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Poliovirus Aggregates and Their Survival in Water

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Inactivation of aggregated poliovirus by bromine is characterized by a continuously decreasing reaction rate. Poliovirus released from infected cells in these experiments by alternate freezing and thawing in water without electrolytes has always been aggregated. The aggregates persist even on 7,000-fold dilution in ion-free water. Virus similarly released into phosphate-buffered saline solution may be well dispersed, but it aggregates when sedimented into a salt-free sucrose gradient or when it is diluted as little as 10-fold in water. Large one-step dilutions of dispersed virus in water remain dispersed. Aggregated virus was not dispersed by one-step dilution (7,000-fold) in distilled or untreated lake water but was dispersed if phosphate-buffered saline or clarified secondary sewage plant effluent was used as diluent. Dispersed virus aggregates at all dilutions in alum-treated, finished water from the city filter plant. This may be the result of complex formation with insoluble material rather than virion-virion aggregation. A simple procedure is described for rendering a very dilute suspension of mixed virion aggregates into a three-part spectrum of sizes.

When enteric viruses make their way into wastewater from the cells of an infected host, it is unlikely that all the virions will be free single particles and available as such to the action of disinfecting agents. There will doubtless be some single virions and others attached to or even buried in foreign matter (6; J. M. Symous and J. C. Hoff, Am. Water Works Assoc. Water Qual. Technol. Conf. Proc. Atlanta, Ga., paper 2A-4a, 1975). There will probably be clumps of virions too because of the origin of these viruses in large, tightly packed cytoplasmic crystals in infected cells and because of aggregation induced as described in our previous paper (4). To understand the course of disinfection kinetics for such a complex mixture, one must determine first the kinetics of the reaction with single virions. This has been done for poliovirus and bromine (3). Then it is necessary to know the physical state of the virus in the polluted water. The quantity of virus in naturally polluted water is too low for direct observation of the virions and their aggregation, but crude lysates of infected cells can be put into water in sufficient quantity for such observation. Data on the behavior of mixtures of single virions and aggregates should be of value in solving the larger problem. In this work, both dispersed and aggregated virus preparations have been put in water of different kinds. The main purpose has been to look for any changes that might occur in the physical state of the virus when it is transferred from the infected cell to the water, which is quite different from the carefully balanced and buffered salt solutions in which it is customarily handled in the laboratory. Particular attention has been paid to the persistence or survival of the original aggregates when crude virus preparations are subjected to high dilution in water. A convenient technique is described for doing this.

MATERIALS AND METHODS

Virus. Poliovirus type I, Mahoney strain, was the same as that used in previous work (3). It was prepared in the same way, except as otherwise indicated in some of the following experiments.

Assay. Plaque assay and physical assay by electron microscopy were exactly as before (3).

Sedimentation velocity analysis. Zonal centrifugation was done in a titanium BXIV rotor as previously described (8). Partition of preparations of virus into three parts containing (i) essentially all singles, (ii) small aggregates, and (iii) large aggregates was done in 5-ml plastic tubes of the Beckman SW-50L centrifuge rotor. Inasmuch as this technique was developed particularly for this work, it will be described later along with the experiments and results.

All the centrifuge runs, both rate-zonal and swinging-bucket partition, were made in a Sorvall OTD2 fluid-drive centrifuge with ARC-1 rate control to insure slow starts and slow stops.

Preparation of the bromine solutions and the demand-free water were the same as in the previous work (3).

RESULTS

Rate-zonal analysis of sedimenting PFU in density gradients of sucrose dissolved in water. Infected cells were harvested in distilled, demand-free water and the poliovirus was released by two freeze-thaw cycles. This crude lysate titrated 8.7×10^8 plaque-forming units (PFU)/ml, and 15 ml of a twofold dilution of it was layered over a 15 to 30% (wt/wt) sucrose gradient in an Oak Ridge type BXIV zonal rotor (1) and centrifuged at 32,000 rpm and 20°C for 112 min ($\omega^2t = 7.95 \times 10^{10}$). The sucrose solutions were made with distilled water containing no buffers or other salts. Fractions were cut from the gradient, and the plaque titer of each is plotted (open circles) on Fig. 1, where it can be seen that there is no sharp peak anywhere. A broad band of infectivity extends over fractions 14 to 21, but there is no sharp maximum in the region of fraction 9, where single virions would be expected in this run. The total of all PFU recovered from all the fractions was 20% of that put in. Apparently 80% of the PFU in the form of large aggregates, either present in the starting stock or formed during the zonal run, have sedimented to the rotor rim. This experiment was repeated several months later with virus prepared the same way, with the same results.

