Inactivation of Coxsackieviruses B3 and B5 in Water by Chlorine

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The inactivation rates of coxsackievirus B3 (CB3) and B5 (CB5) by chlorine in dilute buffer at pH 6 were very nearly the same and about half that of poliovirus (Mahoney) under similar conditions. Purified CB3, like the poliovirus, aggregated in the acid range but not at pH 7 and above. Purified CB5 aggregated rapidly at all pH values; still, the graph of log surviving infectivity versus time was a straight line. No chlorine inactivation data were obtained with dispersed CB5, for it could be dispersed only by addition of diethylaminoethyl dextran, which would react with the chlorine. Addition of 0.1 M NaCl to the buffer at pH 6 did not influence the aggregation of CB5 or the rate of chlorine action on either of the coxsackieviruses, but at pH 10 it increased the disinfection activity of OCl− for both viruses roughly 20-fold. Cesium chloride had a similar but smaller effect. KCl was the most active of the three in this respect, making the inactivating effect of OCl− at pH 10 about equal to that of HOCl at pH 6.

The remarkable advances made in virology in recent years have not provided very much data of immediate use to the sanitary engineer who must inactivate viruses by chlorination of water. The work of Engelbrecht et al. (1) on six enteric picornaviruses is, however, a notable exception. Among the viruses examined, the two that differed most in their resistance to chlorine were two types of coxsackievirus. They found coxsackievirus B5 (CB5) to be 11 times more resistant than coxsackievirus A9 to inactivation by HOCl at pH 6 and 44 times more resistant at pH 10 where the active agent is OCl−. They show CB5 to be 1.6 to 10 times more resistant than four other enteroviruses at pH 6. Inasmuch as we have observed that poliovirus (Mahoney) aggregates at pH 6 and both reovirus and echovirus aggregate at only slightly lower pH (5), it seemed possible that CB5 might aggregate at pH 6 and thereby exhibit high chlorine resistance. Having already developed methods for measurement of virus aggregation by electron microscopy (10, 11) and by ultracentrifugation (5, 19), we have applied these to a study of CB5 and to a lesser extent to coxsackievirus B3 (CB3). The inactivation experiments have been made in such a way that the results can be readily compared with those we have published earlier for poliovirus (3) as well as those of Engelbrecht et al. (1). After purification, CB5 aggregates over the whole pH range 3 to 10. This makes possible a comparison of the inactivation kinetics of this aggregated virus with that of CB3, which does not aggregate in the alkaline range.

MATERIALS AND METHODS

CB3 was obtained from Mark Sobsey, and CB5 was from John Hoff. They were checked by plaque suppression with specific antisera supplied by Microbiological Associates, Walkersville, Md. Both viruses were propagated in monolayer cultures of HEp-2 cells and titrated by plaque formation in 1-ounce (ca. 30-ml) bottle cultures of the same cells. Purification sufficient to remove chlorine-demanding materials and capable of yielding suspensions of poliovirus free from aggregation was done here with both CB3 and CB5 exactly as it was in earlier work with poliovirus (2). The isoelectric point of the CB5 virus was determined in an electrofocusing apparatus, model 8100 (LKB Produktur AB, Stockholm, Sweden), with 1% ampholine carrier ampholytes covering the range of pH 3 to 10.

A buffer of phosphate and carbonate was prepared as previously described (13). This buffer was used for virus inactivation experiments at both pH 6 and 10 with and without the addition of 0.1 M NaCl, CsCl, or KCl. All reagents were American Chemical Society primary standard or enzyme grade. The sodium carbonate was heated at 250°C for 2 h, and the potassium and cesium chloride at 500°C for 1.5 h to remove excessive chlorine demand. Buffer solutions were prepared with deionized glass-distilled water made chlorine demand-free, as previously described (3).

Physical assay (particle counting and aggregation analysis) was done by electron microscopy of virus preparations made by either or both of two quantitative methods, agar pseudoreplication (10), or kinetic attachment (11).

Exposure of virus preparations to chlorine solutions was done in the continuous flow apparatus as previously described (12).

All tubing, glassware, etc., was made chlorine demand-free by methods employed in the earlier work (3), and free chlorine concentrations were determined.
as before by amperometric titration with phenylarsine oxide. Surviving virus infectivity was determined at each point by duplicate plaque titrations.

