Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine

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The initial inactivation of reovirus in water containing 3 to 7 μM bromine as HOBr was very rapid. Electron microscopy revealed extensive physical damage to the virions in as little as 1 min, but none were degraded beyond recognition. As treatment time continued, the reaction rate decreased toward a plateau of resistance, usually at about the 10⁻⁴ survival level; still no particles were lost. Progeny grown from these resistant plaque-forming units (PFU) were no more resistant to HOBr than the parent cultures. Small-number aggregation (adhering groups of two to ten virions counted by electron microscopy) had no detectable effect on the level of persistent PFU. Large aggregates seemed to be involved. Sonic treatment at 20 kHz after bromine exposure increased survival PFU titer 10- to 43-fold. Virus exposed to light centrifugation prior to bromine treatment did not show the plateau of resistance. Surviving PFU sedimented faster in a shallow sucrose gradient than single virions. Large aggregates were apparently too few to be counted by electron microscopy, but their penetration and inactivation must be achieved by any disinfectant chosen to rid water of reovirus.

This is an account of some early findings in an effort to learn the effects of virion aggregation on the survival of reovirus in water containing bromine. Later this work will be extended to include field water of various kinds, but for the initial phases of the investigation the purified virus was added to demand-free water. Virion aggregation has been observed directly by electron microscopy and indirectly by sedimentation velocity of both virions and plaque-forming units (PFU) in density gradients. Speculation about the curvature frequently observed in the graph of log PFU versus treatment time in experiments like these usually cites virion aggregation as probable cause (2), so we have taken special care to observe this parameter —first, as it may provide a means of protection of a few virions from the bromine and, second, as it may influence the detection process through changes in plaquing efficiency. This latter effect may come about in at least two different ways. An adhering group of two or more (a few) damaged virions may produce a plaque by virtue of complementations, sometimes called multiplicity reactivation, whereas none may have been able to do it alone (1, 7). Alternatively, a group sufficiently large to pro-

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MATERIALS AND METHODS

Virus, culture, and plaquing. Reovirus type 3 (Dearing strain) was obtained from W. K. Joklik of the Duke University Medical Center and serially
passed in L cells. Cells were grown as monolayers in 32-oz (0.95-liter) prescription bottles in medium 199 containing 0.07% NaHCO₃ and 5% fetal calf serum. Virus was produced at 37°C in cells infected at a multiplicity of 10 to 20 PFU/cell under a maintenance medium of 199 + 0.15% NaHCO₃ and 2% fetal calf serum. Reovirus from three or four such culture vessels was harvested 20 to 24 h post-infection. For purification, the medium was decanted and the monolayers were washed twice with phosphate-buffered saline (PBS). The washed cells were scraped into a small volume of PBS and pelleted at 250 x g for 10 min. The cells were then resuspended in 5 ml of PBS and homogenized with an equal volume of Freon 113 for 1 min at half speed in a Sorvall Omnimixer. The two liquid phases were separated by low-speed centrifugation (800 x g, 10 min), and the Freon phase was reextracted two or three times more with fresh PBS. The combined aqueous phases were made up to 20 ml with PBS, and one-half was placed over each of two sucrose density gradients (20 to 40%, wt/wt) in the Beckman SW27 rotor. These were spun at 4 C, 25,000 rpm, for 1 h. Virus was collected with a Pasteur pipette from the lower of the two bands that could be seen with a collimated beam of light, pelleted twice to remove the sucrose, resuspended in 0.05 M phosphate buffer at pH 7.2, and stored at 4 to 6 C.

Plaque titrations were performed in tightly stoppered 1-oz (ca. 0.03-liter) prescription bottles under an overlay of 1% agar (Difco) containing Medium 199 with 5% fetal calf serum, 0.175% NaHCO₃, and 0.003% neutral red. Plaques were counted after 6 days of incubation at 37 C.

