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Initial Fast Reaction of Bromine on Reovirus in Turbulent Flowing Water

D. G. SHARP,* R. FLOYD, AND J. D. JOHNSON

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

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An apparatus is described for precise observation of the kinetics of the initial fast reaction of bromine with reovirus in turbulent flowing water. When quantitative electron microscopy shows that virus suspensions are essentially all single particles, the loss of infectivity follows first-order kinetics, the plaque titer falling at the rate of 3 $\log_{10}$ units/s at pH 7, 2 C, and at a 3-μM bromine concentration. Virus suspensions containing small aggregates (2 to 10/clump) exhibit a constantly decreasing disinfection rate with bromine. At a survival level of 10^{-3} for single virions, the aggregated preparations have lost only 99% of their plaque titer and 10^{-4} is reached only after 4 s of exposure. The disinfection rate does not appear to be a simple function of the size and frequency of aggregates in the virus suspension even when the aggregates contain no foreign material. Unpurified virus preparations (crude freeze-thaw lysates of infected cells) are shown, by zonal centrifugation, to contain 50% to over 90% of the infectivity in large, fast sedimenting aggregates. Such aggregates would strongly influence the bromine resistance of virus in polluted water.

In a previous publication (5) we have shown that the inactivation of reovirus by bromine in water is characterized by an initial rapid phase, followed by an extended period of much greater resistance in which infectivity persisted, usually at about the 10^{-4} level of survival. Before examining in further detail the behavior of the resistant fraction of the plaque-forming units (PFU), which appeared to be a few large aggregates, it seemed wise to determine the kinetic features of the initial fast reaction. Here mostly single virions and associated small aggregates (groups of 1 to 10 virions) are likely to be involved, and from them it should be possible to determine the basic inactivation rate for the virus. However, the reaction in this region proceeds so fast that the survival ratio is reduced 2 to 4 $\log_{10}$ units within the first minute at pH 7 and 0 C, even at bromine concentrations as low as 3 to 5 μM. It is clear that the exposure of virus to bromine solutions in stirred beakers cannot yield satsifactory kinetic data for reaction times of the order of 0.5 to 20 s. Consequently, a different experimental approach was designed. Forty-three reaction rate experiments have now been done in this way, and we believe that the results of the last few of these experiments, presented here, will justify a detailed description of the method and apparatus. They also establish the inactivation rate for single reovirions and indicate, to some extent, the effect of small-number aggregates on survival of infectivity.

MATERIALS AND METHODS

Virus, culture, and plaquing. Reovirus type 3 (Dearing strain) was grown in, and all plaque titrations were made on, monolayer cultures of L cells as previously described (5), with the following exceptions. Cells were maintained in 200-ml milk dilution bottles and passaged into 32-ounce (ca. 1.0-liter) prescription bottles for growth of virus. Virus was harvested at 16 to 18 h after infection at 10 to 20 PFU/cell rather than 20 to 24 h as previously noted (5). In addition, the virus was extracted from the cells in 6 ml of phosphate-buffered saline without calcium and magnesium and 4 ml of Freon 113 for 2 min at one-half speed in a Sorvall Omni-Mixer. The virus was placed on 20 to 40% (wt/wt) sucrose gradients in 0.05 M phosphate buffer, pH 7.2, and centrifuged at 25,000 rpm in a Beckman SW27 rotor at 4 C for 1 h. The virus, collected from the lower of two bands seen with a collimated beam of light, was allowed to remain in the sucrose at 4 to 6 C and was not pelleted. Virus stored in this manner retains its infectivity for several weeks and the state of physical aggregation is quite stable. There is no bacterial or fungal growth, and the sucrose does not produce any detectable bromine demand under the conditions of our experiments. Immediately prior to use, the virus

preparation was diluted 1:10; hence the sucrose level as used in an experiment was around 3%.

Plaque titrations were performed in tightly stoppered 1-ounce (ca. 0.03-liter) prescription bottles under an overlay of 1% agar (Difco) containing Medium 199, 5% fetal calf serum, 0.245% $NaHCO_3$, and 0.003% neutral red. Plaques were counted after 6 days at 37 C.

Physical assay of virus and virion aggregation. Single virus particles and aggregates were counted by electron microscopy using the agar pseudoreplica method (5).

Zonal centrifugation. Resolution of both crude and purified virus particle populations into sedimentation velocity spectra (6) was done with a BXIV titanium zonal rotor (provided by Norman Anderson [1], Oak Ridge National Laboratories) operating in a Sorvall OTD2 fluid drive ultracentrifuge.