Estimates of aggregation were made also by either of two sedimentation methods for characterizing a mixture of single virions and groups of many different sizes. The first of these, called the virion aggregation test, separates the mixture into singles, small aggregates, and large aggregates (19). The single particle analysis depends upon the availability of a known monodisperse control suspension of the virus, but with it the fraction of single particles can be determined directly (5). This is particularly useful for comparing aggregation rates with those calculated from such coagulation theories as that of von Smolukowski (6, 16).

RESULTS

CB5. Doubtless the most significant result emerged at the outset. Purified CB5 as found in the sucrose gradient and stored (in the sucrose) unfrozen at 4 to 6°C for as much as 3 days was invariably aggregated as shown by the virion aggregation test; 74 to 100% of the plaque-forming units (PFU) were found in the large aggregate (ca. 18 virions or more) category, whereas similarly prepared poliovirus routinely give 0 to 5% (4). When virion aggregation tests were performed on crude (unpurified) CB5, there was some aggregation (11 to 39% in the large aggregate category), but much less than that of the purified virus. Occasional preparations of virus, if tested by the virion aggregation test immediately after removal from the sucrose density gradient, would show little or no aggregation, but none remained in this condition as long as 3 days and most were aggregated when first tested.

Electron micrographs made from kinetic attachment preparations of freshly purified virus showed very few aggregates and fewer virions than expected, considering the ample plaque titer of this purified virus. Virus particle counts of $3 \times 10^6$ to $8 \times 10^9$ were calculated from electron microscopy pictures of several such preparations, but these were thought to be low because both poliovirus (Mahoney) and echovirus (Farouk) yielded in this laboratory many more particles for comparable plaque titer. After reading the paper by Totsuka et al. (15), we added 100 µl of diethylaminoethyl dextran of 500,000 molecular weight (Pharmacia, Uppsala, Sweden) per ml of 0.05 M phosphate buffer (pH 7) in the sucrose gradient and produced purified CB5 without appreciable aggregation. This was verified by the virion aggregation test in which only 2% of the PFU were found in the large aggregate region. Electron micrographs like Fig. 1 were obtained, showing essentially all single virions in numbers indicating a particle count of

![Fig. 1. CB5 deposited by kinetic bombardment, not drying. The virus suspension contained 100 µg of diethylaminoethyl dextran. No suspension without dextran has given pictures without virion aggregation. Magnification, 20,000x.](http://aem.asm.org/)
3.2 × 10^11/ml, which is similar to counts previously obtained with echovirus and poliovirus. Unfortunately, this well-dispersed virus could not be used in disinfection experiments because of the excessive chlorine demand of even this small amount of diethylaminoethyl-dextran. Attempts to do so did, however, reveal an increased inactivation rate in spite of simultaneous loss in free chlorine.

Electron micrographs showing highly aggregated CB5 were never obtained when the kinetic attachment method of preparation was used, but large aggregates were seen (Fig. 2) when purified virus was diluted 10× with phosphate-buffered saline and the virions sedimented upon agar by the pseudoreplica method (10).

Electrophoretic focusing of CB5 was done as it was with poliovirus (6) in a sucrose gradient containing initially the same amount of virus in all parts and an ampholyte mixture covering the pH range 3 to 10 in an LKB apparatus. After focusing there was a broad peak containing 69% of the PFU in the region of pH 3.5 to 4.1; the rest was scattered, with little evidence of peaks, as far as pH 9. There was no significant peak at or above pH 7.

When CB5 was prepared in a sucrose gradient containing 100 μg of diethylaminoethyl dextran per ml, there was no aggregation as shown by electron microscopy and, on electrophoresis, the PFU accumulated in two places (Fig. 3). A low broad peak appeared in the pH 3 to 4 region just as it did with untreated virus, but a second, sharper peak appeared at pH 7.2.