Another zonal centrifuge run was made with well-dispersed, purified poliovirus. This stock virus had been stored in 0.05 M phosphate buffer at pH 7.2 containing 20% sucrose. It was diluted with an equal volume of PBS, and 15 ml was layered over the 15 to 30% (wt/wt) sucrose gradient and centrifuged exactly as above. The total particle count in the 15-ml starting volume was 3×10^{11}. This was not titrated, but such stocks have routinely produced one plaque on HEp-2 cell monolayers per 100 particles counted, so the PFU input was judged to be (within a factor of 2) quite similar to that used in the crude virus experiment above. Plaque titers of these fractions are plotted also on Fig. 1 (filled circles) and now a small sharp peak appears where none was before, in fraction 9. There is no peak in the deeper region where most PFU appeared in the crude preparation, but substantial numbers of PFU were found at all levels in the density gradient, indicating that aggregation was present to an extent much greater than that present in the stock virus (3).

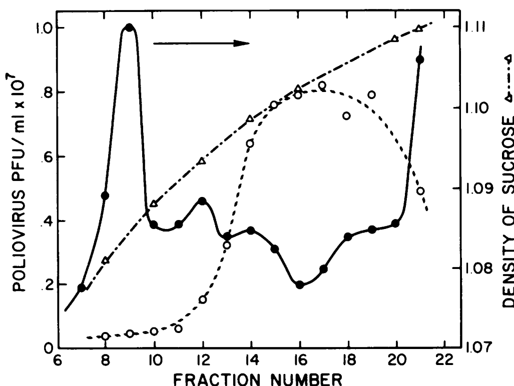

FIG. 1. *When a well-dispersed purified suspension of polio virions sediments into a salt-free sucrose density gradient (at no point more dense than the virus), some of the PFU are found in a sharp peak characteristic of single virions but most of the PFU move faster. Apparently aggregates are being formed (solid line). Unpurified virus released by freeze-thaw cycles into distilled water (dotted line) has no slow-moving peak. Almost all of the PFU are aggregates.*

A third zonal centrifuge run was made, this time with purified virus in a 15 to 30% (wt/wt) sucrose gradient containing phosphate-buffered saline (PBS) (those above contained no salt). The PFU were found in a very narrow band. One fraction contained 63%, with 35% divided about equally between the two on either side. The combined titer of all the other fractions was only 2%. This virus was certainly well dispersed.

Aggregated virus and its survival in the presence of bromine. One way to get all the virus (singles and aggregates) from a crude cell lysate and still render it sufficiently free of halogen demand for disinfection experiments is to pellet it repeatedly in the centrifuge, discarding soluble material in the supernatant fluids. Unfortunately, this process tends to compound the ambiguity of interpretation by inducing aggregation among even those virions that might have been free and by promoting added complexity with particles of foreign matter. We have layered crude cell lysate (freeze-thaw) over a 5% sucrose solution in a swinging-bucket centrifuge tube (SW-27 rotor) and sedimented the virus into a pellet (25,000 rpm, 4.75 h at 4°C). This should leave all the soluble material near the top of the supernatant fluid, and it did. Fiftyfold dilutions of the resuspended pellet material had no appreciable bromine demand. There was still enough particulate foreign matter present to make any quantitative estimate of virion aggregation by electron microscopy impossible. Exposure of this virus to 10 μM bromine at pH 7 and 10°C revealed the strong influence of aggregation (Fig. 2, triangles). The initial slope of the semilog plot of the survival ratio is approximately the same as that for

single particles (3), showing that many single virions must have been present, but after 12-s exposure to the bromine there were 110 times more survivors in the pelleted virus than there were in the dispersed preparation (Fig. 2, circles).

To avoid whatever virion aggregation might be induced by pelleting and still minimize halogen demand, we dialyzed about 100 ml of a freeze-thaw cell lysate in the Amicon model 202 ultrafiltration cell, using an XM 300 filter disk and continuous agitation while passing through 2 liters of demand-free water. With a nitrogen pressure of 30 lb/in^2, this took 24 h at 4 to 6°C. On exposure to 10 μM bromine, the $\log_{10}$ PFU titer of survivors was quite linear while it declined by a factor of 1,000 (Fig. 2, squares). After 12 s of exposure, the survivor titer in the dialyzed virus preparation was 288 times greater than that found in similarly treated dispersed virus. This clearly demonstrates that a linear semilog decline in survival titer can be obtained with aggregated virus, but the slope, of course, is very much less than that for singles. Independent evidence of aggregation in this preparation was provided by electron microscopy, Fig. 3, 4, and 5 showing a few single virions, aggregates associated with foreign material, and multilayer virion aggregates completely free of other material. We were not able to obtain a meaningful frequency distribution chart of aggregate sizes, but it must have been different from that in the pelleted virus.

