Effect of Ionic Environment on the Inactivation of Poliovirus in Water by Chlorine

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The rate of inactivation of poliovirus in water by chlorine is strongly influenced by the pH, which in turn influences the relative amounts of HOCl and OCl⁻ that are present and acting on the virus in the region of pH 6 to 10. The distribution of HOCl and OCl⁻ is influenced to a lesser extent by the addition of NaCl. The major part of the sharp increase in disinfection rate seen with this salt is thought to be due to its effect on the virus itself resulting in an increased chlorine sensitivity, especially at high pH.

Laboratory experiments to determine the kinetics of inactivation of viruses by chlorine are usually made in water containing a dilute buffer system to maintain a definite pH during the reaction and to insure a known composition of the reacting agents. Chlorine dissolved in water at pH 6 is essentially all HOCl. At pH 10, it is predominately OCl⁻, and at all pH values between, there is some of each. The relative proportions of the two can be determined from the pH, temperature, and ionic strength (9). Still there is little published data showing how the inactivation rates of viruses change with pH in the region of 6 to 10.

One of the reasons for the paucity of comparative reaction rate data is doubtless the fact that, in most disinfection experiments, the inactivation rate does not remain constant, even in cases where the concentration of chlorine does remain constant and the virion population has been shown to be genetically homogeneous. Often such departure from first-order reaction kinetics has been attributed to aggregation among the virus particles, but little data have been provided until recently in support of this possibility. We have been making an extensive study of virion aggregation in which it has been found that both pH and ionic strength are critically involved (6, 7). From these results, it is clear that in any series of experiments to determine the inactivation rate of virus by chlorine in the pH range 6 to 10 care must be taken to avoid virion aggregation at each step.

Two difficulties were encountered in our present efforts to make such a series of experiments. Our sucrose-banded virus concentrates became physically unstable and tended to aggregate in

borate buffer at pH 9, and they tended to aggregate also in slightly acid phosphate buffer at pH 6. The first difficulty was avoided by using a phosphate-carbonate combination buffer system over the whole pH range, and the second was apparently solved when 0.1 M NaCl was added to the buffer. The salt raised the ionic strength of the suspending solution from approximately 0.025 (Table 1) to approximately 0.125, still substantially less than that of phosphate-buffered saline.

Here we give an account of some results obtained with chlorine and poliovirus type 1 (Mahoney) in a series of inactivation experiments at pH 6, 7, 8, 9, and 10. Beyond the fact that the data obtained here are of immediate practical value, there is a further reason for making these experiments. Several years ago, in a paper by Scarpino et al. (12), evidence was presented showing that poliovirus type 1 (Mahoney) was inactivated more rapidly by chlorine in the form of OCl⁻ at pH 10 than by HOCl at pH 6. This was contrary to existing concepts as expressed earlier by Weidenkopf (16), and the discrepancy has remained unexplained in the published literature. The present work, which has been vitally concerned with the effect of sodium chloride on the virus-chlorine reaction, provides a likely explanation for that apparent contradiction.

MATERIALS AND METHODS

The virus used was poliovirus type 1 (Mahoney) prepared in cultures of human epidermoid carcinoma (HEp-2) cells, purified and concentrated as previously described in this journal (4). Plaque assay was made in monolayer cultures of the same cells, and physical assay (virion counting) was done on preparations for the electron microscope made by the kinetic attachment method as previously described (13). Storage of

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TABLE 1. Composition of phosphate-carbonate buffer

pH	KH$_2$PO$_4$ (M)	Na$_2$CO$_3$ (M)	Ionic strength
6.0	0.0176	0.0012	0.021
7.0	0.0116	0.0042	0.024
8.0	0.0079	0.0061	0.026
9.0	0.0067	0.0067	0.027
10.0	0.0045	0.0078	0.026

purified concentrates of the virus was at 4 to 6°C in the approximately 20% sucrose of the density gradient in which it was prepared. This sucrose solution was made in 0.05 M phosphate buffer at pH 7.2. Virus concentrates prepared and stored in this way have remained fully infectious and practically free of aggregation for several months.