Inactivation of the CB5 at pH 6 and 20°C by several different concentrations of HOCl (10, 15, 20, and 30 μM) has shown very little evidence of the departure from first-order kinetics (Fig. 4) that might have been expected in such experiments with aggregated virus. The slopes of the lines of Fig. 4 yield the reaction rates plotted in Fig. 5 which increase with HOCl concentration in a linear fashion except that a fifth experiment (not shown on Fig. 4) at 37 μM HOCl indicated

![Fig. 2. CB5 without dextran is aggregated. This virus was prepared for electron microscopy by sedimentation upon agar from which it was transferred to collodion by pseudoreplica. Magnification, 20,000×.](http://aem.asm.org/)

![Fig. 3. By electrofocusing CB5 extracted and purified with diethylaminoethyl dextran two PFU peaks were observed. Only the accumulation in the pH 3 to 4 region was seen when no dextran was present.](http://aem.asm.org/)
that a maximum had been reached with no further increase above 30 \( \mu M \) HOCl.

Careful attention to the inactivation kinetics during the first 10 s of reaction with 20 \( \mu M \) HOCl has revealed a curious transient when 0.1 M NaCl was present. This salt was used in previous experiments with poliovirus to prevent aggregation. It has no effect on the aggregation of CB5, but when it was present in chlorine experiments the plaque titer dropped immediately (within 2 s) by 1.3 logs (20\times). Figure 6a shows all the data from several experiments which show a 15-s period of delay and suggest a slight increase before falling into a linear decline. We have no evidence of such transient changes in reaction rate when there was not NaCl present, and the linear inactivation rate was about the same after the first 10 s whether there was 0.1 M NaCl present or not (Fig. 6b). When CsCl was substituted for NaCl the reaction rate was semilog linear with about the same slope. No attempt was made to detect short-time transients with CsCl.

At pH 10, 20°C, and 20 \( \mu M \) OCl\(^-\), the inactivation rate (Fig. 7) was much slower than at pH 6. When 0.1 M NaCl was added to the pH 10 buffer, the time for 99% plaque reduction was reduced from 7.9 min to 17 s. The effects of 0.1 M CsCl and 0.1 M KCl were similar to that of NaCl except in magnitude (Fig. 7). The cesium salt was much less effective in reducing inactivation time than the sodium salt, and the KCl was more effective.

Finally, inactivation experiments were made at 7 \( \mu M \) HOCl and 5°C for purposes of direct comparison with the experiments of Engelbrecht et al. (1). Again, the inactivation rate was essentially the same whether NaCl was present or not, but when it was present there was an initial 1-log plaque titer drop followed by a slight rise and subsequently by a slow steady drop beginning at 60 s (Fig. 8). This behavior is somewhat

![Fig. 4](image1)

**Fig. 4.** Inactivation of CB5 at 20°C in dilute phosphate-carbonate buffer at pH 6. Free chlorine concentrations were 10 \( \mu M \) (□), 15 \( \mu M \) (○), 20 \( \mu M \) (●), and 30 \( \mu M \) (△).

![Fig. 5](image2)

**Fig. 5.** Inactivation rate of CB5 as a function of HOCl concentration. Beyond 30 \( \mu M \) concentration of free chlorine, there is no further increase.

![Fig. 6](image3)

**Fig. 6.** (a) Inactivation of CB5 when 0.1 M NaCl was added to the buffer. After the initial transient which was complete in 10 s, the inactivation rate was the same as that of 20 \( \mu M \) HOCl with no NaCl. (b) There is no initial transient where no NaCl is added to the buffer.

![Fig. 7](image4)

**Fig. 7.** Inactivation of CB5 at 20°C by 20 \( \mu M \) OCl\(^-\) in dilute phosphate-carbonate buffer at pH 10 (○). The reaction proceeds faster when 0.1 M CsCl is present in the buffer (△) and faster still if 0.1 M NaCl is present (□) or KCl (●).
like that seen with the higher concentration of HOCI (Fig. 6), but it takes place more slowly. This transient irregularity was not present when the NaCl was omitted. The HOCI data from the Engelbrecht paper (1) are indicated by the dotted line on the graph (Fig. 8) where the time for 99% inactivation was slightly longer (39%) than that shown by our data.