Response of poliovirus aggregates to acoustic energy (20,000-Hz waves). Attempts to disperse aggregated poliovirus with acoustic energy (sonic waves at 20,000 Hz) have not been very successful. The Branson Sonifier model LS-75 (Heat Systems Inc., Plainview, N.Y.) with the Micro Tip was used, and the rate of energy input to the sample was determined by measuring the rate of increase in temperature produced in 5 ml of water in a well-insulated glass bottle. This proved to be quite reproducible and we used dial setting no. 1, which gave about 10.5 J/s or 10.5 W. This power level heats small samples very quickly and inactivates the virus before observable deaggregation takes place if the temperature rises above 40°C. Heating the virus to 50°C for 30 s in a water bath produced a comparable loss in titer. However, the same 10.5-W power input produced no loss in titer when the sample was kept in an ice bath so that the temperature did not rise above 31°C during the treatment. In spite of this high level of acoustic power absorption in a 5-ml volume of virus suspension for 30 s, clumps of 100 or more virions could still be found by electron microscopy. In one case, a sixfold increase in titer was observed when bromine-treated survivors were sonically treated at the 10^{-3} survivor level, but in several other instances no changes occurred. Sonic treatment of aggregated poliovirus before treatment with bromine did not alter its inactivation rate.

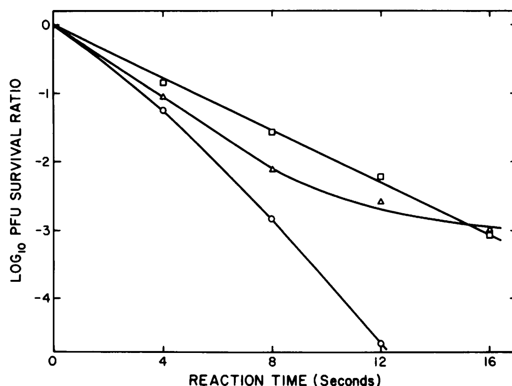

FIG. 2. *Disinfection of poliovirus by 10 μM bromine reaches $10^{-4.8}$ survival in 12 s when only single particles are present (○). Preparations containing aggregates usually show a curve of increasing resistance, as singles and small groups are inactivated first (△). Sometimes a mixture of singles and aggregates produces a straight line (□), but the slope is much less than that for singles.*

Spectrum of aggregate sizes in a mixture, separated into three arbitrary parts. The above experiments were made with virus preparations containing at least 10^{10} virions (ca. 10^8 PFU) per ml. This was done so that sufficient virus would be present to permit direct observation by electron microscopy. The physical state of poliovirus at low concentrations in water is not observable directly by electron microscopy, and PFU titration of the many fractions from rate-zonal centrifugation is a laborious process. Therefore, we have devised an abbreviated rate-zonal experiment that gives an approximation of the percentage of single virions in the preparation as well as a measure of the frequency of small and large aggregates.

A small centrifuge tube (5 ml for Beckman SW-50L rotor) is prepared by placing 1 ml of 50% sucrose solution containing neutral red in the bottom. A 2.5-ml amount of 20% sucrose containing trypan blue is layered over this, and 2 ml of the viral suspension to be tested is put on the top. This is spun at a speed and time sufficient to sediment about half of the single virions out of the top (white) section. Preliminary experiments with the best available monodisperse virus showed this to be accomplished in 20 min at 30,000 rpm and 20°C ($\omega^2t = 1.28 \times 10^{10}$). As the virions move into the 20% sucrose

