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Aggregation of Poliovirus and Reovirus by Dilution in Water

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Poliovirus and reovirus were found to aggregate into clumps of up to several hundred particles when diluted 10-fold into distilled water from a stock preparation of minimal aggregation in 0.05 M phosphate buffer, pH 7.2, plus 22 to 30% sucrose. Reovirus was also found to aggregate when diluted into phosphate-buffered saline. The aggregation was concentration dependent and did not occur when either virus was diluted into water 100-fold or greater. The aggregation of poliovirus was reversible by further addition of saline and produced a dispersed preparation of virus. Reovirus aggregation was not reversible. Both viruses aggregated when diluted into buffers at pH 5 and 3, and poliovirus aggregated at pH 6, and this aggregation of both viruses was reversible when returned to pH 7. Aggregation did not occur at alkaline pH values. Aggregation at low pH could be prevented by suitable concentrations of sodium or magnesium ions, but neither caused aggregation of either virus at pH 7. Calcium ions, however, were found to aggregate both viruses at a concentration of 0.01 M.

The influence of virion aggregation on the survival of infectivity in water containing halogens and their compounds has long been suspected (1). Direct observation of the virion aggregation in disinfection experiments with bromine has been made recently with both poliovirus and reovirus (2, 7, 8), in which it was found that aggregated virus was substantially more resistant than suspensions of single particles. This resistance was, in one case with reovirus (7), shown to be due to the presence of large aggregates exerting a protective effect on interior particles. Other effects of aggregation such as complementation and/or multiplicity reactivation could also conceivably occur with small aggregates after inactivation by bromine, chlorine, or other compounds. Although this possibility has not yet been rigidly documented, it has been suggested in an earlier paper (8).

Ideally, any comparison of the relative resistance of two different viruses to one disinfectant must be made first on suspensions of single particles. However, the aggregated state is difficult to avoid in the laboratory, and certain precautions must be taken to insure a monodispersed suspension of virus. Animal viruses are usually gathered and maintained in salt solutions chosen for best maintenance of infectivity. Optimum ion kinds and concentrations, usually similar for different viruses, and ranges of pH stability have been established for different viurses. On the other hand, drinking water, and the lake and river waters that are the usual sources for drinking water, do not provide the optimum ions that are used in the laboratory. Therefore, it is reasonable to expect that viruses may not react with predictable behavior in raw or finished water, or in water passing through sewage or water treatment plants. This paper establishes, by electron microscopy and sedimentation velocity, some of the aggregation changes that two common laboratory viruses undergo when placed in water and under other non-physiological conditions.

MATERIALS AND METHODS

Viruses and cell lines. Reovirus type 3 (Dearing) and poliovirus type 1 (Mahoney) were produced in, and plaque titrations were performed in, L and HEp-2 cells, respectively, as described previously (2, 8). The extraction procedure and velocity banding in sucrose density gradients, and storage of virus stocks, have also been described (2, 7). Particle concentrations of stocks ranged from 10^{10} to 2×10^{12} particles/ml.

Physical assays of the viruses. Reovirus was prepared for electron microscopic particle count and aggregation analysis by the sedimentation and agar pseudoreplication method (5) and by the kinetic attachment method (6). Poliovirus was prepared for similar assays by the kinetic attachment method.

Virion aggregation analysis by sedimentation velocity. A single particle approximation (SPA) test has been used to determine the concentration of single particles in a virus suspension. Figure 1 shows the geometry of the test. A cellulose nitrate tube, which fits the Beckman SW50.1 rotor (0.5 by 2 inches [1.27 by 5.08 cm]), is filled uniformly with the

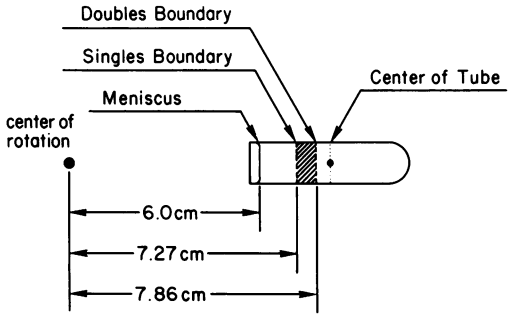

FIG. 1. *This diagram gives the basic measurements for the SPA test. Although any rotor can be used, the values given here are for the Beckman SW50.1 rotor utilizing 0.5- by 2-inch (1.27 by 5.08 cm) tubes. The terms "singles boundary" and "doubles boundary" refer to the distance traveled by single and double particles, respectively, from the meniscus of the fluid under the influence of the centrifugal force.*