CB3. The behavior of CB3 was in some respects quite different from that of CB5 especially with regard to aggregation. Although preparation, purification, and storage of the two viruses were the same, electron microscopy, virion aggregation tests and single particle analyses have shown no evidence of virion aggregation of CB3 in 0.05 M buffers from pH 6 to 8.2. Below pH 5, purified preparations of this virus aggregated rapidly (Fig. 9). This closely resembles the behavior of echovirus (18) in the acid region.

These data are logs to the base 10 of the ratio of PFU titers of two supernatant fluids from single particle analysis centrifuge tubes. In each case the titer of the supernatant fluid from the test sample was divided by that of an unaggregated control sample. Virus at pH 7 was used for this control. The centrifuge runs were made after the stock virus had stood at 25°C for 4 hours after 50-x dilution in the appropriate buffer.

The inactivation rate of CB3 at pH 6 by HOCI in the absence of NaCl is shown in Fig. 10. When 0.1 M NaCl was added, there was no significant change in either the initial transient or final steady inactivation rate.

At pH 10 the inactivation of CB3 was very slow (Fig. 11). At 20°C in 20 μM free chlorine (OCl⁻), the PFU survival ratio of 10⁻⁴ was reached in 15.5 min. When 0.1 M NaCl was added to the buffer, the time was reduced to 25.5 s. Here again, as with CB5 the presence of 0.1 M NaCl at pH 10 makes a major increase in the inactivation rate by OCl⁻.

Sugam and Helz (14) have observed significant ion pairing in seawater, and they have suggested that this might increase the toxicity of chlorinated waters at pH greater than 8.0. With this in mind we determined the extent of ion pairing for three concentrations of KCl and NaCl in water containing approximately 200 μM OCl⁻ at pH 10. Solutions of 0.50 M KCl and 0.50 M NaCl first were made chlorine demand-free. Then suitable dilutions in chlorine demand-free water were made to obtain 0.05, 0.10, and 0.50 M solutions to which 0.05 M NaOCl was added, and the pH was adjusted with dilute NaOH to obtain approximately 200 μM OCl⁻. These chlorinated salt solutions were tested in a Cary model 219 scanning spectrophotometer to determine the peak absorbance at 292 nm. The total chlorine concentration also was determined by amperometric titration. From these data the
molar absorptivities (ε) at 292 nm were calculated from Beer’s law for OCl⁻ in solution with various concentrations of Na⁺ and K⁺. The calculated extent of ion pair formation between OCl⁻ and these cations can be seen in Table 1, where the ε values for the salt solutions are compared with the expected value of ε = 350 for free OCl⁻ at its absorbance maximum. These calculations are based on the assumption that paired OCl⁻ ions have a much lower molar absorptivity than unpaired OCl⁻ ions. Table 1 shows that the potassium ion is sightly better at pair formation than sodium, particularly at the low concentrations.

DISCUSSION

If enough facts relevant to the inactivation of viruses by chlorine in water are to be assembled to provide a basis for the generalization that is needed by those who must make reused water safe for drinking, many viruses must be examined. Of the two viruses chosen here, one (CB5) has been reported to have high chlorine resist-

Our finding that CB5 aggregates rapidly after purification, at all pH values from 3 to 10 even in the presence of 0.1 M NaCl, which has a dispersive effect on some other viruses (3), would seem to supply a reason for its reported high chlorine resistance were it not for other apparently conflicting facts revealed here. The CB5 strain aggregated also in our experiments at pH 3 to 5 but not at pH 6 and above. We have seen no evidence of the aggregation of CB3 reported by Totsuka et al. (15). The chlorine resistance of the two was about the same at pH 6 (compare 10⁻², 10⁻³, or 10⁻⁴ plaque survival levels in Fig. 6 and 10). This means that the aggregated CB5 has the same chlorine resistance as the dispersed CB3 at pH 6, and this is apparently true also for OCl⁻ at pH 10 (compare Fig. 7 and 11).

We were not able to measure the resistance of dispersed CB5 to chlorine and so detect the effect of aggregation directly. Only by using diethylaminoethyl dextran were we able to see this virus in dispersed form, and the minimum amount required was too much for a “demand-free” experiment. Experimental efforts to do this resulted in increased inactivation rates in spite of simultaneous loss of free chlorine from diethylaminoethyl dextran demand, possibly due to dispersion of the virus or to changes in the surface properties evident in the isoelectric-focusing data. Only these meager qualitative results suggest that virion aggregation may account for part of the resistance of CB5.