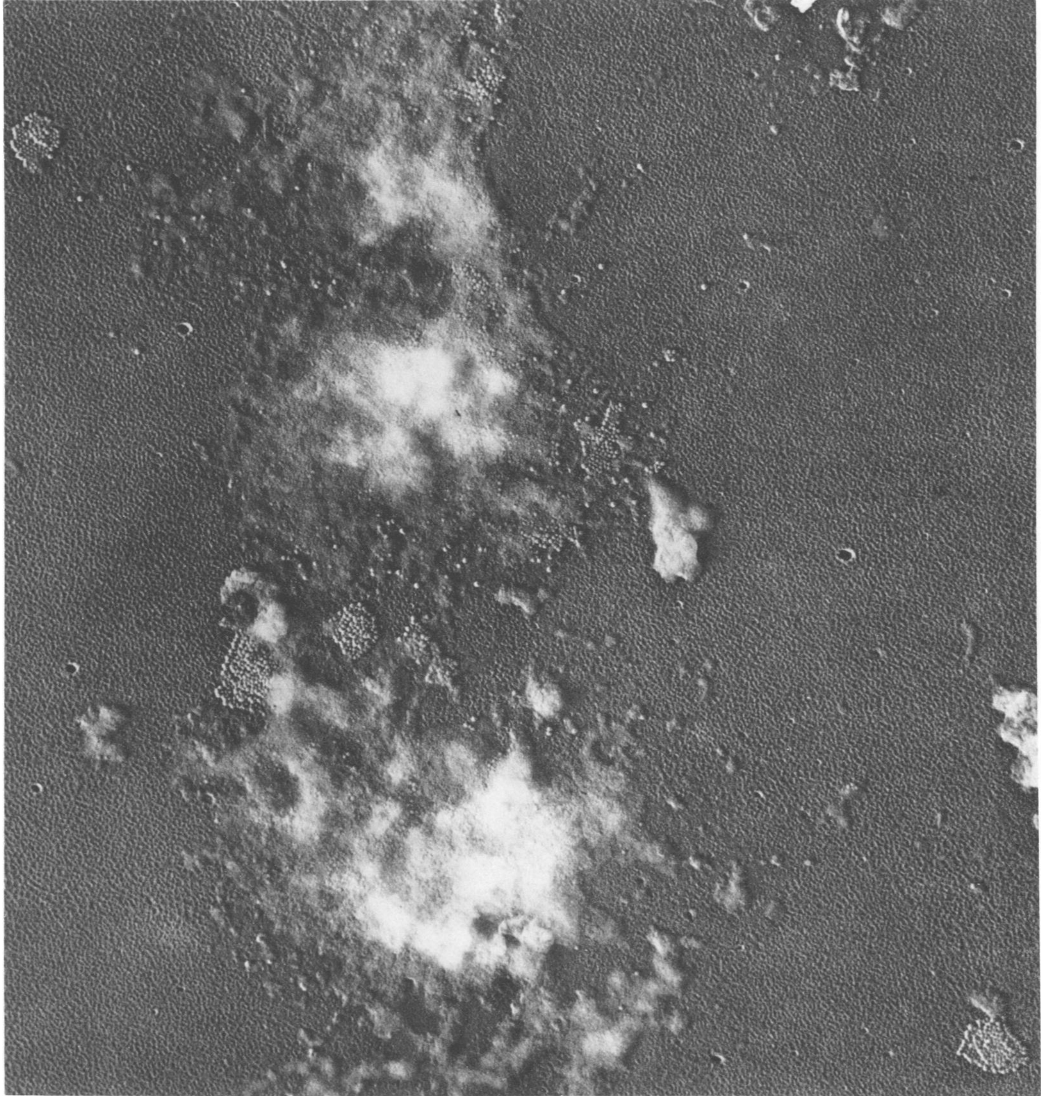

FIG. 3. *Typical poliovirus aggregates found in a dialyzed but unpurified freeze-thaw lysate of infected HEp-2 cells. ×20,000.*

(blue) section, they sediment slower, so that single particles in the original 2-ml volume will be compressed into a smaller volume (shaded, Fig. 6). Pairs, which sediment approximately 40% faster than singles, should be 70% removed from the white section and negligible numbers of larger aggregates should remain. So with a monodisperse poliovirus suspension one should find half of the PFU remaining in the white, the other half in the blue, and none in the red section. With polydisperse preparations we estimate, without experimental evidence at this time, that the largest compact adhering group that could remain in the blue section would be eight particles. All larger groups would be in the bottom red section. In actual practice, better reproducibility in removal of the top (white) section for PFU titration was obtained when a small amount of the blue section was taken with it. In the experiments reported below, a total of 2.5 ml was taken with the top section, and the average of eight repeat tests with a highly dispersed virus preparation gave 66% (55 to 75%) of the original titer remaining in the white fraction. In these same tests the bottom (red) averaged 3%, with a range of 0 to 7%. This might have been a few aggregates or it might have been a small amount of virus left by in-