**Physical assay of virus.** Virus was assayed by electron microscopy of preparations made in two different ways. For virus concentrations of 10⁹ particles/ml or greater the kinetic attachment method was used (8). Very briefly, this consists of placing a high drop of the virus suspension on an aluminum-coated collodion film covering a standard disk (1/4-inch [ca. 0.32-cm] diameter) wire grid. Humid conditions are provided to prevent any drying. Virus particles impinge and are retained by the aluminum coating for an appropriate time (10 min to 2 h), after which the drop is completely washed away, and washing is continued for at least 30 min to insure the removal of all unattached virus before drying is permitted. After drying, the grids may be shadowcast for ease of counting. The number of particles (NC) counted per unit area of picture at magnification (M) will depend upon the number (No) per ml of the suspension, the diffusion constant (D), the viscosity (η), the absolute temperature (T), and the time (t) in seconds of contact in the following manner: NC = 1.15 No√D t M² (Valentine and Allison [11], Equation 1), in which DTet = 1.38 x 10⁻¹⁰ T⁻⁶ cm² s⁻¹, so DTet at 25 C in water is 7 x 10⁻⁸. Equation 1 is valid for reovirus for collection times up to 2 h if the drop is at least 1-mm deep over the entire surface. A convenient apparatus for making these kinetic attachment preparations is shown in the reference cited (8).

For virus suspensions of concentration about 10⁸ particles/ml, the agar pseudoreplica method was used (6). This involves sedimentation of the virus in a special centrifuge rotor, and it is 100 to 1,000 times more sensitive than the kinetic attachment technique.

**Bromine inactivation experiments.** (i) Water. All the water used in these experiments was made free of halogen demand and stored protected from the atmosphere by 50% sulfuric acid scrubbers. Deionized water was chlorinated to about 5 mg/liter for 4 days and dechlorinated the day of the experiments with an ultraviolet lamp.

(ii) Glassware. Glassware used for preparing, containing, or analyzing bromine solutions was soaked in alkaline bromine solutions greater than 200 mg/liter for 1 to 3 days before the experiments.

(iii) Buffer. Buffer (KH₂PO₄-K₂HPO₄, pH 7.0, 0.1 M) was filtered through a 22-μm membrane filter (Millipore Corp.), chlorinated to about 5 mg/liter for about 4 days, and dechlorinated the day of the experiments with an ultraviolet lamp.

(iv) Neutralizer solution. Solution of 2 x 10⁻⁴ N sodium thiosulfate and 0.001 M phosphate buffer (KH₂PO₄ - NaOH) (pH 7.4) were filtered through 0.22-μm membrane filters (Millipore Corp.), autoclaved, and stored at 4 C. They were reautoclaved the day of the experiment.

(v) Bromine analysis. Bromine concentrations were analyzed by the method of Taylor (10). Samples (30 ml) were drawn from the test beaker and mixed with 0.15 ml of 0.4% potassium iodide in a 50-ml volumetric flask. Fifty-five seconds after the addition of the KI, the sample was poured over 1.5-ml quantities of each SNORT reagent and magnetically stirred in a 250-ml beaker (5). The samples were read 1 min after mixing in 10-cm cells in a Cary 14 spectrophotometer.

(vi) Procedure. The system used was similar to that used by Scarpino et al. (5). A 100-ml control beaker containing only buffer and a 250-ml test beaker containing 2 to 6 μM bromine were used for each experiment. A 5-ml amount of halogen demand-free water and 5 ml of 0.1 M buffer were put in the control beaker. A 50-ml amount of buffer and some halogen demand-free water were put in the test beaker. These beakers were put in an ice bath, covered with stainless-steel lids, and agitated with glass stirrers (four glass paddles, 0.5 inch [ca. 1.3 cm] wide and 0.75 inch [ca. 1.9 cm] radius) attached to an overhead stirrer set at 81 rpm. After 30 min, the temperature was below 2 C, and enough precooled bromine was added to the test beaker to give a total volume of 100 ml at the desired bromine concentration. After 10 more min the bromine concentration was measured. Virus was then added to both test and control beakers. At chosen time intervals 5-ml samples were removed from the test beaker and finally from the control beaker. These samples were blown into test tubes containing 2.5 ml of pH 7.4 buffer and 2.5 ml of thiosulfate solution. Plaque assay was made the same day.