virus sample. Under the centrifugal force developed by the spinning rotor, each aggregate size has a characteristic velocity of sedimentation. Singles sediment slowest, whereas pairs sediment approximately 40% faster. Groups of three, four, and more each sediment somewhat faster. If the run is terminated at the proper time, those single particles that were at the meniscus of the fluid will sediment one-third of the distance down the tube. Pairs will sediment about one-half the tube length, whereas larger aggregates will all enter the bottom half of the tube. Therefore, in that volume of fluid between one-third and one-half the length of the tube will reside single particles only (the shaded area in Fig. 1), and removal and titration of the top half of the contents of the tube (ca. 2.3 ml) will yield a measure of the singles population. The more aggregation present, the fewer singles remaining in this volume of fluid. For poliovirus, the conditions that place single particles at this level are: rotor speed, 30,000 rpm; time, 20 min; and temperature, 20°C; integrated time (t) at top speed, $\omega^2 t = 1.23 \times 10^{10}$ rads^2/s. Reovirus can be treated in a similar manner, the only exception being that the rotor speed is reduced to 15,000 rpm ($\omega^2 t = 3.13 \times 10^9$ rads^2/s).

RESULTS

Poliovirus: electron microscopy. The examination of virus aggregation requires a stock virus preparation in a well-defined monodispersed state. The poliovirus stocks used in this work were prepared by Freon extraction followed by sucrose density gradient sedimentation in 0.05 M phosphate buffer, pH 7.2 (2). The virus band, collected from the gradient, was stored at 4°C without removal of the sucrose, which resulted in a final concentration of approximately 22%. The particle count of these preparations was 7×10^{11} to 2×10^{12} particles/ml in the dispersed state. Dilution at 1:10 with phosphate-buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, and 0.01 M KH_2PO_4-Na_2HPO_4, pH 7.2) permitted kinetic attachment of single virions and aggregates to aluminized films for electron microscopy (6). Four pictures were taken of such a dilution and divided into five equal areas, and counts of single particles, pairs, triplets, etc., were made on all 20 areas. A part of one picture is shown in Fig. 2a, and the group frequency data are shown in Table 1, where it can be seen that the mean singles count per area was 124 with standard error σ = 15, and a mean frequency of pairs was observed to be 3.4%. Inasmuch as 124 particles would exclude 0.7% of the total area of the picture, we estimated that an average of five pairs per picture would have been produced by coincidence (2). The expected accidental frequency of triplets and groups of four was not calculated since the number of pairs observed indicated that aggregation was quite low in this preparation. That this stock of virus was quite stable is indicated by the increase of only 2% in pairs over a period of 60 days.

Dilution of poliovirus into water. When a poliovirus stock of 7×10^{11} particles/ml was diluted 10-fold into PBS or 0.14 M NaCl, it was revealed to be dispersed (Fig. 2a), but when the same preparation was diluted 10-fold into distilled water the particles formed aggregates (Fig. 2b). These aggregates were made of up to several hundred particles, but most often consisted of 10 to 50 particles. The aggregates appeared to be tightly bound together and did not spread out flat, but rather remained piled up on the aluminized film.

These aggregates were produced under the conditions of reduced ionic strength as revealed by the fact that there was a rather sharp cut-off level in ionic strength above which aggregation did not occur, and below which it did. This point was approximately 10 mM for phosphate buffer (ionic strength, $\Gamma/2 = 0.02$) and 60 mM for saline ($\Gamma/2 = 0.06$). Thus, the aggregation could occur with appreciable salts in the water. An increase in ionic strength, as by further dilution in PBS or 0.14 M saline, led to the dispersion of these aggregates, and electron microscope examination of these preparations revealed a picture similar to Fig. 2a.

The formation of these aggregates was markedly dependent on particle concentration. When a preparation of poliovirus was diluted 1:10 into distilled water, aggregates formed as described above. However, when the same preparation was diluted 1:100 or 1:1,000 into distilled water, the preparation remained dis-