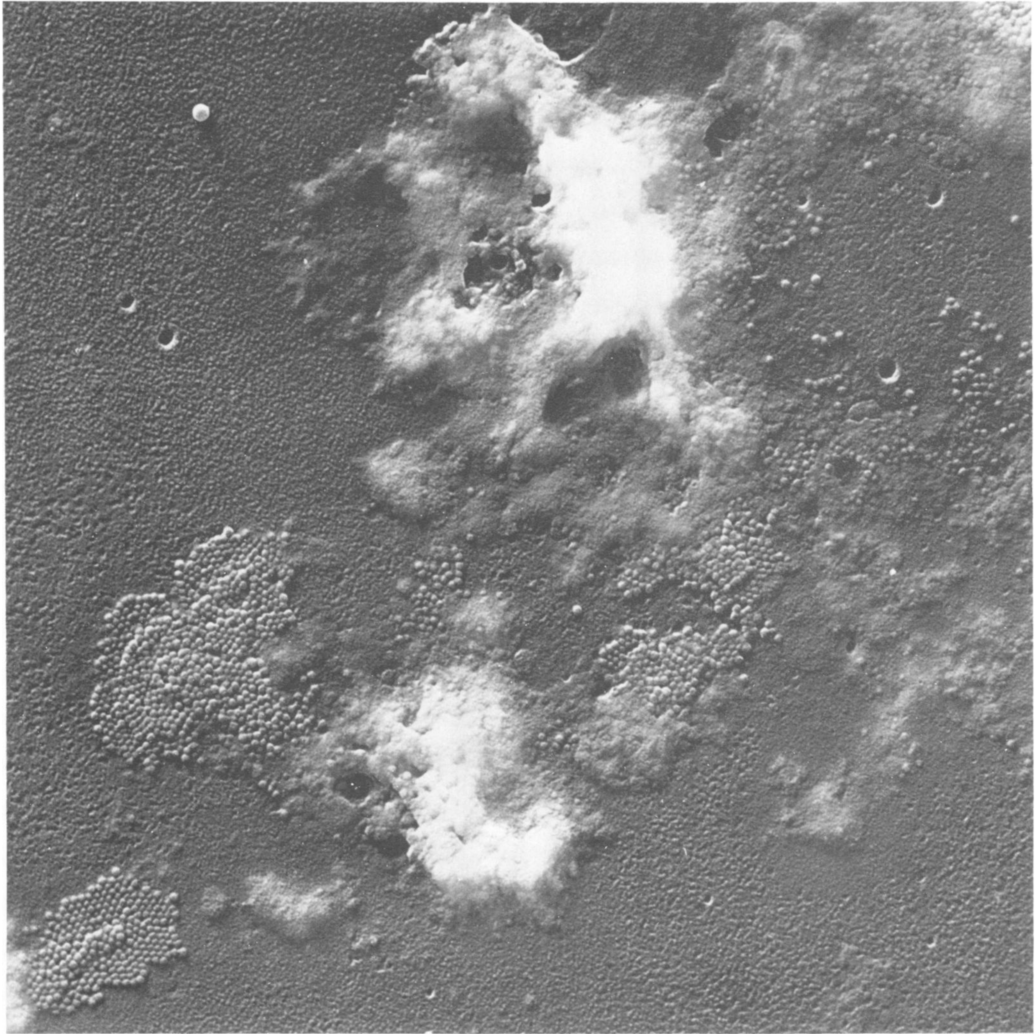

FIG. 4. *A closer view of poliovirus aggregates mostly associated with foreign material, probably host cell debris. ×33,600.*

complete removal of the overlaying fractions.

Fate of poliovirus aggregates when diluted in a large volume of water. The virus from 2 ml of a freeze-thaw lysate of infected cells was separated into three parts as described above. The two top fractions were discarded. The bottom red section provided a stable stock of aggregated virus. Dispersed virus of comparable titer was prepared by diluting a purified concentrate with PBS. The experiment consisted of adding 1 drop of either the dispersed or the aggregated virus to 200 ml of the water sample under test. With constant mixing, this approximately 7,000-fold dilution was kept at 20°C, and samples were removed after 1 min and again after 60 min. Each of these samples was centrifuged again, and the red, white, and blue parts were titrated separately.

Water of several kinds was tested in this way for its effect on the virus. From the Chapel Hill water purification plant, the incoming reservoir water was tested before and after centrifugation sufficient to remove particles of poliovirus size. Finished water from the purification plant (dechlorinated by ultraviolet light) and secondary trickling filter effluent from the sewage plant (clarified in the same way as the reservoir water and unclarified) were tested as well as several controls, including distilled, deionized water and PBS.