Bromine, glassware, and water. Bromine measurement and preparation of glass apparatus and demand-free water were the same as previously reported (5), except that amperometric titrations were used together with the method of Taylor and Johnson (7) and all phosphate buffers were made with Fisher primary standard potassium salts.

Kinetic experiment. (i) Object. The apparatus must provide a large enough volume of water to insure stability of temperature, bromine concentration, and flow rate through the reaction siphon tube (Fig. 1) for a period of about 1 min. The reaction tube is 3/8-inch (ca. 0.96-cm) ID, and the bottle (20 liter) height is adjustable so that turbulent flow at the desired rate can be achieved. Turbulence is essential, so the Reynolds number has been kept equal to 3,000 in all experiments. The reaction tube has five access ports covered by disposable serum bottle stoppers. Five 5-ml syringes with needles are inserted. The first of these (at the left) carries the virus inoculum (5 ml), which, when triggered, starts the weight-driven plunger, which delivers the inoculum steadily for 5 s into the turbulent stream of buffered bromine water. There is a 6-mm-diameter disk located on the axis of the tube just downstream of the inoculum needle tip to aid in mixing.

The time of transit from injection point to sampling point will be determined only by the flow rate and the distances, D_1, D_2, etc. It is not in any way dependent on when the sample is taken as long as the sample is taken within the 5-s interval during which the "polluted" water passes the sampling port in question.

Each of the four sampling syringes contains 1 ml of 2 mM sodium thiosulfate solution, 1 ml of air, and a small Teflon-coated magnetic stirring bar. The

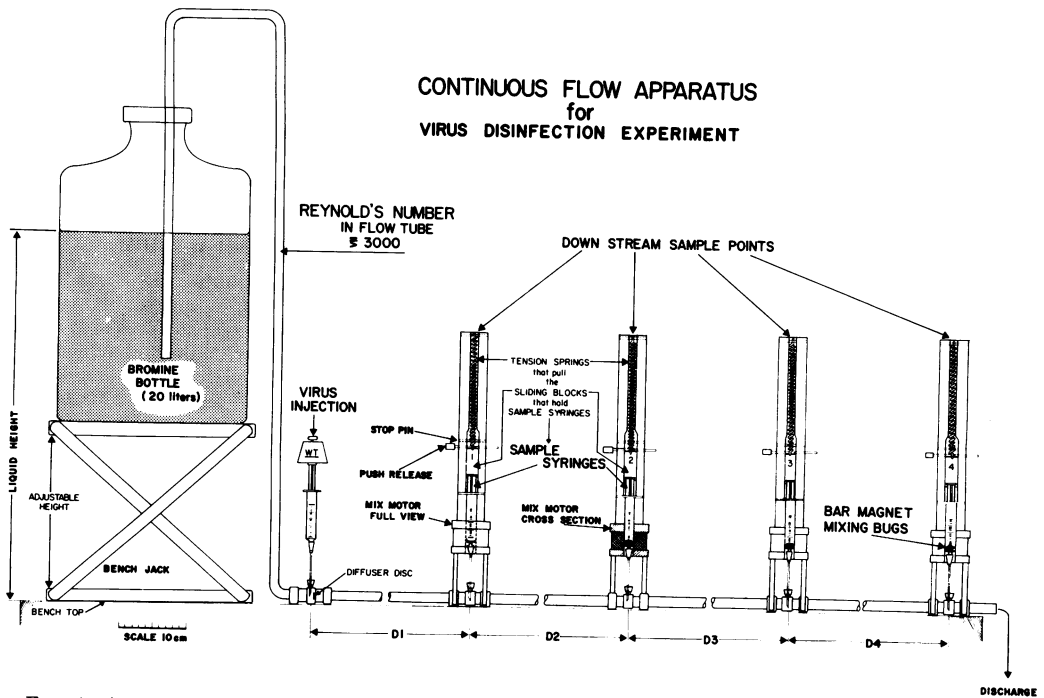

FIG. 1. *Apparatus for injection and recovery of virus for measurement of short-time inactivation by bromine in water. After constant turbulent flow of bromine-water was established through siphon and treatment tube, virus was injected steadily for 5 s from the weighted syringe. Four spring-loaded syringes containing continuously mixed sodium thiosulfate solution were then released in turn to withdraw 1 ml and quench the bromine at exposure lines determined by the velocity of flow in the stream and the distance, D1, D2, etc. Bromine demand was determined by measurement of the discharge fluid, and samples were assayed for surviving virus by the plaque method.*

syringe plungers are held in metal blocks attached to helical springs, each under tension and restrained by a release pin that can be pushed at the desired time. When the spring is released, the syringe draws exactly 1 ml from the flowing stream. This is mixed with the thiosulfate solution to stop the reaction of the bromine on the virus. For virus-bromine exposure times of 1 s or less, additional precautions were taken to insure instant mixing. This is done by means of the magnetic stirring bar, which is made to rotate at 1,800 rpm by 2-pole AC motor windings surrounding each sampling syringe. These are stator windings used in small electric servo-motors kindly provided for us by the Sperry Rand Co., Durham, N.C.