Preparation of apparatus and chlorine solutions was the same as described in previous work (5). In some of the experiments, it was necessary to observe the effect on the virus of chlorine exposures for times as short as 1 s, and many data points were taken within 15 s after contact so the continuous flow apparatus (14) was used for all the inactivation experiments. Briefly, 20 liters of buffered, chlorine demand-free water was adjusted to the temperature and chlorine concentration required for the experiment. After constant turbulent flow was established through the treatment tube, the virus sample was injected steadily for 5 s with a synchronous motor-driven plunger. Spring-loaded syringes containing continuously mixed sodium thiosulfate solution were released in turn to withdraw 1 ml of the chlorine-virus mixture and quench the reaction at exposure times determined by the velocity of flow in the stream and the distance from the injection point. When exposure times greater than 15 s were required, the well-mixed chlorine-virus mixture was collected as it emerged at the end of the treatment tube after 15 s of exposure and held at reaction temperature, and samples were taken for titration of virus and chlorine when sufficient additional time had elapsed.

The buffer utilized in the maintenance of the desired pH value was prepared by mixing crystalline KH$_2$PO$_4$ (Fisher Scientific Co., catalog no. P-382, primary standard grade) and Na$_2$CO$_3$ (Fisher S-281, alkalimetric standard) together in 20 liters of deionized glass distilled water in a Pyrex glass bottle (the large vessel described in [14]). The final molarities of each reagent are given in Table 1. The solution was then made chlorine demand free as previously described (5). Table 1 also shows the final ionic strength of the buffer at each pH (exclusive of NaCl). When NaCl was also used, it was at 0.1 or 0.2 M (ionic strength, 0.1 and 0.2, respectively) and was obtained from Fisher (S-671, biological grade).

RESULTS

Previous work in this laboratory has shown that poliovirus type 1 (Mahoney) remains dispersed as single particles in 0.05 M phosphate buffer at pH 7, but at pH 6 it tends to aggregate (6). This aggregation can be prevented by adding 0.1 M NaCl to the pH 6 buffer. To avoid any possibility of aggregate formation in this work, 0.1 M NaCl was included in pH 6 buffer as it was in previous work (5) and, for uniformity, in all the others as well. Accordingly, phosphate-carbonate buffer (Table 1) was prepared with 0.1 M NaCl at pH 6, 7, 8, and 9, and virus was exposed in each of these buffers to 2.5 μM HOCl at 20°C. This means that at pH 6, 7, 8, and 9, the concentrations of OCl$^-$ present were 0.11, 0.90, 9.1, and 91 μM, respectively. In this series, pH 10 was omitted because the expected 900 μM OCl$^-$ seemed impractical. The pH 10 experiments were made at lower chlorine concentration.

The progress of the inactivations can be seen in Fig. 1. There is a remarkable increase in reaction rate between pH 6 and 7 and successively smaller increases at pH 8 and 9, whereas the OCl$^-$ concentration increased steadily by about a factor of 10 at each step. A second series of experiments was made like the first except that the 0.1 M NaCl was omitted from the buffer (Fig. 2). This reduced the ionic strength of the suspending fluids from 0.125 to 0.025, and it also reduced the reaction rate by a factor ranging between 2× and 4×, depending on the pH (compare Fig. 1 and 2). The effect of salt at pH 10 was sought by making experiments at a lower total free chlorine concentration (10 μM) which is now in the form of OCl$^-$. Here the inactivation rate with 0.1 M NaCl present was of the order of 50 to 100 times faster than without it (Fig. 3). Pressing further with this extraordinary result, we observed that in the presence of the salt the inactivation rate of the virus increased with chlorine concentration only up to 10 μM; at 40 μM it
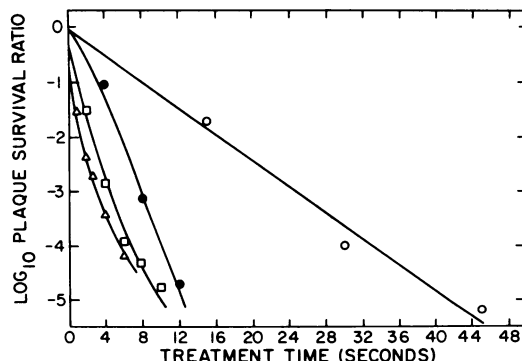