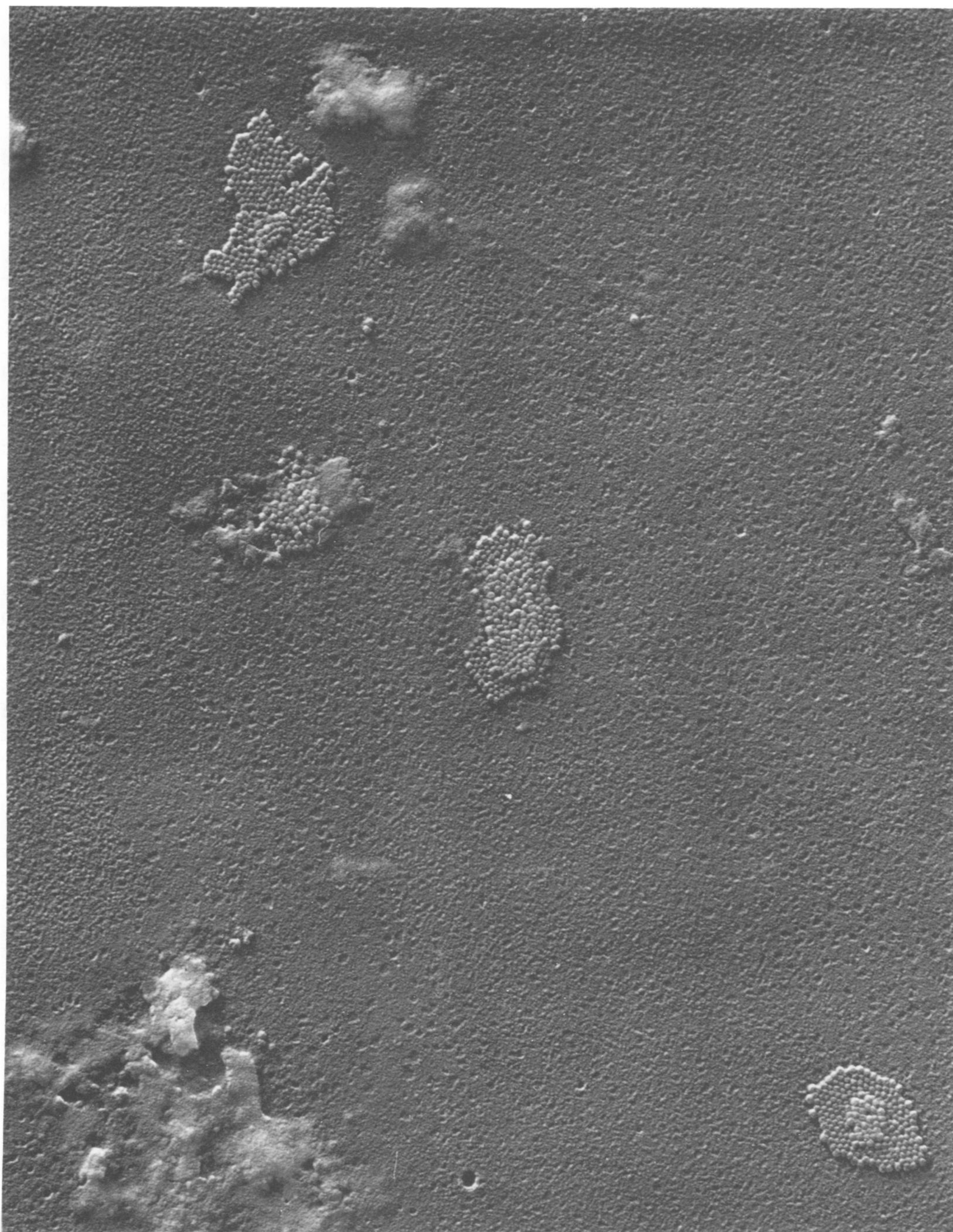

FIG. 5. *Some poliovirus aggregates, even in unpurified lysates of infected cells, appear to have been reconstituted from dispersed single particles. ×33,600.*

The results are shown in Table 1. In all the tests the plaque titer of the starting virus mixtures in the 200-ml reaction vessel was in the region of 10^4 PFU/ml. The degree of aggregation of the virions in each test can be judged from the fraction (percent) of titer in the white, blue, and red parts of each centrifuge tube.

Aggregated virus remained aggregated in

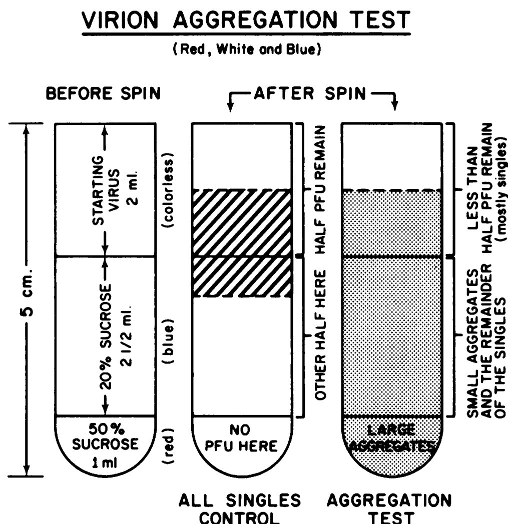

FIG. 6. *Abbreviated method of analysis of virion aggregation by separation of the sedimentation velocity spectrum into three arbitrary parts in a step gradient for PFU titration. After centrifugation there are mostly single particles remaining in the top (white) section of the 5-ml swinging-bucket tube. The middle (blue) section has the rest of the singles and small groups, whereas all the large groups are in the bottom section (red).*

distilled, reservoir, and finished water, leaving only 7 to 15% in the white supernatant region and 43 to 74% in the aggregated red region. Dispersed virus remained dispersed in all the samples except the finished water from the filter plant. In this finished water only 6% of the PFU remained in the white supernatant layer. About 50% were in the small aggregate blue region and the rest were in the red bottom. This result was confirmed by three more similar tests. Either this virus has become aggregated or it has formed complexes with insoluble particulate matter in the finished water and so increased in sedimentation rate.