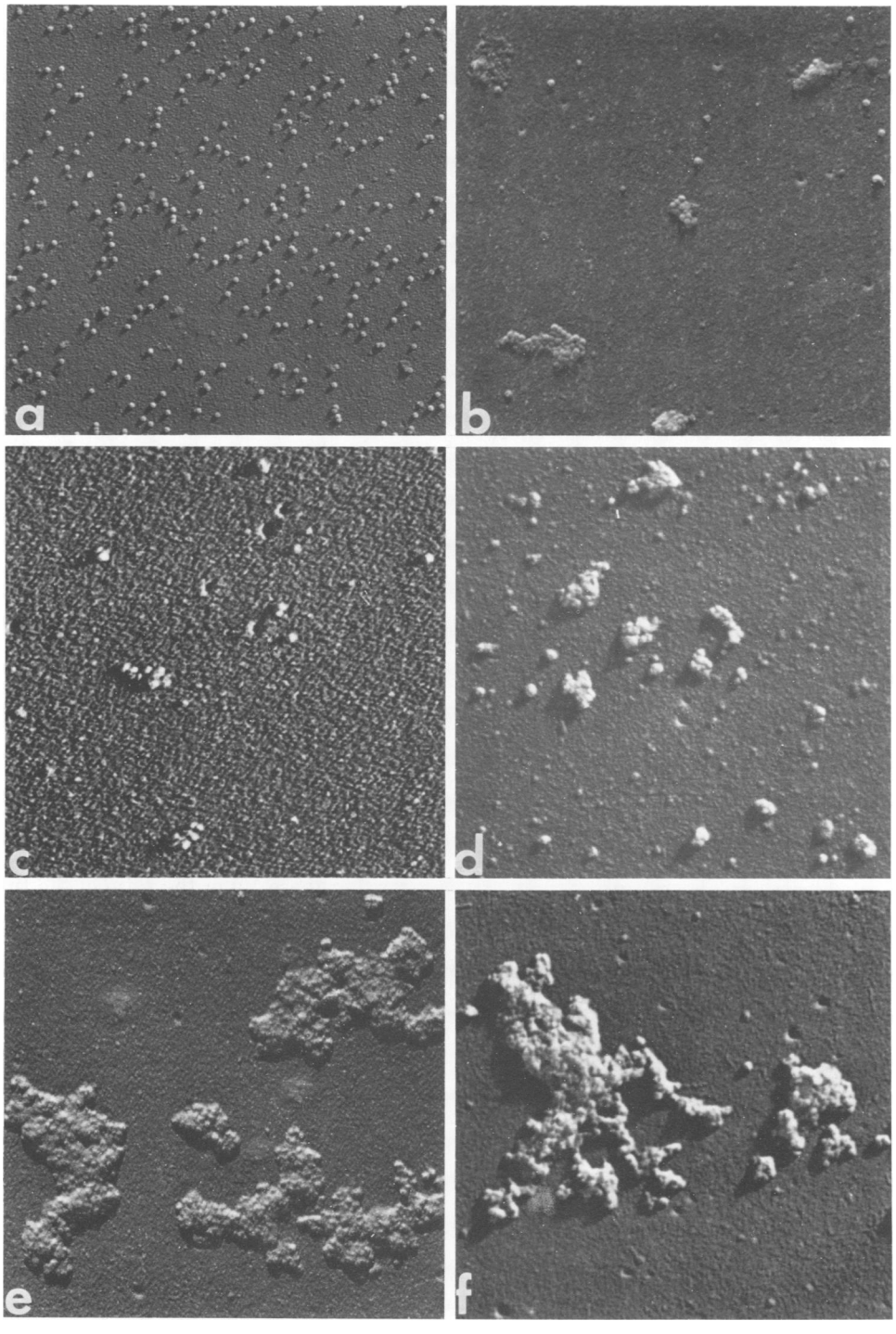

FIG. 2. *A poliovirus stock concentrate at 7 × 10^{11} virions/ml was diluted 10-fold in (a) PBS; (b) distilled water; (c) 0.01 M $CaCl_2$; (d) 0.05 M phosphate, pH 6.0; (e) 0.05 M acetate, pH 5.0; and (f) 0.05 M glycine-HCl, pH 3.0. All were prepared for electron microscope examination by the kinetic attachment method. (a) ×24,800; (b)-(f) ×29,000.*

TABLE 1. *Distribution of group sizes of one poliovirus preparation*

Group size	Group frequency per area[a]	Standard deviation (σ)	Total groups counted
1	124	15	2,480
2	4.5	2.1	89
3	0.35	NC[b]	7
4	0.05	NC	1

[a] Twenty areas were counted. This column gives the average per area.

[b] NC, Not calculated.

persed, as revealed by an SPA test as described in Materials and Methods.

Aggregation of poliovirus by ionic involvement. Three cations, Na^+, Mg^{2+}, and Ca^{2+}, were chosen for examination of their effect on poliovirus aggregation because of their physiological importance and widespread presence in water.

(i) NaCl. Dilution of poliovirus 10-fold to 7×10^{10} particles/ml in NaCl of concentrations up to 5.0 M had little effect on aggregation and led only to the formation of small numbers of triplets and aggregates of four, five, and six virions.

(ii) $MgCl_2$. A 10-fold dilution of poliovirus to 7×10^{10} particles/ml in $MgCl_2$ of up to 0.25 M had effects similar to those of Na^+ and resulted in very little aggregation.

(iii) $CaCl_2$. A 10-fold dilution of poliovirus to 7×10^{10} particles/ml in $CaCl_2$ of 0.001 M did not produce aggregation, but in $CaCl_2$ of 0.01 M aggregation occurred (Fig. 2c). [Higher concentrations of $CaCl_2$ could not be tested due to the formation of insoluble $Ca_3(PO_4)_2$ with the residual phosphate in the virus sample.]

Aggregation of poliovirus by pH. Quite important in the disinfection process is the effect of pH, particularly low pH, since drinking-water processing involves lowering the pH with alum, and the subsequent raising of it with lime. Therefore, we investigated the effects of pH on aggregation in some detail.