FIG. 1. Log$_{10}$ plaque survival ratio versus time for poliovirus in phosphate-carbonate buffers containing 0.1 M NaCl. Total ionic strength was approximately 0.125 and HOCl concentration was 2.5 μM in all four experiments. The OCl$^-$ concentration was 0.11 μM at pH 6 (○), 0.90 μM at pH 7 (●), 9.1 μM at pH 8 (□), and 91 μM at pH 10 (△).

went very little faster (Fig. 4). A similar satura-tion effect was observed in previously reported experiments (5) without salt. Under these con-ditions, the concentration for maximum rate was about 15 μM.

In the absence of 0.1 M NaCl at pH 10, the inactivation rate of the virus was only about 1 $\log_{10}$ unit in 60 s (Fig. 3). Although the concen-tration of OCl^- is four times greater than that of HOCl at pH 6 (Fig. 2), the inactivation rate is much slower. Apparently OCl^- is only about one-tenth as effective in the inactivation of this particular virus as HOCl. Still, at pH 7 where the OCl^- concentration is only 0.9 μM, the rate of inactivation is roughly three times as great as it was at pH 6 (Fig. 1). This must be due, at least

FIG. 4. *Rate of inactivation of poliovirus in phos-phate-carbonate buffer containing 0.1 M NaCl as a function of the concentration of OCl^- at pH 10. The ordinate is the number of log_{10} units of plaque titer lost in 6 s.*
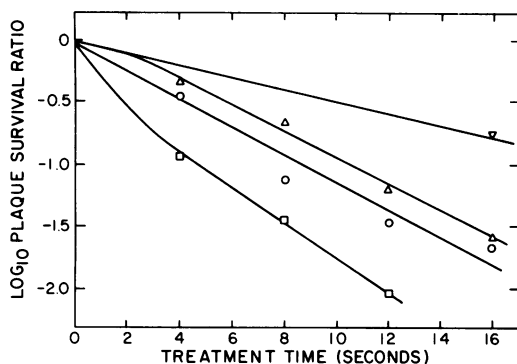

FIG. 2. *Log_{10} survival ratio versus time for polio-virus in phosphate-carbonate buffers without 0.1 M NaCl. Total ionic strength was approximately 0.025 and HOCl concentration was 2.5 μM in all four ex-periments. The OCl^- concentration was 0.08 μM at pH 6 (∇), 0.77 μM at pH 7 ($\triangle$), 7.75 μM at pH 8 ($\bigcirc$), and 80.7 μM at pH 9 ($\square$).*
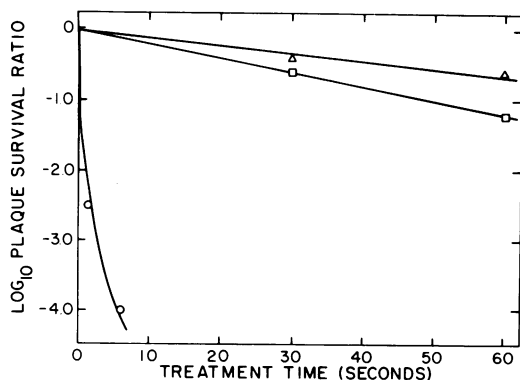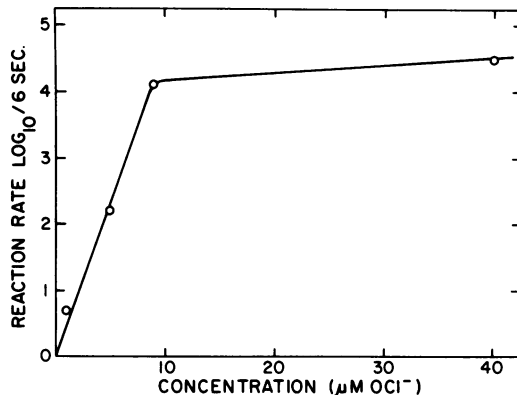

FIG. 3. *Log_{10} plaque survival ratio versus time for poliovirus in phosphate-carbonate buffer at pH 10 containing 0.1 M NaCl ($\bigcirc$) and without 0.1 M NaCl ($\triangle$). When no buffer and no NaCl were used (ionic strength, 0.0001), the squares ($\square$) were the result. The concentration of OCl^- was 10 μM in all three cases.*

in part, to a decrease in the resistance of the virus in the presence of 0.1 M NaCl. The further increases in rate at pH 8 and 9 may be attrib-utable to the rapidly increasing OCl^- concentra-tions as well as the increased sensitivity of the virus to OCl^- in the presence of salt and may involve the saturation effect observed with OCl^- at pH 10 (Fig. 4) as well.