A surprising result was observed with PBS and with the effluent from the sewage plant. In both of these the aggregated virus became dispersed so that the PFU titer in centrifuged supernatants was indistinguishable from the controls.

Some small changes can be seen (Table 1) when 1-min samples are compared with 60-min samples, but it seems that most of the action has occurred within the first minute after the 7,000-fold dilution whether it be aggregation or deaggregation. One result, with aggregated input virus and unclarified sewage plant effluent, may be a significant exception. Here the evidence for continuing dispersal of aggregates appears as a 2× titer increase from 1 to 60 min in the white supernatant and a simultaneous decrease (twofold) in the red.

Two more controls were added in an attempt to learn the reason for the apparent dramatic aggregation of dispersed virus by finished water from the filter plant. Inasmuch as lime and alum are used in treatment of this water before filtration, we tested a calcium chloride solution containing 6.3 mg of Ca^{2+} per liter (the concentration of Ca^{2+} found in the finished water by atomic adsorption spectroscopy) and another dosed with 20 mg of $Ca(OH)_2$ and 32 mg of $Al_2(SO_4)_3 \cdot 18H_2O$ per liter (the dosage used at the Chapel Hill water treatment plant), adjusted dropwise to pH 7 with 1 N HCl and centrifuged to remove aluminum hydroxide floc at a speed sufficient to pellet particles of poliovirus size. Both solutions were allowed to stand for 6 h at 25°C before addition of the virus. These data are included in Table 1, where it can be seen that calcium alone had no effect but when the alum was present about 80% of the virus sedimented to the bottom.

The virions in the drop of concentrate may have aggregated instantly when they fell into the 200 ml of alum-treated water, they may have reassembled after the 7,000-fold dilution, or their high sedimentation rate may not be due to aggregation but rather to formation of dense complexes with particles already present in the water. Instant aggregation would have to be critically dependent on the virion concentration in the drop, so a drop was put into 200 ml of distilled water (about 7,000× dilution). Previous experiments have shown that this diluted virus is dispersed. When 5 ml of this was mixed with 15 ml of alum-treated water and analyzed 15 min later, only 8% of the PFU were found in the combined white and blue fractions; 92% sedimented to the bottom red fraction. This indicates complex formation rather than virion-to-virion aggregation.

DISCUSSION

In kinetic studies of disinfection, one would like to observe the decline in titer over as wide a range as possible so that the emergence of a relatively few resistant units of infectivity may be detected. This means that the starting virus concentration must be high and the initial dilution into the test environment must be low. And if the test environment is to be natural water, these conditions are very difficult to meet without compromising the result. The foregoing experiments show that aggregates of polio virions are not easy to break mechanically, even by vigorous application of acoustic

TABLE 1. *Effect of different waters on the physical state of poliovirus*[a]

Input virus	Time[b]	State	University lake water (%)		Filter plant finished water (%)	Sewage plant secondary effluent (%)		Controls (%)			
			Raw	Clarified		Raw	Clarified	PBS	Distilled water	Distilled water + Ca^{2+}	Distilled water + Ca^{2+} and Al^{3+}
Dispersed	1 min	W	57	54	6	73	63	60	36	65	17
		B	31	39	51	20	33	37	62	35	6
		R	12	7	43	7	4	3	2	0	77
	1 h	W	58	51	6	76	60	55	32	75	10
		B	33	45	50	15	34	44	62	25	7
		R	9	4	44	9	6	1	6	0	83
Aggregated	1 min	W	8	15	10	28	61	54	7		
		B	22	34	19	31	32	42	21		
		R	70	51	71	41	7	4	72		
	1 h	W	16	14	7	45	69	60	5		
		B	30	43	19	36	29	37	26		
		R	54	43	74	19	2	3	69		

[a] The fractions of PFU found in the top (white, W), middle (blue, B), and bottom (red, R) parts of the tube after centrifugation are given as percentages of the total. The titer before centrifugation was about 10^4 PFU/ml. The three categories of aggregation are: W, essentially all singles; B, singles and small aggregates; R, large aggregates.

[b] The time is from the moment of mixing of 1 drop of virus in 200 ml of test water or control solution to withdrawal of 2 ml of the mixture for centrifugation. The centrifugation took 20 min for all samples, and the three fractions were titrated immediately.

waves. They show that poliovirus released from infected cells by freeze-thaw cycles, directly into water, is highly aggregated. Virus released into suitable buffered salt solutions, velocity banded in a sucrose density gradient containing the same salts and stored unfrozen in the same solution, remain dispersed. But when this dispersed stock virus is made to sediment into a sucrose solution without salt, the PFU sediment faster than single virions, indicating that aggregation occurs when the virions move into the salt-free environment.

Pelleting of crude (freeze-thaw) lysates in water or dialysis over Amicon XM 300 filter membranes, reduces halogen demand to tolerable levels, but both techniques leave the virus in a highly aggregated state. Electron microscope pictures show some of these aggregates completely free of foreign material, as though they had been reconstituted from dispersed single virions, and others complexed with what is probably host cell debris.