Figures 2d, e, and f show electron micrographs of aggregation of poliovirus as induced, respectively, at pH 6, 5, and 3. The aggregation at pH 6 was marked, but the aggregation at pH 5 and 3 was so massive that very few single particles could be seen in pictures taken at these pH values. This aggregation was quite similar to that shown for adenovirus-associated virus under low pH conditions by Johnson and Bodily (4).

The SPA test was used in the investigation of aggregation by low pH since it allowed a kinetic curve to be established for examination of the rate of aggregation of the virus, as well as providing a measure of the total amount of aggregation that took place. The tests were conducted as follows. A stock solution of virus was diluted to various particle concentrations in a final volume of 0.5 ml in the buffer at the pH under study, in 0.5- by 2-inch cellulose nitrate centrifuge tube. The tube was allowed to remain at room temperature (24°C) for 60 min. Similar dilutions in other tubes were made and also incubated at room temperature for 40, 20, 10, and 5 min. The continuing aggregation in each tube was reduced 100-fold by the addition of 4.5 ml of the same buffer at 20°C, and the contents of each tube were thoroughly mixed. (A 100-fold reduction in aggregation rate results in an effective stopping of the reaction since very little aggregation takes place during the next phase of the experiment.) A sixth tube at pH 7 in 0.05 M phosphate buffer was included in each test to serve as a control titer of a nonaggregated preparation, since poliovirus did not aggregate in this buffer. All six tubes were centrifuged under conditions described in Materials and Methods in a DuPont-Sorvall OTD-2 ultracentrifuge, using the Reograd mode at the termination of the run. It was found to be extremely important to allow the rotor to come to a slow, coasting stop, to prevent mixing within the tubes from destroying the separation of aggregate sizes established during centrifugation.

The top 2.3 ml of each tube was then removed as carefully as possible with a 5-ml pipette, thoroughly mixed, and titrated on HEp-2 cells. The results of each titration were plotted as the logarithm (base 10) of the ratio of the titer in the low pH tubes to the pH 7 control.

Figures 3, 4, and 5 show kinetic curves of poliovirus aggregation at pH 6, 5, and 3, respectively. Each curve could be divided into two distinct phases. The first phase was an initial

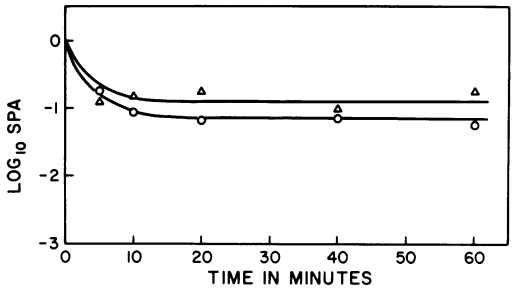

FIG. 3. *Kinetics of aggregation of poliovirus at pH 6.0 in 0.05 M phosphate buffer as determined by the SPA test. Initial single particle concentrations:* △, 1.4×10^{10}; ○, 7×10^{10}.

rapid aggregation as the virus was first subjected to the low pH conditions; the second phase, usually after 20 min, was a more level, stable phase wherein very little further aggregation took place.

Under the conditions where aggregation took place, the appearance of large aggregates must have been the result of the clumping of single particles. As the aggregates formed, the remaining single particles were left further apart, making the formation of aggregates less likely. This is revealed by the fact that curves produced by more dilute preparations of virus show less rapid aggregation during the first phase and a level second phase at a higher ratio value.

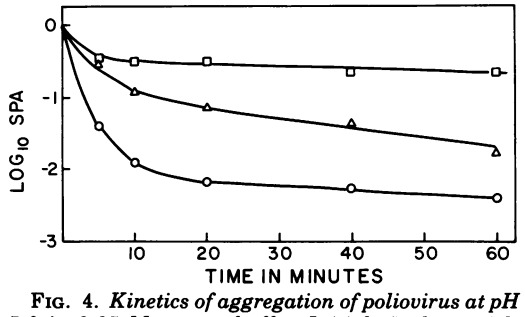

FIG. 4. *Kinetics of aggregation of poliovirus at pH 5.0 in 0.05 M acetate buffer. Initial single particle concentrations:* □, 1.5×10^{10}; △, 3×10^{10}; ○, 1.5×10^{11}.

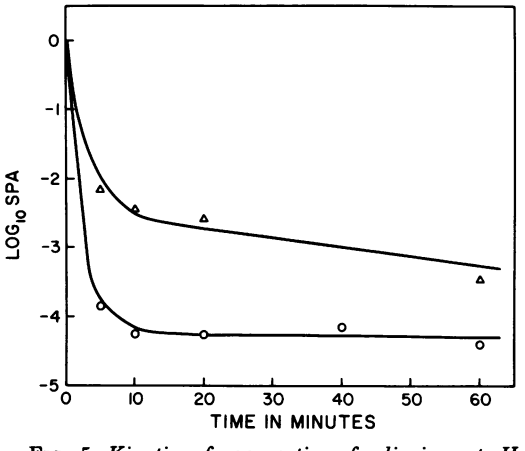

FIG. 5. *Kinetics of aggregation of poliovirus at pH 3.0 in 0.05 M glycine-HCl buffer. Initial single particle concentrations:* △, 7×10^9; ○, 7×10^{10}.

