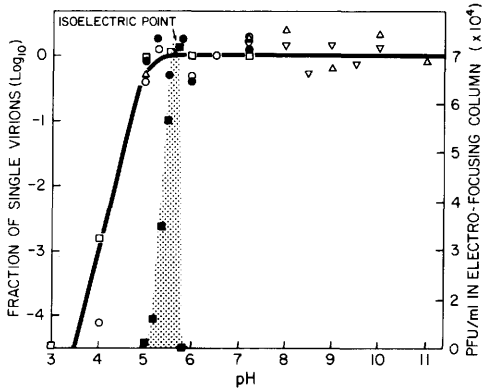

FIG. 2. ECHO-1 virus has an isoelectric point of pH 5.7 (shaded region, PFU scale on right), but SPA tests (solid line, scale on left) show no evidence of aggregation at this pH; aggregation occurs only in the acid region below pH 5.

idly in a virus suspension containing a disinfecting agent, then the reaction slows or even stops, one looks for depletion of the active agent, or a fraction of the virus in large protective aggregates, or possibly a resistant fraction of the population that may be genetically different. Inasmuch as the reaction with ECHO virus begins again after a 14-sec delay at the 2% survival level, there is no question here of depletion of the HOCl.

It would take at least 16 spheres (virions) to surround one and so provide it with even one layer of protection. No aggregates this big were observed in any of the electron micrographs containing several thousand virions each. Furthermore the resistant PFU sedimented at the same rate as single virions. Still furthermore, virus progeny from resistant survivors have the same initial rapid reaction rate, the same plateau of resistance (survival level ca. 10^{-2}), and the same delay time until disinfection is resumed. This pattern was not altered when the virus concentration was reduced by a factor of 10. If aggregation were induced by destabilization of the virion suspension when it entered the HOCl solution, the rate of formation of aggregates would have to be proportional to the square of the virion concentration (9). Reducing this by a factor of 10 would have reduced the aggregation rate by a factor of 100.

These observations are sufficient to send us looking for a different survival mechanism of ECHO virus in HOCl. A few percent of the virion population is, or becomes, sufficiently resistant to sustain for 15 sec the concentration of HOCl that inactivated 98% of the population in 1 sec. If the resistant ones are just more difficult to penetrate, then the observed resumption of disinfection indicates that sufficient penetration of the HOCl has finally been achieved. Evidence has been presented by Mandel (10) and also by Fujioka and Ackermann (11) supporting the hypothesis that the poliovirus capsid proteins may exist in two or more different, possibly metastable, conformational states. This evidence comes from observations of electrophoretic mobility in Mandel's experiments, in which he finds not one but two isoelectric points for poliovirus. We find only one pI for ECHO virus, but Fujioka and Ackermann find their evidence in the effects of cations on the reactivity of poliovirus with guanidine. They recognized four conformational states of the virion which were reversibly interchangeable and yet retained infectious potential.

We are therefore encouraged to propose that ECHO-1 virus may exist in two different conformational states, one of which is substantially more resistant than the other to penetration by HOCl. But even the resistant state is ultimately penetrated under the conditions of these experiments. Virions in the resistant state may be present initially or a fraction of the population may be converted to the more resistant conformation by the change in environment when they enter the flowing stream of chlorine water. Our experiments do not distinguish between these two alternatives, but they do suggest a new avenue of investigation of the complex problems involved in the inactivation of viruses in water by halogen compounds.

Summary. A fraction of the virions in a monodispersed suspension of ECHO virus is temporarily much more resistant than the rest to disinfection by chlorine (HOCl). This fraction is not genetically different. It may, however, owe its temporary resistance to a different conformation of the protein capsid that retards penetration by the HOCl.

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