**RESULTS**

**Stability of a reovirus suspension.** Virus taken from the prominent band formed in the
sucrose gradient was pelleted twice to remove sucrose and suspended in 80 ml of 0.005 M phosphate buffer at pH 7.0. Five electron microscope (EM) pictures were taken at random on agar sedimentation preparations made immediately. The average number of particles per picture was 173, \( \sigma = 25 \), and the \( \log_{10} \) of the total number of singles, doubles, triples, etc., is shown plotted against \( \log_{10} \) group size in the lowest of the three lines of Fig. 1. The straightness and slope of this plot was examined again more critically after the suspension had stood 4 days at 4 to 6 C. This time 25 random pictures were taken, and the average total count per picture was 175, \( \sigma = 22 \). The log-log frequency distribution of this series is shown in the top line of Fig. 1. The middle line of the figure came from 10 EM pictures taken 21 days after the first set. The total count was 178, \( \sigma = 21 \). Less complete data of the same kind show that the number and frequency distribution of aggregates is equally stable in 0.05 M phosphate buffer at the same pH. Apparently the degree of aggregation that existed when the virus was first resuspended from the last pellet was not altered in 21 days, and no significant loss of virus to the glass walls of the storage bottle was noted even though no special precautions were taken to prevent it.

The effect of dilution on the state of aggregation was observed using virus at a concentration of \( 3 \times 10^9 \)/ml. This count was verified and the frequency distribution of aggregates was measured in EM pictures made by the kinetic attachment method. These virions became attached to the collodion film during Brownian motion in this suspension at high concentration. Then this suspension was diluted \( 50\times \) with 0.005 M buffer, and another set of EM pictures was made by the agar sedimentation method. Approximately the same number of virions was present on both sets of pictures, and the log-log plots of group frequencies were as near alike in slope as those of Fig. 1. Thus, within the limits available for direct observation of reovirus particle aggregation in 0.005 M phosphate buffer at pH 7, the state of dispersion is independent of virion concentration.

**Yield and physical appearance of the reovirus.** When Freon-extracted virus from about 80 million L cells was velocity-banded on the sucrose gradient, about \( 5 \times 10^{11} \) virions were regularly recovered from the prominent, rapidly moving band. These are shown shadowcast and negatively stained in Fig. 2a and b, respectively. In a faint, slower sedimenting band, as many as \( 1.3 \times 10^{11} \) particles have been recovered. These “top component” (9) particles are shown in Fig. 2c and d. Many of them are flattened in the shadowcast pictures and appear empty in the negatively stained preparations. These pictures are shown here for comparison with the bromine-treated virus below.

**Effects of bromine on the reovirion.** The survival ratio of PFU is shown plotted as a logarithm against the time of treatment for several concentrations of bromine as HOBr (Fig. 3). The virion concentration was in the range of \( 10^4 \) to \( 5 \times 10^4 \) particles/ml, and in 12 such experiments the degree of aggregation among the in-going virions ranged from a slope of \(-178 \) to \(-3.12 \) on the log-log plot of frequency versus group size for groups of size from 1 to 10. In spite of this substantial amount of aggregation, the reaction rates were too fast for accurate measurement during the initial stages even at HOBr concentrations as low as \( 3 \) \( \mu \)M. Below this value it was difficult to maintain a constant bromine concentration. In the experiments reported here the bromine concentration from the beginning of the disinfection run never varied by more than \( 1 \) \( \mu \)M. Nevertheless, all the lines were curved. Curvature was usually detectable at the \( 10^{-2} \) survival level but always before the \( 10^{-4} \) level was reached, indicating that the resistance of surviving PFUs was continuously increasing. This effect might have been due to depletion of the free HOBr during the reaction, but experiment (Fig. 4) shows that, after the reaction had essentially stopped, fresh virus