Additionally, these curves show that the amount of aggregation, as measured by the residual single particles in the top half of the centrifuge tube, becomes greater with decreasing pH. This is consistent with electron microscope data at pH 6, 5, and 3, which have revealed much more massive aggregation, and fewer single particles, at the lower pH values (pH 5 and 3) than at the higher one (pH 6).

All aggregation experiments at low pH were performed in buffer, each at a concentration at 0.05 M. This aggregation was found, however, to be sensitive to the ionic strength of the solution and could be prevented by appropriate concentrations of NaCl or $MgCl_2$. Table 2 shows the inhibition of poliovirus aggregation at pH 6, 5, and 3. As the H^+ ion concentration was increased, higher concentrations of monovalent Na^+ ion were required to prevent aggregation, for example, 2.5 M at pH 3 compared to 0.1 M at pH 6. The concentrations of the divalent cation Mg^{2+} required to prevent aggregation were generally lower than that of Na^+ at any given pH (except for pH 5) but reached a plateau of 0.25 M at pH 5 and 3. Higher concentrations produced no further change in the state of aggregation.

The alkaline range of pH had little effect on the aggregation of poliovirus, as determined by an SPA test. At pH values up to 11, no significant aggregation took place, although noncentrifuged controls of virus showed that viability was sensitive to pH 11 and lost plaque titer at approximately 1 $\log_{10}$/h. This is in contrast to low pH, were poliovirus viability was found to be stable at pH 3 for periods of time up to 1 h.

Aggregation at low pH was found to be a reversible phenomenon. Thus, when aggregates at pH 5 were returned to pH 7 by the addition of an equal quantity of pH 9 borate buffer, they broke up into single particles and small aggregates and presented a picture similar to Fig. 2a.

Reovirus: electron microscopy and aggregation upon dilution. The physical state regularly seen with reovirus concentrates prepared

TABLE 2. *Ionic inhibition of poliovirus and reovirus aggregation at low pH*

Virus	pH 6		pH 5		pH 3	
	Na^+	Mg^{2+}	Na^+	Mg^{2+}	Na^+	Mg^{2+}
Poliovirus	0.1	0.01	0.2	0.25	2.5	0.25
Reovirus	Na[a]	NA	0.6	0.25	>1.0	0.25

[a] NA, not applicable, since reovirus did not aggregate at pH 6.0.

by Freon extraction and sedimentation velocity banding in sucrose is shown in Fig. 6a. This picture was obtained when the stock virus in 30% sucrose was diluted into PBS in two steps: 10-fold and then immediately 20-fold; it was then treated for electron microscope count by sedimentation on agar for pseudoreplication. The numbers of single particles, pairs, etc., are plotted as circles on the frequency chart (Fig. 7). The single particles comprised about 79% of a total of 1.5×10^{10} virions/ml in this preparation, and this percentage was consistent in each preparation. This type of preparation is similar to that shown as the dotted line in Fig. 5 of an earlier paper (8) in which a crude, unpurified preparation of virus was subject to sedimentation velocity centrifugation in a zonal rotor (B-XIV). Both the purified and crude preparations had few aggregates of the smallest sizes, i.e., pairs, triplets, and so forth.

A markedly different picture was obtained when the stock solution was diluted, first, 10-fold into water or PBS and allowed to stand at room temperature for 2 to 3 h and then further diluted 20-fold into PBS. These conditions produced the aggregation seen in Fig. 6b, and it is apparent that, in contrast to poliovirus, the aggregates did not disperse when diluted further with PBS in the final 20-fold dilution. Their frequency is plotted as squares in Fig. 7. The percentage of single particles has dropped to 29% of the total.