(ii) Operation. Twenty liters of buffered chlorine-demand-free water is prepared and adjusted to the temperature required for the experiment. Enough bromine is added 17 h before the experiment to bring the concentration in the bottle to approximately the right value. Just before the experiment, more bromine is added to adjust the concentration to exactly the required level. A previous experiment has established the exact level of liquid in the bottle to produce the desired flow rate (range, 20 to 40 ml/s in different experiments). This level is marked for easy reference, and all experiments start at this level. Beginning with the liquid at a higher level, the siphon is started and the flow is continued for a few seconds to allow tubing to adjust to the temperature in the bottle; when the liquid level reaches the marked starting level, the virus injection is started. The stirring bars in all four sampling syringes are already running in their 1-ml volumes of thiosulfate solution, and it only remains to trigger each of the syringes as the flood passes and also catch a sample of the virus-bromine mixture at the discharge end to determine its bromine concentration for comparison with that in the bottle. Neutralized bromine-virus mixtures from the sampling syringes and the virus from the inoculation syringe are then assayed for virus by the plaque method.

The dilution of virus as it enters the flowing stream is just the ratio of the rates of injection and stream flow. The injection syringe is timed by direct observation and stopwatch. The stream flow rate is easily obtained by timing the outflow of some conveniently measured volume, such as 500 ml. Variation in flow rate with liquid level in the bottle is negligible during the experiment time, which has not been over 20 s, because of the large (ca. 700 cm^2) surface area.

Tygon tubing is convenient for the flowing stream, but we have found that it absorbs substantial quantities of bromine. Glass is difficult to manage without catastrophic accidents. However, "high-density" polyethylene tubing has performed well. It is readily cut to desired lengths and exerts negligible bromine demand.

RESULTS

Several experiments were made at 10 C with samples taken at 4-s intervals. These were made with virus prepared in the same way as that of our earlier publication (5), which involved pelleting of the purified virus to remove sucrose. These were done at 3, 5.8, and 5.9 μM bromine concentrations (Fig. 2), and they all show approximately the same level of persistant resistance previously reported in exposures of longer duration. They show also that the initial fast reaction is taking place during the first 4-s interval. Virus was titrated after passing through the apparatus in exactly the same way, without any bromine in the flowing stream. Virus dilution was equal to the ratio of stream velocity (39.6 ml/s) to injection velocity (1.11 ml/s) times 2 for the dilution with the sodium thiosulfate ($2 \times 39.6/1.11 = 71$). Each titer was multiplied by this factor and then divided by the titer of the starting virus, and the logs of these ratios are plotted as filled circles at the top of Fig. 2. They show, by their small deviation from the dotted horizontal line drawn through them, that no changes in plaque titer occur as the control virus passes through the apparatus, except those due to dilution.

At this point we added the magnetic mixing devices to the sampling syringes and reduced the length of the flow tube to give samples at 1-s and later at 0.5-s intervals. The temperature was reduced also, from 10 to 2 C, to reduce the reaction rate and still permit the use of bromine concentrations high enough to hold constant

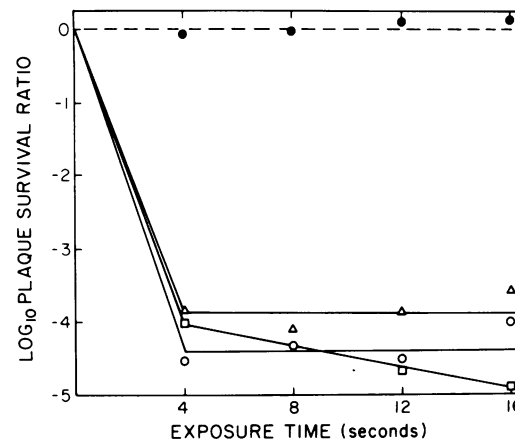

FIG. 2. *Survival of reovirus infectivity in water containing 3 μM (triangles), 5.8 μM (circles), and 5.9 μM (squares) bromine at 2 C and pH 7.0. The rapid phase of the reaction was done in less than 4 s, and the slower phase has been shown to be caused by a few large aggregates. Without bromine, virus passes through the apparatus with no significant change in plaque titer (filled circles), except for dilution at injection and sampling points for which correction has been made.*

when confronted with expected bromine demand of the virus.