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**Fig. 1.** The state of small-number aggregation of \( 10^8 \) reovirions/ml of 0.005 M phosphate buffer (pH 7) is shown in terms of group size and frequency. Data are from electron micrographs made immediately (\( \Delta \)), after 4 days (O) and after 3 weeks of storage at 4 to 6 C (\( \Box \)).
FIG. 2. Fresh reovirus (untreated) shadowcast (a) and negatively stained (b). Virus-like particles from the slower sedimenting "top component" shadowcast (c) and negatively stained (d) are usually hollow in the center and their shadows are usually shorter, indicating defective structure.
persistent resistance observed enough thiosulfate, added for the tional AM in 43-fold increase of water of phosphate.

brominated after 98 Ui. 3 V.-3 Virus FIG. 1/ 2.5 to 54 M to 5.4 M to 3. > 2 3. 4. The expected number, mixture was made 2.5 to 54 M to 3. > 2 3. The virions induced aggregation analysis by bromine experiment at 50,000 rpm at 5 C. The total integrated effect of this procedure was equivalent to 33 min at 40,000 rpm, and this would have been quite sufficient to sediment all single virions and clumps to the bottom of the tube. Less than 1% of the surviving PFU were found in the supernatant fluid after this centrifugation. Certainly there were no significant numbers of plaques produced by ribonucleic acid released from the virions by the HOBr. At this point it seemed unlikely that aggregates sufficiently large to provide protection for even one virion could have accumulated soon enough after the virus was added to the HOBr solution. Therefore, the next experiment sought to remove hypothetical aggregates, too few to be noticed by electron microscopy but presumably present before HOBr treatment. Inasmuch as at least 16 spheres are required to form a protective coat one sphere thick around one of equal diameter, and such a clump must have a radius about three times that of one of the spheres, we chose first to remove them by centrifugation at a speed of 11,300 rpm in an SW50 rotor for 18 min. A 5-ml amount of the starting virus was subjected to this treatment, and 4 ml of the supernatant fluid was carefully removed for HOBr treatment done in parallel with uncentrifuged starting virus. EM counts showed that the number of virions per milliliter of the
FIG. 5. Reovirus, after treatment with bromine, has lost both substance and structural form as shown here shadowcast (a) and negatively stained (b), but the number of these heavily damaged particles remains the same. No detectable fraction of the input particle count is lost.

supernatant fluid used was not significantly less than that of the control. After treatment of both with 3.3 μM bromine for 1 min, the control virus produced \(10^{3.23}\) plaques/ml, whereas the centrifuged virus produced less than \(10^{1.23}\) plaque/ml. Essentially the same result was also achieved by treating the starting virus preparation with 20-kHz waves prior to bromine treatment. Apparently the potential surviving PFU are large and exist before bromine treatment, and they are few enough to escape detection when only a few hundred virions are observed on EM pictures used for routine counting.

Isopycnic banding of virus after 4-min exposure to 5.4 μM bromine was done by adding CsCl crystals to the reaction mixture after neutralization with sodium thiosulfate. One tube of the SW50 (Beckman) rotor was filled with this homogenous virus suspension at a starting density of 1.22 g/ml and spun at 35,000 rpm for 20 h at 5°C. The virus was located by EM count in a single peak at \(p = 1.25\) g/ml (Fig. 6).

Velocity banding was tried by placing 10 ml of bromine-treated reovirus over a 20 to 40% (wt/wt) sucrose gradient in an SW27 Beckman centrifuge tube and spinning at such speed that single virions might move appreciably into the sucrose before PFU were all sedimented to the bottom. Even with the established isopycnic density of the bromine-treated virus, we were not able to predict nor even measure accurately the relative sedimentation velocities because of the difficulty of precisely locating the starting position of the particles. Nevertheless, the experiment did clearly establish the fact that the virus particle peak located by electron microscopy contained no PFU, and no particles (or clumps) could be found by electron microscopy in the region of the much more rapidly sedimenting PFU peak (Fig. 7). Although the two peaks are drawn (for convenience) with approximately equal area, the number of virions per milliliter and the number of PFU per milliliter in the respective peak regions
are actually in the ratio of about 1,000,000 to one.

Bromine-resistant virus has not yet been found. Virus populations, grown from plaques isolated at the \(10^{-4}\) survival level in bromine experiments, have shown the same sensitivity to bromine as the original population.