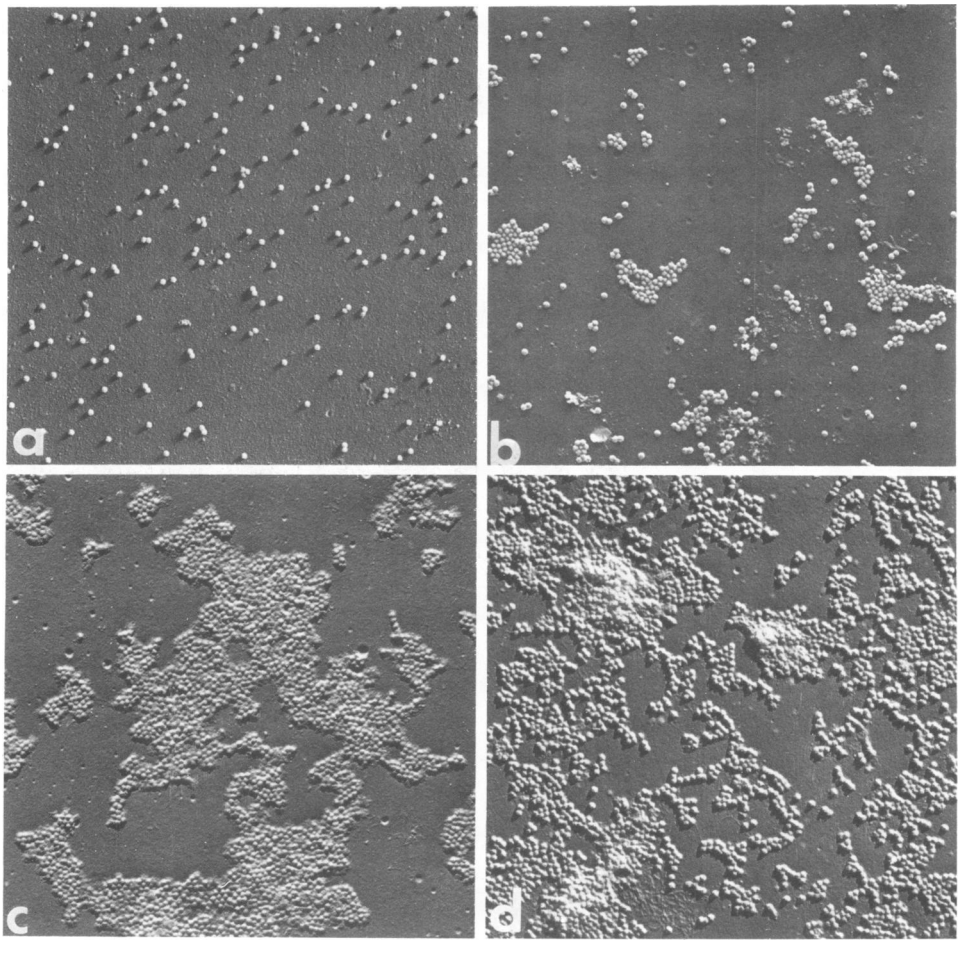

FIG. 6. *Reovirus stock at 2×10^{10} virions/ml diluted (a) 200-fold in PBS and (b) 10-fold in distilled water, allowed to remain at room temperature for 2 h, and then further diluted 20-fold in PBS. Both were prepared for electron microscope examination by the spin-down method. (c) Stock at 4.6×10^{11} virions/ml diluted in 0.05 M acetate at pH 5.0. (d) Same stock as (c) diluted in 0.05 M glycine-HCl, pH 3.0. Particles in (c) and (d) were collected by the kinetic attachment method.* ×8,500.

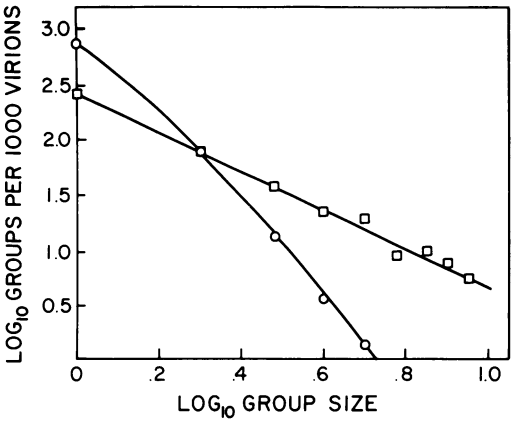

FIG. 7. *Dual-log plot of the frequency of aggregates in the stock reovirus preparation of Fig. 6 (○) and after extensive aggregation induced by dilution in water (□), as shown in Fig. 6b. The slope of each line is a quantitative measure of the aggregation, and the difference in slope between the two lines is a relative measure of the aggregation produced by the dilution in water.*

Freshly prepared stock concentrates of reovirus containing 10^{10} or more particles per ml regularly aggregated in water or PBS as described above, but they gradually lost this property, and after about 2 weeks of storage at 4 to 6°C they failed to aggregate under the above conditions.

The physical state of the virus in the undiluted concentrate cannot be examined by electron microscopy, but pictures of virus deposited on an aluminized collodion film from a fivefold dilution in PBS were similar to Fig. 6a, and the frequency distribution plot was the same as the circles in Fig. 7. Thus, a 5- or 200-fold dilution made no significant difference in the state of aggregation.

Aggregation of reovirus by ionic involvement. Reovirus behaved in a manner similar to poliovirus with respect to aggregation in NaCl, $MgCl_2$, and $CaCl_2$.

(i) NaCl. No significant aggregation occurred when reovirus was diluted 10-fold to 5×10^{10} particles/ml in NaCl solutions up to 1.0 M.

(ii) $MgCl_2$. Solutions up to 0.25 M $MgCl_2$ did not produce any significant aggregation over the control preparations when reovirus was diluted 10-fold to 5×10^{10} particles/ml.