Three experiments were made at 2.8 to 3.0 μM bromine concentration with virus prepared without pelleting. Two were done with 1-s time intervals and one with the intervals reduced to 0.5 s. The frequency of aggregates observed by electron microscopy in this virus preparation is shown in Fig. 3. Of the whole particle population, 73% are singles, and the groups appear to be distributed in such a way that the log frequency of each group size is a linear function of the log of the number in that group. We (3) and others (2) have observed this same distribution with other viruses and also with polystyrene and acryl particles of comparable size. The inactivation of the virus by bromine in these three experiments is shown in the semilog plot of survival ratio versus time in Fig. 4. It is curved all the way. A single line has been drawn by inspection through all three sets of points. Correlation of this result with the aggregation distribution (Fig. 3) will be made later (see Discussion).

Zonal centrifuge experiments. (i) Experiment 1. An effort was made to obtain a preparation of virus containing only single particles. This is needed to provide a frame of reference

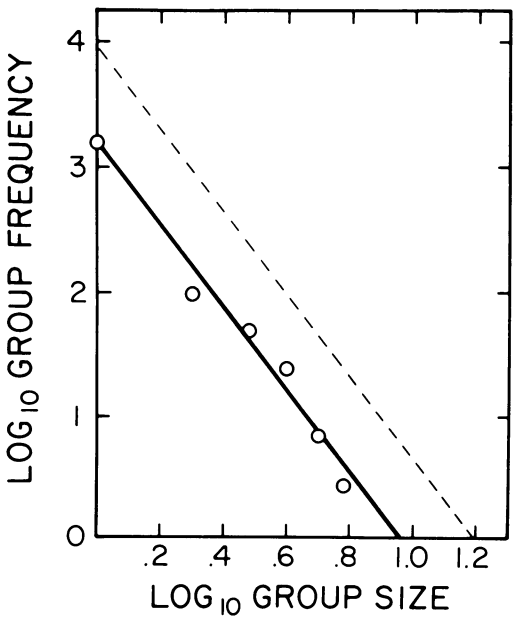

FIG. 3. *Frequency distribution of aggregates observed by electron microscopy (circles). The dotted line indicates the same distribution for a more concentrated suspension containing a total of 10,000 particles.*

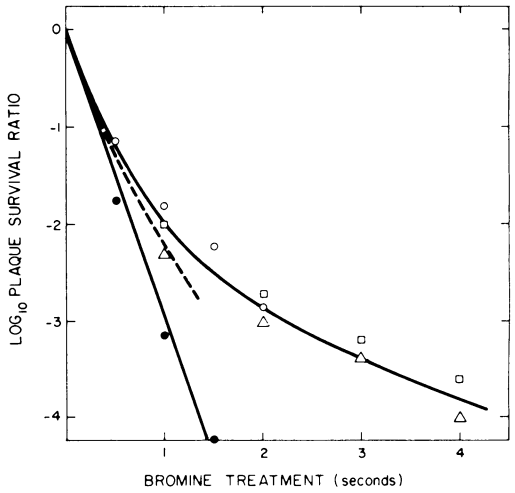

FIG. 4. *Inactivation of reovirus during the first 4 s of contact with 2.9 μM bromine in water at pH 7 and 2 C. Three separate experiments are shown with different symbols. The frequency of aggregates of different sizes is that shown in Fig. 3. Filled circles show the fast inactivation rate, where the suspension contains essentially all single particles. Dotted line shows survival calculated from the observed frequency of aggregates and the Poisson limit as described in Discussion.*

for comparison with the above results with aggregated virus. A 15-ml amount of a purified virus preparation, aggregated as shown in Fig. 5, was layered over a 15 to 30% (wt/wt) sucrose gradient with the BXIV zonal centrifuge rotor running at 2,000 rpm. The total number of virions put into the rotor was 10^{11} by electron microscope count. After operating at 25,000 rpm for 30 min ($\Sigma\ \omega^2 t = 1.44 \times 10^{10}$) at 20 C, the 640-ml volume of the rotor was completely displaced by 35% sucrose piston fluid, and 25-ml fractions were collected in 1-ounce bottles. The virus particle count on each was made by electron microscopy after dilution with 0.85% filtered sodium chloride solution. A dilution of at least 1/10 is required; otherwise the agar block that receives the sedimented virions in the pseudoreplica counting process will float to the surface because of residual sucrose. Particle counts are plotted against fraction numbers, and a scale of radial distances is included on Fig. 5. Densities in the sucrose gradient were measured by direct pyknometer weighings.