**DISCUSSION**

The decline in plaque titer of virus treated in water with any of several agents, including nitrogen mustard and formaldehyde, usually departs from the straight semi-log line predicted for a first-order reaction. Sometimes the departure occurs only after the survival ratio has been reduced by several factors of 10 (4), but in others (7) increasing resistance of survivors was apparent before the \(10^{-2}\) level was reached. In the poliovirus work of Salk and Gori (4), the persistant infectivity was attributed to clumps of virions which could be removed by filtration. Their function was presumed to involve protection of one or more virions intact within the lump. The behavior of vaccinia virus was different. The number and frequency of aggregates of two to 10 virions was determined and found to be closely correlated with the slope and shape of the survival curve. Furthermore, dispersive treatment of vaccinia virus survivors with sonic waves produced a decrease in plaque titer which was reversed when the virions were permitted to reaggregate (7). Complementation or multiplicity reactivation can account for these observations.

In the present work the state of aggregation of the reovirus in groups of one to ten was carefully measured, but no correlation was found with the level of persistant survival in the presence of bromine. The physical appearance and the decreased density of the virions indicated that they lost ribonucleic acid and possibly other structural material as well. Virions in this condition would not be likely to benefit from complementation in plaque formation by small groups. Still the decline in PFU in 5 \(\mu\)M bromine practically stops (Fig. 4) at the \(10^{-4}\) survival level, and sonic treatment raised the residual titer of such virus as much as 43-fold. The plaquing efficiency of fresh virus has been about \(1 \times\), so \(0.01 \times 10^{-4}\) or one large clump in each million virions could account for this titer. Such a small number would doubtless escape notice in routine counts by electron microscopy. Most of these aggregates must have been present before bromine treatment began, because both 20-kHz waves and precentrifugation sufficient to remove spherical close-packed clusters of at least 16 fresh virions (\(\rho = 1.36\)) greatly reduced the number of surviving PFU when subsequently treated to the same degree with bromine.

Revival of PFU titer of a suspension containing bromine-treated aggregates might come about in several ways. If such a plaque-forming clump contained as many as 43 plaque-forming particles, scattering them with sonic waves could produce 43 PFU where one existed before. This seems unlikely because at least 4,300 intact virions would have to be protected to produce 43 plaques from one dispersed clump. It seems more probable that sonic waves enhance the plaque titer of such protected aggregates by making the one or few protected virions more available for cell infection. In this case the degree of enhancement would not depend very much upon the actual number of protected virions per clump. It would depend mainly upon the efficiency of removal of the protective coating material prior to titration.

The residual PFU titer of bromine-treated reovirus cannot be caused by free infectious ribonucleic acid. If it were, it would not be so easily removed by centrifugation. Virus aggregates are likely to be present even though there may have been none in the velocity band selected in the sucrose gradient during purification. They could have been produced during the two pelleting operations that were employed to remove the sucrose. This is not to say that such aggregates are unlikely to occur in polluted water. The large cytoplasmic crystalline aggregates so frequently seen in cells infected by
enteric viruses are a likely source, and it is doubtful that all these are completely dispersed in the cycle of pollution. Indeed, such aggregation may constitute a means of virus survival outside the host. Something of this kind certainly happened in these experiments, and it may occur in other water treatment processes as well.

It appears that the sterilization of water containing reoviruses will depend upon the ability of the active agent to penetrate aggregates as well as to inactivate single free virions. This will require further investigation along lines heretofore rarely considered in the literature. Also, there is the other part, the early rapid part of the inactivation process that has been largely omitted here except for the presentation of small-number aggregation data. These aggregates must exert an influence during the first few seconds of experiments such as those of Fig. 3. Work is now under way to obtain essentially all single particles and to use such suspensions in experiments of short duration for comparison with aggregated virus like that of Fig. 1 but without large aggregates. These experiments should help reveal the true nature of the primary reaction of bromine on single virions and aid in the effort to fully appraise the infectious or plaque-forming unit as it exists in polluted water and as it may survive purifying treatment.

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