(iii) $CaCl_2$. Reovirus (at 2×10^{11} particles/ml) aggregated into groups of up to 100 particles when diluted 10-fold into $CaCl_2$ at a concentration of 0.01 M, but no significant aggregation occurred in $CaCl_2$ at 0.001 M.

Aggregation of reovirus by pH: SPA tests. Aggregation due to lowered pH took place with reovirus in a manner similar to that with poliovirus, with the exception that no significant aggregation occurred at pH 6. Figures 6c and d show electron micrographs of aggregates of reovirus at pH 5 and 3 as prepared by the kinetic attachment method. As with poliovirus, the aggregation was quite marked at these low pH values. SPA tests were performed with reovirus, as described above, to measure the kinetics of aggregation at low pH. Figures 8 and 9 show kinetics of aggregation of reovirus at pH 5 and 3. The curves are similar to those of poliovirus and likewise can be divided into two phases, the first of rapid decrease in single-particle titer and the second of leveling off after 10 to 20 min with little or no further increase in aggregation. At pH 5, the kinetic curves (Fig. 8) show that at a higher particle count aggregation was more rapid than at lower concentrations, consistent with the results found for poliovirus. However, at pH 3 (Fig. 9) the aggregation kinetic curves show that the most concentrated virus preparation (4.6×10^{10} particles/ml) gave the least reduction in titer due to loss of single particles into groups, whereas the least concen-

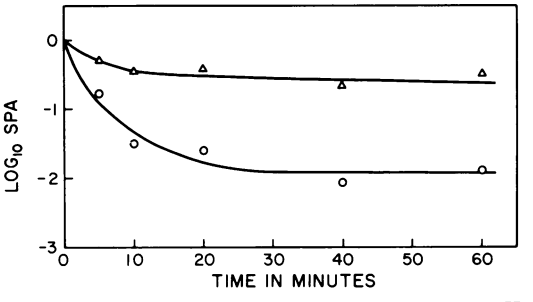

FIG. 8. *Kinetics of aggregation of reovirus at pH 5.0. Initial single particle concentrations: △, 4.6×10^9; ○, 10^{10}.*

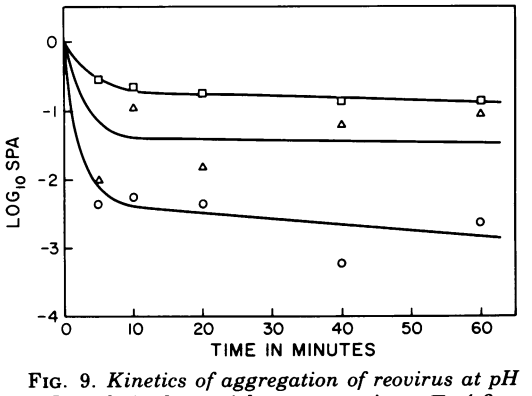

FIG. 9. *Kinetics of aggregation of reovirus at pH 3.0. Initial single particle concentrations: □, 4.6×10^{10}; △, 1.8×10^{10}; ○, 1.8×10^9.*

trated preparation (1.8×10^9) gave the greatest aggregation. The reasons for this apparent reversal of behavior are unknown but are presently under investigation.

As with poliovirus, aggregation of reovirus at low pH was sensitive to the ionic environment. Table 2 shows the minimal amount of NaCl and $MgCl_2$ required to prevent aggregation at pH 5 and 3. Somewhat larger concentrations of Na^+ are required to prevent aggregation at pH 5 for reovirus than for poliovirus, 0.6 M as compared to 0.2 M, but Mg^{2+} inhibited aggregation at pH 5 at 0.25 M for both viruses. Aggregation inhibition at pH 3 required larger concentrations of Na^+ than at pH 5, but the concentration of Mg^{2+} required was the same as for pH 5 inhibition, 0.25 M. Higher concentrations of Mg^{2+} ion at pH 3 produced massive aggregation; these were the only conditions under which Mg^{2+} ions caused aggregation of reovirus.

Aggregation of reovirus at low pH was found to be reversible when the pH was returned to neutral. When a heavily aggregated suspension of virus at 5×10^{10} particles/ml at pH 5 was treated with sufficient pH 11 glycine buffer to raise the pH to 7 and examined immediately by the kinetic attachment test, it was found to be dispersed.

Aggregation at pH 9, 10, and 11 at 5×10^{10} particles/ml was minimal with reovirus and was not significantly greater than that of the control at pH 7. Viability was sensitive to pH 10 and 11, and the plaque titers fell at the rates of 0.52 and 0.58 $\log_{10}$/h, respectively.

DISCUSSION

Interpretation of the results of disinfection experiments with virus in water by the observation of plaque formation of survivors requires knowledge of the state of aggregation of the particles at the time they were exposed to the disinfectant, and also at the time they were put upon the cells for titration. Our results indicate that both poliovirus and reovirus, at least the strains used here, will tend to maintain the aggregated state in fresh water. Viruses generally, and picornaviruses in particular, are produced intracellularly in a tightly packed formation, which, although not necessarily crystalline in nature, certainly results in a close arrangement of the particles which could be described as aggregated. It is likely that the virus particles are released into fresh water in this state. Hence, with the reduction in ionic strength that accompanies dilution into fresh water, any viral aggregates will be induced to maintain the clumped state.