Fractions 4 through 10 contained 69% of the recovered virus. In the peak fraction (no. 9), there were 93% single particles. Six percent of the remainder was in pairs, and the remaining 1% was in triplets and groups of four (Fig. 6, bottom line). Thirty-one percent of the total

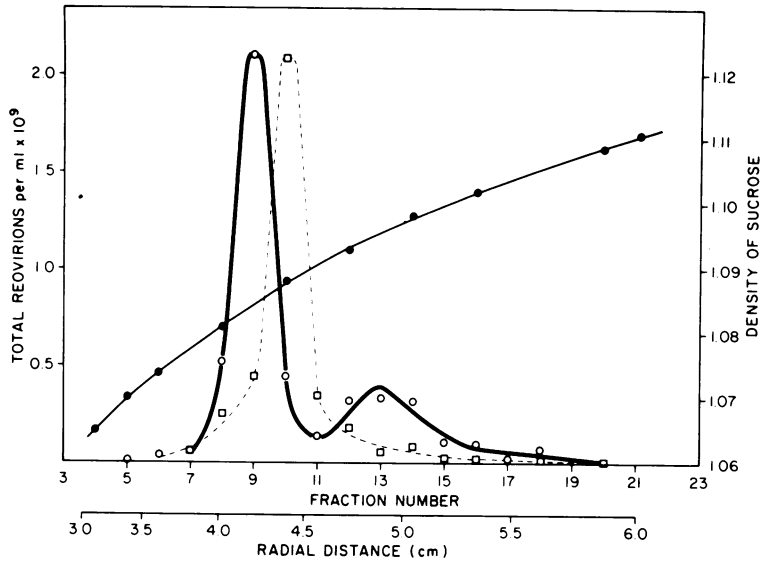

FIG. 5. *Sedimentation velocity spectrum obtained in the zonal centrifuge of spontaneously aggregated reovirus (solid line). Ordinates are particle counts made by electron microscopy. Sixty-nine percent of the virus is in the major peak, which has 93% single particles and a few remaining small aggregates, as shown in Fig. 6. The secondary peak contained mostly pairs and triplets in fraction 13. The dotted line shows a similar spectrum of crude (unpurified) reovirus showing that the original state of freeze-thaw-released virus contains very few small aggregates. Large aggregates have sedimented to the rotor rim.*

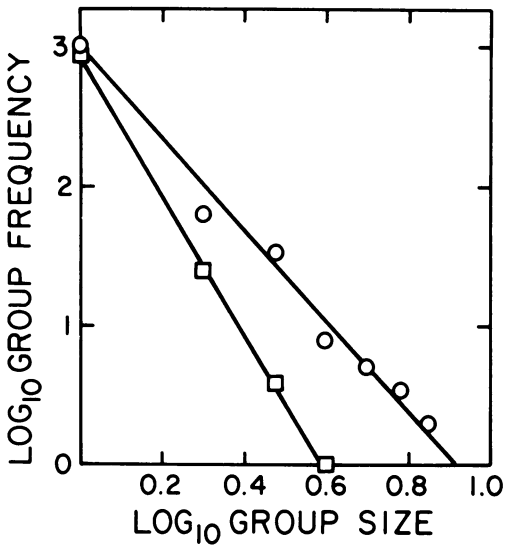

FIG. 6. *Frequency distribution of aggregates observed in starting virus preparation for zonal centrifuge experiment no. 1 (circles). The squares show how the distribution was altered by removal of most of the aggregates from the region of the principle peak (Fig. 5).*

recovered virus made a broad secondary peak with maximum count in bottle no. 13, in which 63% of the particles it contained were in pairs. The total count of all the fractions indicated 110% of the 10^{11} particles that were put in. Apparently there was no detectable loss in the partition process, and calculations from the electron microscope frequency distribution chart (Fig. 6, upper line) predict that 71% of the in-going population was single particles, a figure in excellent agreement with that observed in this actual sedimentation velocity spectrum from the zonal centrifuge.