Sodium chloride added to the buffer at pH 6 prevented aggregation, although in 0.05 M phos-phate buffer at pH 6 this aggregation was quite slow (7). To get the reaction rate of chlorine on the dispersed virus without salt, experiments were made that showed that it could be done even at pH 6 if the inactivation experiment was done within a few minutes of the time the dis-persed stock virus at pH 7 was diluted and adjusted to pH 6. This is the method by which the top line of Fig. 1 was obtained and also the "no salt" line of Fig. 5. Figure 5 also shows the slower chlorine inactivation rate that was ob-tained if the virus was permitted to aggregate before the run was made. In addition, it shows that the accelerated rate produced by 0.2 M NaCl is little, if any, more than that of 0.1 M salt.

DISCUSSION

The plaque titer of our purified preparations of poliovirus has not declined to any measura-ble extent on standing 4 h in water, at 25°C in the pH range 3 to 10, in any of several buffer systems, or in phosphate- or tris(hydroxy-methyl)aminomethane-buffered saline. Others have reported the pH range of maximum heat stability to be pH 3 to 7 (1, 11). Thermal degra-dation occurs more rapidly in the alkaline region, and it is augmented in the presence of sodium chloride particularly at the higher pH's (1), but
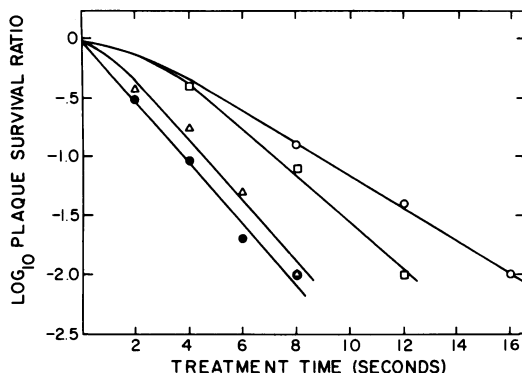
FIG. 5. Log_{10} plaque survival ratio versus time of poliovirus in phosphate-carbonate buffer at pH 6 of ionic strength 0.021 containing 20 μM HOCl. □, Experiment made within 5 min of dilution of the stock virus; ○, results with virus that has had time to aggregate. The triangles and filled circles are for virus dispersed by adding 0.1 M NaCl or 0.2 M NaCl, respectively, to the reaction mixture.

we are not aware of any previous report showing that chlorine destroys viruses more rapidly when NaCl is present.

Free chlorine inactivated this virus under all conditions that we have examined, but it is clear that when no NaCl was added to the buffer, a given concentration was much more effective at pH 6 than at pH 10 (compare Fig. 2 and 3). On the other hand, when 0.1 M NaCl was added to the buffer, the rate of inactivation at pH 10 (Fig. 3) was increased 50- to 100-fold (depending on the survival level chosen for comparison), and the rate for a given concentration was more effective at pH 10 than pH 6. Now the complexity of the situation becomes apparent; the partition of the free chlorine between HOCl and OCl⁻ depends primarily on the pH and, to a lesser extent, on the salt concentration, and the sensitivity of the virus varies also with pH and with the amount of salt present.

It is clearly not a simple matter to determine the relative effectiveness of HOCl and OCl⁻ in the disinfection of poliovirus, but an initial step would seem to be to perform experiments at pH 6, 7, 8, 9, and 10, keeping the concentration of HOCl constant. This was done at 2.5 μM HOCl, at pH 6, 7, 8, and 9, where the corresponding concentrations of OCl⁻ are calculated to be 0.11, 0.90, 9.1, and 91 μM, respectively. At pH 10, we reduced the total free chlorine to 10 μM to keep it within a practical range. Calculations of relative amounts of HOCl and OCl⁻ were made by the method of Davis (2) as applied by Morris (9) and by Sugam and Helz (15). The acid ionization constants of HOCl given by Morris (9) were used.