A second factor contributing to the maintenance of the aggregated state in water is the presence of calcium salts (and also aluminum salts; see reference 15) in many drinking and flushing waters. Therefore, there seems sufficient reason to believe that if virus clumps are introduced into fresh water they will remain in that state.

The presence of large aggregates of viruses in water can be of significant public health importance if these aggregates are capable of protecting viable particles within the interior of the aggregate. We have previously demonstrated (7) that reovirus aggregates are capable of such protection against the action of bromine as a disinfecting agent. Whereas the disinfection of water supplies under conditions in which the virus is in the dispersed state may lead to a more nearly complete inactivation, it may be of little practical utility because of the relatively high ionic strength needed to disperse the virus particles.

On the other hand, if single particles of poliovirus or reovirus are present in fresh water, aggregation will take place if two or more particles approach close enough. However, as we have shown qualitatively in the data presented here, and as demonstrated quantitatively in the predictions of von Smoluchowski (10, 11), the collision frequency is dependent on the square of the particle concentration. A 10-fold reduction in particle concentration results in a 100-fold drop in collision frequency. For example, a 1:10 dilution from 7×10^{11} particles/ml in the stock to 7×10^{10} particles/ml in distilled water allowed for collisions to occur at a rate detectable by aggregate formation in 1 to 2 h. A further 10-fold dilution reduced the collision frequency a further 100-fold, making the time necessary to detect aggregate formation excessively long. With the very low concentrations of single particles expected in water, the collision frequency would be so low as to be nonexistent, unless measures were taken to concentrate a large sample of fresh water.

Our results also show that both poliovirus and reovirus can be prepared and stored under refrigeration for a substantial time in a nearly monodispersed condition without loss of infectivity. This method of Freon extraction and sucrose density gradient sedimentation avoids induced aggregation of virus, which may occur during pelleting (7), freezing and thawing, dialysis, or adsorption to filters at low pH (9) or in the presence of Al^{3+}, Mg^{2+}, or Ca^{2+} ions (12, 13), to polyelectrolytes (14), or to celite (3), techniques used in virus concentration and/or purification. It does not insure against induced aggregation when the stock virus is diluted for disinfection experiments, particularly into water with very low salt content. There appears to

be a critical range in the dilution process when both viruses aggregate rapidly. The physical state of the suspension becomes unstable as the sucrose and buffer salt concentrations decrease during dilution, and if the virion concentration is high enough when this point is reached, rapid clumping will occur. The aggregation of reovirus appears to depend at least partly on the drop in sucrose concentration upon dilution, since dilution into 0.14 M NaCl or PBS will initiate aggregation, as will dilution into water. Freshly prepared reovirus regularly exhibits this behavior but gradually loses it after 2 to 3 weeks. Poliovirus at the same concentration has never exhibited any aggregation in PBS or 0.14 M NaCl.

Electron microscopy has revealed the presence of virion aggregation induced by water dilution, $CaCl_2$, and acid buffers, as reported here, but all have been done at the high ($>10^9$ particles/ml) concentrations necessary for the instrument. At lower concentrations, where the electron microscope cannot be used, it has become necessary to use the ultracentrifuge to obtain a measure of aggregation as revealed by sedimentation rate. The sedimentation rates of single spherical particles of known size and density are highly predictable, and those of the smallest aggregates (i.e., pairs) are substantially greater. (Virion concentration, at the levels used here, does not affect sedimentation rate.) Hence, by carefully removing a definite quantity of supernatant fluid from a swinging-bucket rotor tube that has been centrifuged just enough to bring the single virions part way down, it is possible to sample the single particles that were in the heterogeneous mixture. Whereas it is theoretically possible to sample only the singles population, in practice that is quite difficult due to the fact that no gradient is used for stability. Careful handling is the rule. We compare only one sample with another of the same volume taken from another bucket of the same set of six in the rotor and containing the best dispersed virus as a control. Strictly speaking, this may not be a singles population analysis, but it remains useful as an SPA test.

The observations that (i) water-induced aggregates of poliovirus are dispersed by dilution in PBS, whereas those of reovirus are not, and that (ii) acid-induced aggregates of poliovirus are dispersed when returned to pH 7 are particularly significant in water disinfection experiments. They mean that the disinfecting agent might be acting upon a heterogeneous population of aggregates and single particles, and if the survivors are subsequently diluted with PBS for plaque titration the cells of the monolayer may receive redispersed virus in the case of poliovirus, or possibly clumped virus in the case of reovirus. Some of our past experiments have been compromised in this manner, and we suspect that others may have encountered similar difficulties.

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