(ii) Experiment 2. Inasmuch as zonal selection provides a method of obtaining a very high percentage of single reovirus particle suspensions, we prepared a fresh quantity of purified virus containing about 50 times more virus than that of the above zonal fractionation. This preparation yielded the velocity spectrum with single peaks like that of Fig. 5, but fractions 12 through 19, which contained aggregates of two to eight particles constituting 31% of the first population examined, now contained aggregates in the same size range but relatively few of them (less than 10% of the total). Apparently, freshly prepared Freon-extracted virus has very few aggregated particles. In the peak fraction (no. 10) there were 87% singles. It is possible that at this high concentration (2.5×10^{10} virions/ml) some spontaneous aggregation took place before preparations could be made for electron microscopy. A part of this fraction was

frozen immediately to preserve both infectivity and physical dispersion for subsequent bromine inactivation experiments.

(iii) Experiment 3. One more zonal velocity spectrum experiment was made with a crude freeze-thaw lysate of infected L cells without even low-speed clarification. This crude preparation contained the virus from the same number of infected cells as that providing virus for the last experiment, described above. Again a single particle peak was observed (Fig. 5), and again there was very little virus in the region in which aggregates of two to eight were seen in substantial numbers in the first experiment. This unexpected result drew attention to the fact that the total quantity of virus recovered from the crude starting material was only about 1/5 of that usually recovered by Freon extraction of an equal number of infected cells. It would appear that an excellent fraction of single particles can be obtained in this way with no previous purification and that there are very few small aggregates in such a preparation, but that the major part of the virus must have been in large aggregates or combined with larger cell debris which sedimented beyond the sampling range and reached the rim of the zonal rotor.

Bromine inactivation of "singles" fraction from velocity spectrum. The singles peak fraction from zonal velocity spectrum (ZR2 above) was frozen at −40 C in 1-ml vials to preserve infectivity and state of dispersion. Subsequently thawed samples had not lost infectivity, and the relative numbers of single particles and groups in the size range of two to eight had not changed, but bromine inactivation showed that changes had nevertheless taken place. After a rapid start, the reaction became slower and appeared to reach a resistant level at about 10^{-3} survival after 2 s at 3 μM and 2 C. If these surviving PFU are aggregates, they must have "grown" from single particles during the short time interval before freezing or in the thawing process.

Five milliliters of the same virus was treated with bromine in the same way, except that it was centrifuged for 17 min at 20,000 rpm at 20 C in a Beckman SW50 rotor. Only the top 4 ml was used in the bromine experiment. All large aggregates should have been removed from this preparation. The resulting rapid linear decline in PFU (Fig. 4) provides excellent confirmation.

Partition of crude reovirus between singles and large aggregates by means of a sucrose gradient in the swinging bucket centrifuge rotor. Zonal sedimentation velocity spectra of crude reovirus (ZR3 above) yields a prominent sharp band of single virions and relatively few small aggregates, but most of the virus seems to have sedimented much faster then either. An experiment was done in the large (37-ml) buckets of the Beckman SW27 centrifuge rotor to determine, if possible, the quantity and physical state of virions and the plaque titer of the two major components of this particle population, the singles band, and the pelleted material. To do this, a 20 to 40% sucrose gradient was established and 10 ml of the freeze-thaw lysate (the same as that used in experiment ZR3) was layered over it. After spinning at 25,000 rpm (mean centrifugal field, 81,000 × g) for 1 h, the supernatant fluid above the visible singles band was discarded. A 10-ml amount, including the singles band, was collected, and then the pelleted material was resuspended in the remainder of the supernatant fluid by pumping with a pipette. These two fractions will be called band 1 (B1) and pellet 1 (P1).

An equal quantity of the crude virus preparation was extracted with Freon and layered over a similar sucrose gradient, centrifuged, and harvested in the same manner, band 2 and pellet 2.

One-half of the resuspended pellet 1 material was extracted with Freon, made up to the same volume (10 ml), and banded and harvested like the previous ones, band 3 and pellet 3.

A part of both fractions B1 and P1 were treated for 30 s with 20-kHz acoustic waves from the microtip of a Branson Sonofier model LS-75. Sample volumes treated were 5 ml each, and they were immersed in ice water during treatment. The maximum temperature attained was 15 C. There were eight samples in all prepared for virion count and observation of aggregation in the electron microscope and for plaque titration. The results of physical assay are shown in Fig. 7. Particle count of pelleted fractions from Freon-extracted preparations contained a large amount of cellular debris, which made the virions difficult to count. Nevertheless, the total particle yield (sum of counts from pellet and band) was approximately the same (Fig. 7). Freon extraction yielded 57% of the particles in the band, but with the crude virus only 28% was in the band. Freon extraction of virus from the pellet fraction of the crude virus experiment (P1) yielded 67% of the particles in the band. Apparently the Freon extraction process is quite efficient for extraction of reovirus in monodisperse form from the infected cells.