In the absence of NaCl at pH 10, where essentially all of the 10 μM free chlorine (Fig. 3) is OCl⁻, approximately 10% of the plaque titer survived 60 s of exposure. At pH 6, 2.5 μM HOCl reduced the survival ratio to approximately the same level in 16 s. It appears from this alone that OCl⁻ is poor compared with HOCl as a disinfecting agent. When NaCl is present, there is a 50 to 100× increase in virus sensitivity at pH 10 where the reacting species is OCl⁻, compared with an increased sensitivity at pH 6 of threefold, where the reacting species was HOCl. It was not clear from these results whether the increased sensitivity to OCl⁻ in the presence of NaCl extends to the lower pH values of 6 and 7. If it does, the inactivation rate for 1 μM OCl⁻ at pH 10 in the presence of NaCl (Fig. 4) still does not appear to account completely for the increases from 0.11 to 0.9 μM. It is possible that the virus becomes more sensitive to the 2.5 μM HOCl with increasing pH, a trend that may continue at pH 8 and 9.

The effect of added salt (0.1 M NaCl) on the rate of degradation of plaque titer by HOCl can be seen by comparing Fig. 1 and 2 to be about a threefold increase at pH 6. In this case, the virus will aggregate without the salt (6) so the inactivation experiments were performed quickly, before appreciable aggregation could occur (7). The much greater increase in inactivation rate of chlorine produced by salt at pH 10 might be suggested but not predicted in view of the similar effect that has been observed (1) with NaCl on the thermal degradation of this virus. It is, nevertheless, a fact whose practical significance cannot be ignored, for it is apparent that, at high pH, OCl⁻ destroys this poliovirus more rapidly in the presence of 0.1 M NaCl than HOCl does at pH 6 in the same buffer with or without the added salt.

At very low ionic strength (0.0001, pH 10 without buffer) plaque titer was reduced more rapidly by 10 μM OCl⁻ than in phosphate-carbonate buffer of ionic strength 0.027 and the same pH (Fig. 3), although the difference is not very great. This suggests that the greatly increased rate at pH 10 in the presence of 0.1 M NaCl may be a specific effect of the sodium or chloride ions. Potassium chloride, included in borate buffer at pH 10 by Scarpino et al. (12), yielded a more rapid poliovirus (Mahoney) inactivation by chlorine than they observed at pH 6. This may mean that KCl and NaCl act similarly, but the comparison cannot be made accurately because it is not clearly stated in their paper what concentration of KCl was used, and no attempt was made in their experiments to control virion aggregation which is not negligible (Fig. 5). The most effective concentration of

NaCl seems to have been approached at 0.1 M, since the reaction rate with 0.2 M (Fig. 5) is very little greater. Other salts and other viruses have not been tested in this respect, but work in progress at this writing indicates that the reaction rates of chlorine with coxsackie B5 at pH 10 are increased not only by NaCl but also by KCl and by CsCl. Doubtless other salts commonly found in hard waters should be investigated as well.

The ability of OCl⁻ in the presence of 0.1 M NaCl to inactivate poliovirus (Mahoney), is subject to similar limitations that have been observed (4) in the data of Weidenkopf (16). After increasing linearly to 10 μM, the rate increased very little at 40 μM (Fig. 4).

In conclusion, it has become clear from the results of recent work on viral disinfection (4-9, 11, 14, 15) that many more variables are present than were previously suspected (12, 16). Therefore, we strongly urge that in the future all workers in viral disinfection control for and clearly state in the published paper the following variables: (i) the time of contact of the virus with the disinfectant, (ii) the temperature of the reaction, (iii) the total ionic strength of the reaction mixture, (iv) the pH of the reaction mixture, (v) the type of buffer used to maintain the pH, (vi) the total chlorine concentration and the relative amounts of HOCl and OCl⁻, and (vii) the state of aggregation of the virus. Only when all of these parameters are known will it be possible to make adequate comparisons between data from different laboratories.

ACKNOWLEDGMENT

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