Disinfection of such aggregated preparations of poliovirus with bromine generally reveals a continuously decreasing reaction rate, indicating a range of resistance among the PFU qualitatively consistent with the observed aggregation. One conspicuous exception (Fig. 2) was a straight-line 3-$\log_{10}$ reduction in survival. This must mean that PFU of different degrees of resistance, probably different degrees of aggregation, were present in exactly the right proportions in the mixture. This condition has been proposed on a hypothetical basis but has not often been observed in experiments (7). Of course the slope of this straight line was much less than that observed with monodisperse virus.

Unless some other way is found to introduce poliovirus into water at high concentrations, it seems likely that such disinfection experiments with poliovirus in water will yield ambivalent results. Apparently the removal of salt from the virus suspension, even at neutral pH, leads to colloidal instability and increasingly frequent adhesion when two or more virions collide in their constant Brownian motion. According to von Smoluchowski (9, 10), single particles should disappear under these conditions at a rate proportional to the square of their concentration. Dilution of such an aggregating suspension with more water might not alter the instability, but it certainly would quickly change the rate of coagulation. This was verified by the

mixing of 1 drop of purified virus concentrate with 200 ml of water; no detectable aggregation occurred. Nor was any expected, if the theory is correct, for the dilution factor is about 7,000-fold, so the Brownian collision frequency should have been reduced by about 10^{-7}. Quasi-stable suspensions of poliovirus can doubtless be produced in water with less dilution. Still, there is no evidence of reversal of the aggregation at this high dilution. Aggregated virus did not disperse when diluted the same way. Aggregated virus did remain aggregated in all waters tested, except secondary effluent from the sewage plant. Even with particulate matter present, this effluent reduced virus aggregation, and when clarified by ultracentrifugation it dispersed the aggregates as effectively as PBS. The reason for this is not known.

The remarkable aggregating effect of the filtered finished water after the lime and alum treatment process must be somehow achieved before the drop of concentrated input virus is dispersed in the whole 200 ml, unless there are other insoluble particles present or still forming in this water to which dispersed virus may quickly attach, gain increased sedimentation rate, and so act like virion aggregates in the red-white-blue experiment. The aggregating effect of this finished water is not temporary; samples held for 6 days in the laboratory had undiminished aggregating power and simulated ones were effective after 2 months. Dilution (7,000-fold) of the input sample of dispersed virus did not change the reaction rate when it was put into finished water. This dilution factor should have reduced the rate of disappearance of single polio virions, if every Brownian collision resulted in adhesion, such that the time required to reach 10% survival of singles would have increased from 1.5 min to 175 h (9). Therefore, we must conclude that particles, presumably insoluble salts, are attaching to the virions and making them sediment faster. Direct observation of these hypothetical complexes by electron microscopy has not been achieved because poliovirus in sufficient concentration in water to be seen by electron microscopy aggregates even when there are no particles present. However, the response of such aggregates to halogen treatment should be quite different from that of virion-to-virion aggregation, and this we expect to investigate soon.

Another fact observed in passing is that the PBS of Dulbecco is an exceedingly good solvent for poliovirus. Virus released into PBS by freeze-thaw action deposited only 20% of the PFU in the aggregated category when tested in the concentrated form, and even these aggregates were dispersed when the test was repeated a second time at high dilution in PBS (Table 1). There does not appear to be any substantial fraction of the original virus crystal fragments or other "original" aggregates that do not disperse in PBS. This, of course, means that any dilution series made in PBS for plaque titration will be likely to disperse any aggregates and bring only single virions to the titrating cell monolayer. This presents an uncomplicated readout, but it means that one cannot expect to distinguish, by plaque titration alone without some selection process such as centrifugation, between an aggregated and a dispersed preparation of poliovirus.

Hamblet et al. (5) found a four- to sixfold increase in plaque titer when nutrient broth was added to a poliovirus suspension in Hanks balanced salt solution. We are not surprised that soluble proteins would have a dispersive effect, but the presence of aggregates in Hanks' solution and not in Dulbecco's (2) is quite unexpected considering the small differences in their composition. Doubtless this point requires further investigation.

Our experiments show that the normal state of poliovirus in fresh water is the aggregated state. Even when there are no other particles in the water to which virions can attach, they tend to attach to each other. In subsequent experiments the formation of complex aggregates such as those encountered by Schaub and Sagik (6) will be examined further by our methods. But even without considering the further complexity that must certainly exist in naturally polluted water, it is clear that the time and concentration of bromine necessary to inactivate poliovirus will be dictated by these aggregates. They are doubtless a major part of the mechanism for the survival of poliovirus infectivity in water.

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