Plaque titration of banded and pelleted fractions from several identical partition experi-

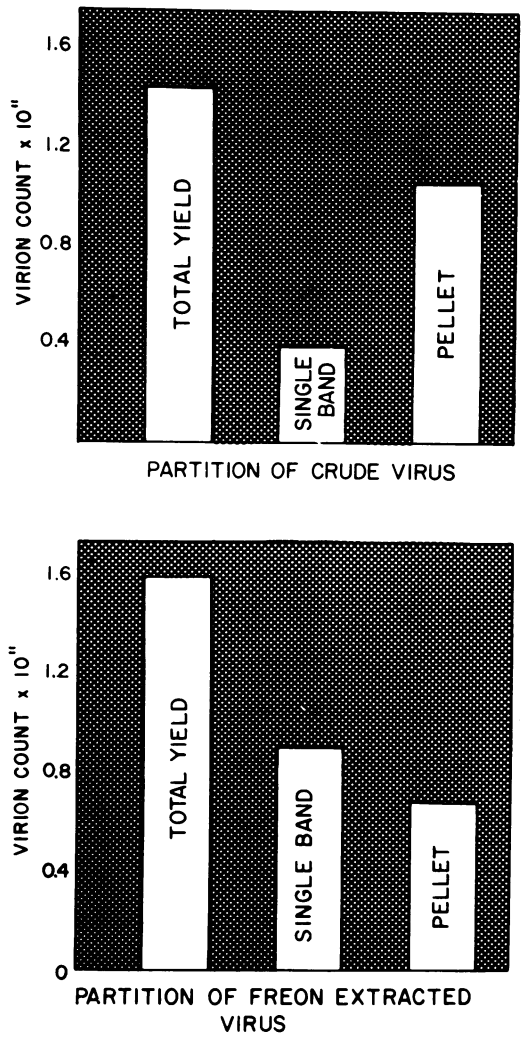

FIG. 7. *Total yield of reovirus extracted from a given number of infected cells is about the same whether it is done by three freeze-thaw cycles (top) or by Freon extraction (bottom), but only 27% of it appears as a band of single particles when the crude virus is sedimented in a 20 to 40% (wt/wt) sucrose gradient. The rest is in the pellet. For Freon-extracted virus the partition is 53 to 47%. Fig. 5 shows that there are very few small aggregates present.*

ments have shown 1/7 of the Freon-extracted virus to be in the pellet and 6/7 in the band. The partition of PFU in crude preparations has been erratic. The pellet fraction always has much more than the band, and once there were 50 times as many PFU in the pellet as there were in the band. Treatment of banded and pelleted fractions with 20-kHz acoustic waves have not made any substantial change in particle count, but in this case it reduced the plaque titer of the pelleted fraction by a factor of 10. The same treatment made no change in the titer of the well-dispersed virus in the banded fraction.

DISCUSSION

Reovirus was chosen for this work from among the many water viruses because of its size, which makes it readily amenable to physical assay by two essentially independent methods. Earlier work has shown (5) that the reovirus particle counts and aggregation analyses made by the kinetic attachment method of preparation for electron microscopy yield the same results with suspensions of 10^{10} virions/ml as those obtained by the sedimentation or agar pseudoreplica method on the same suspension after aproximately 100-fold dilution. Demonstration of this fact is essential, of course, for if it were not so the dilution of suspensions of aggregated virus for plaque assay would yield no information about the bromine resistance or plaquing efficiency of the aggregates seen in the electron microscope. For this reason reovirus may provide a useful model of reference when experiments of this kind are made with the many smaller picornaviruses that are of greater significance as water pollutants but are more difficult to assay by physical means.

The reaction of bromine (3 μM) with preparations of reovirus in water at pH 7 is shown here to destroy 99.9% of the PFU in 1 s at 2 C, if the preparation contains predominately single virions. After the most strenuous efforts to remove all aggregated virus, the logarithm of the survival ratio is a linear function of the time of exposure to the bromine at least to the level of 1:10,000 surviving PFU, the titer falling at the rate of 3.0 $\log_{10}$ units/s. This is 25 times faster than the rate observed with poliovirus under similar conditions of bromine exposure (1a). It is more than 100 times faster than rates indicated by Scarpino et al. (4) and Weidenkopf (8), respectively, for chlorine at pH 6 and the same molar concentration and approximately the same temperature. Comparison of bromine rates at pH 7 with those of chlorine at pH 6 seem most appropriate for comparison of the effects of HOCl and HOBr.