Both types of the coxsackievirus differ substantially from poliovirus in their chlorine sensitivity in the presence of NaCl. At pH 6 the inactivation rate of poliovirus (Mahoney) was increased threefold when 0.1 M NaCl was added to the buffer (13). Neither coxsackievirus was

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<th>Salt concn (M)</th>
<th>pH</th>
<th>Amperometric Cl₀ (µM)</th>
<th>Absorbance</th>
<th>ε</th>
<th>% OCl⁻ involved in ion pairing</th>
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<td>NaCl</td>
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affected in this way by NaCl at pH 6; however, both were strongly affected at pH 10. In both cases, the otherwise weakly active OCl− ion gave rapid inactivation comparable with that of HOCI at pH 6. Similar enhancing effects have been seen with NaCl and poliovirus at pH 10 (13), and we would expect that the superiority of OCl− over HOCI in the inactivation of poliovirus (Mammone) reported by Scarpino et al. (9) was due to the KC1 that was present in their pH 10 buffer but not in their pH 6 buffer. Our experiments with 0.1 M KCl showed its effect to be greater than that of NaCl. Still another monovalent chloride (CsCl) was tested briefly here. It too increased the inactivation rate of CB5 at pH 10 but not to the extent observed with either the sodium or the potassium salt (Fig. 7). This suggests the metal ion either is having some effect on the virus or is reacting with the OCl− and forming ion pairs which may be as effective as HOCI. No divalent ions were tested here, but some experiments with Ca2+ by Kenyon and Schaub (8) indicate that 250 mg of CaCl2 per liter increased the rate of inactivation of poliovirus in the presence of OCl−.

While considering Fig. 7 it is worthy of note that the virus was aggregated in all three experiments, although not necessarily to the same extent. Still, some lines are straight and others are curved. Straight lines are seen also with aggregated virus in Fig. 4 even where only 10−4 of the PFU survive, and curved lines have been obtained in similar experiments with well-dispersed poliovirus (3). In the presence of all these facts, the practice of deriving from the shape of a disinfection curve the distribution of aggregate sizes in the virion suspension becomes a doubtful activity. Apparently there are chloride disinfection curves of a variety of shapes emerging from experiments with monodisperse virion suspensions, some of which closely resemble shapes expected from a mixture of aggregate sizes, and there are also straight lines observed in some cases in the present work where aggregation is known to exist.

The inactivation rate at pH 6 of CB5 increases linearly with increasing HOCI concentration but only up to about 25 μM, above which it remains constant (Fig. 5). Saturation effects of this kind have been observed with several picornaviruses and several halogen agents (1, 2-4, 17).

Transient departures from linear semilog disinfection rate plots such as those of Fig. 6 (CB5, pH 6, 0.1 M NaCl present) and Fig. 10 (CB3, pH 6, no salt added) are not yet understood. They have been seen before with echovirus and with poliovirus (8, 18). Further study of this phenomenon is continuing in this laboratory in the hope that it will reveal something of the mechanism of the disinfection process. At present, it seems to be of little practical significance because the time involved is usually small, but the frequency of its appearance in recent work suggest that the simple straight line may be the exception rather than the general rule among viruses.

Doubtless the item of greatest practical significance in this work on coxsackievirus is the observation previously reported for poliovirus (13) that the comparatively weak disinfecting action of OCl− at pH 10 is enhanced many times (in this case 20- to 30-fold) by the addition of 0.1 M NaCl. Under these conditions these viruses are destroyed at a rate comparable with that of the same amount of free chlorine, as HOCI, at pH 6. The effectiveness of the three different chlorides that have been tested in this respect are similar but different in magnitude, with CsCl the least, NaCl the next, and KCl the most active. The large heavily hydrated cesium ion might be expected to have the greatest difficulty penetrating the virus capsid, but the reason for the difference between the effects of Na+ and K+ must be sought elsewhere. A difference in ion pairing has been demonstrated here, with the potassium ion showing a greater tendency to pair with OCl− than sodium. If the pairs are more active in the inactivation of virus than the ions, as suggested by Sugam and Helz (14), the explanation may be at hand.

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