Whereas reovirus, velocity banded in a sucrose gradient, is usually 90 to 95% single particles, it gradually aggregates in such a way that the log of the frequency of any group size becomes and remains a linear function of the log of the number of particles in the group (Fig. 3). Inactivation by bromine of such aggregated virus is characterized by a continuously decreasing decay constant. It would appear from this that plaque-forming aggregates are more re-

sistant to bromine than single virions.

If the probability of survival of a single virion after a mean dose (m) of bromine is taken to be e^{-m}, and the survival of one of the particles of an adhering pair as $(1 + m)e^{-m}$, etc., and for a group of i particles according to the Poisson function $\{1 + m + [m^2/2!] \ldots m^{i-1}/(i - 1)!\}e^{-m}$, then the titer of a mixture of $N1$ singles + $N2$ pairs + $N3$ triplets, etc. would be given by: $T = N_1e^{-m} + N_2 (1 + m)e^{-m} + N_3 (1 + m + [m^2/2!])e^{-m}$. . . in which the coefficients N_1, N_2, N_3, etc. can be supplied by the EM pictures (Fig. 3). Now the unit dose m can be taken from the experimental inactivation of an essentially singles population (Fig. 4). Thus we can calculate and plot the expected survival curve for this particular mixture of aggregates. The result is the dotted line in Fig. 4, which has the approximate shape of the observed curve but not quite enough departure from the straight line to match the experimental curve. Inherent in the above analysis is the assumption (i) that inactivation of a virion is the result of a single vital event which either does or does not occur during the time interval specified, (ii) that survivors are undamaged, and (iii) that the other virions in a clump containing one survivor do not help or hinder its chances of making a plaque. We are doubtful that all of these assumptions are valid, particularly the last one, and we point out also that before bromine treatment there are approximately 40 times as many virions as there are PFU in reovirus preparations. This means that none of the group sizes from two to ten would have over a 25% probability of containing more than one plaque-forming particle before the inactivation began. It appears that the survival of aggregates is substantially greater than that predicted by the Poisson theory. As yet we are unable to account quantitatively for the experimental results on a basis of either protection or complementation.

Preparations of reovirus containing 90 to 95% single particles can be obtained easily from either Freon-extracted lysates of infected L cells or directly from crude lysates in which virus has been released from the cells by three cycles of freezing and thawing. The Freon extracts yield about twice as much virus but both appear equally well purified when harvested from the sucrose gradient in the singles band in either the swinging bucket or the zonal centrifuge rotor. Slow spontaneous aggregation occurs in preparations of reovirus stored at 4 to 6 C, and in these mixtures of aggregates there is a continuum of sizes the logarithm of whose frequency is a linear function of the logarithm of the number of virions in the group. As aggregation continues, the slope of this line changes but the line remains straight. These aggregates appear to contain nothing but virions (like those in the experiment of Fig. 4). They are "pure" aggregates. But the major part of the virus released from infected cells by freezing and thawing alone, without homogenization with Freon, must consist of aggregates of a different kind. One difference is immediately seen in the sedimentation velocity spectrum of such crude virus, which is produced in the zonal centrifuge rotor. Whereas about 1/5 of the total virions and 1/50 of the PFU appear in a prominent "singles" band, there are very few groups of two to ten such as those seen in spontaneously aggregated preparations (Fig. 5). The supporting experiment in the swinging bucket rotor supplied the quantitative data on the partition of the virus in the crude preparations. It indicates clearly that the fast-reaction kinetics established in this report, and doubtless required for understanding of the bromine-virus reaction, have been obtained with about 1/5 of the virions normally present in crude extracts of infected cells, whereas the remaining 4/5 is in quite a different physical state, a state that appears to be much more resistant to bromine action. One can reasonably expect that the virus found in polluted water will be in a state more like the crude than the purified preparations used in this work. Thus it will be necessary to examine in some detail the nature of the naturally occurring aggregates as well as the spontaneous or "pure" aggregates and their resistance to halogen ions if conditions in polluted water are to be understood. The data from the initial fast reaction with single virions and small groups provides a step in this direction.

ACKNOWLEDGMENTS

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Florence B. Stubbs prepared the many virus samples and made the count and aggregation analyses by electron microscopy. We appreciate this excellent technical work.

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