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# Viral Aggregation: Mixed Suspensions of Poliovirus and Reovirus

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The aggregation of mixtures of two dissimilar viruses, poliovirus I (Mahoney) and reovirus III (Dearing), was followed by electron microscopy under conditions known to induce either aggregation or dispersion of each virus separately. Neither virus aggregated at pH 7 in an appropriate buffer, and no mixed aggregates were formed. Under conditions of lowered ionic strength (by dilution into distilled water) poliovirus became aggregated, whereas reovirus did not, and again no mixed aggregates were formed. At pH 6, however, poliovirus again aggregated and, although reovirus did not, it attached to poliovirus aggregates. Thus, some inducement toward aggregation was necessary to cause formation of mixed aggregates. This inducement probably took the form of a reduction of the ionic double layer surrounding the particles, which is known to occur at low pH. At pH 5 and below both viruses aggregated severely, and large mixed aggregates were formed. These mixed aggregates could be broken up by neutralization of the suspension, although small aggregates of poliovirus remained. Reovirus showed a marked tendency to attach to large clumps of poliovirus, but the reverse tendency was not observed. The results indicate that mixed aggregates may be of significance in the isolation of viruses from water or wastewater.

In the examination of waters and wastewaters for the presence of pathogenic viruses, isolates have been made from several virus groups, including poliovirus, echovirus, and coxsackievirus of the enterovirus group, as well as from the reovirus and adenovirus groups (12, 13, 15-17, 27). Members of the parvovirus group have also been isolated from fecal material (2, 3, 11), which indicates their probable presence in wastewaters, and the agent of acute infectious nonbacterial gastroenteritis has been placed into this group, denoting the importance of this group of viruses to water virologists. The virus of hepatitis A has been suggested to belong either to the parvovirus group (5) or, more likely, to the enterovirus group (20, 21), which puts further emphasis on the importance of the latter group in water virology. Several investigators have reported isolating viruses from more than one major virus group in samples of water or wastewater (13, 15, 17), and the isolation of these agents is usually accompanied by considerable concentration of the sample of contaminated water. Thus, there is a real possibility of clumps or aggregates of viruses containing more than one representative of the various virus groups being present in the final concentrate as it is prepared for titration. Conceivably, if an aggregate of viruses contained, say, several particles of poliovirus and echovirus, the more rapidly growing poliovirus might well outgrow the slower echovirus in the initial unicellular infection in the plaque titration. The final plaque would in turn be scored as "poliovirus," masking the presence of infectious echovirus. If this possibility is true, then tests for viruses isolated from water or wastewater might be artificially low. In this paper I have examined some of the conditions for the formation of aggregates containing particles from more than one virus group.

Because the mechanism of formation of such aggregates must be due to interaction of the ionic double layers surrounding each particle, a knowledge of the conditions under which mixed aggregates can form may lead further to a better understanding of the mechanism of aggregation or adsorption of virus particles to one another and to other particulate matter.

## MATERIALS AND METHODS

**Viruses and cell lines.** Poliovirus type 1 Mahoney strain was grown in roller bottle cultures of HEp-2 cells, and stocks were purified by Freon extraction and sucrose density gradient centrifugation as previously described (6). The Dearing strain of reovirus type 3 was grown in roller bottle cultures of L cells and

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purified in a manner similar to that for poliovirus (23). One purified preparation of each virus was used throughout this work; the poliovirus stock had a particle count of $10^{12}$ particles per ml, and the reovirus stock had a particle count of $5 \times 10^{11}$ particles per ml. Both preparations were suspended in 0.05 M phosphate buffer, pH 7.3. Particle counts of purified virus stocks were obtained by the use of the kinetic attachment method (22).

**Mixed aggregation tests.** The basic test for the examination of viral aggregation in mixed suspension was the following. Both viruses were diluted 20-fold by simultaneously placing 0.05 ml of each virus stock suspension in 0.9 ml of the particular buffer being used in any given experiment, and the mixture was allowed to remain at room temperature (22°C) for 1 h. All buffer concentrations were 0.1 M. Control tubes of 0.05 ml of each virus alone in 0.95 ml of the same buffer were prepared and also allowed to remain at room temperature for 1 h. One drop of each suspension was then placed on an aluminum-coated collodion-covered electron microscope grid in the kinetic attachment apparatus (22). All suspensions were allowed contact with the grid for 30 min further, and the grids were thoroughly washed, dried, shadowed with chromium, and examined as previously described (22). The two separate control tubes insured that the viruses aggregated or remained dispersed according to previous studies in the nonmixed state. Variations on this basic protocol are given in Results.

**Buffers.** The composition and pH of buffers used in the mixed aggregation tests were the following: at pH 7.4, tris(hydroxymethyl)aminomethane (Tris)-buffered saline containing: NaCl, 0.14 M; KCl, 0.003 M; and Tris-hydrochloride, 0.01 M; at pH 6.0, 0.1 M potassium phosphate ($KH_2PO_4$-NaOH); at pH 5.0, 0.1 M acetate ($CH_3COOH$-NaOH); at pH 3.0, 0.1 M glycine ($NH_2CH_2COOH$-HCl), with or without 0.5 M $MgCl_2$; and Tris-citrate buffer, prepared at pH 3.0 from 0.1 M Tris base and 0.04 M citric acid as previously described (10). When dilutions were made into distilled water, the pH of the final mixture was 7.1 to 7.2, which resulted from the buffering activity of the residual phosphate buffer carried over from the virus samples.

## RESULTS

The examination of the aggregation of two viruses when mixed in the same environment was carried out by diluting each virus 1:20 into suitable buffers at low pH. The buffers were 0.1 M to guard against any alteration in pH of the final mixture due to the residual buffers in the virus stock suspensions. For all experiments, two control tubes, one for each virus, were also prepared with the same buffer as for the mixed suspension and at the same particle count.

The results of several experiments are shown in Fig. 1. At pH 7.4 in Tris-buffered saline, both viruses remained dispersed in the control tubes, as has been reported for poliovirus and is the most usual case for older preparations of reovirus (7). When mixed (Fig. 1A), the two viruses showed no tendency to aggregate either together or separately and in fact formed a uniform distribution over the electron microscope grid. Occasional aggregates of reovirus consisting of groups of two and three particles are present in Fig. 1A, but these are characteristic of preparations of this virus and appear in all micrographs at pH 7 (7). At the concentration used here, poliovirus aggregates of more than two particles were extremely rare.

When both viruses were diluted 20-fold into distilled water, the control tubes yielded an aggregated preparation of poliovirus, whereas reovirus remained in the dispersed state. These results are also typical of poliovirus and older (>2 weeks) suspensions of reovirus under conditions of low ionic strength at pH 7 (7). In the tube of mixed virus (Fig. 1B), the poliovirus aggregates and reovirus particles were present well distributed over the surface of the electron microscope grid, but no evidence of mixed aggregation could be found. Although there are several reovirus particles attached to the large poliovirus aggregate shown in Fig. 1B, there appears to be no specificity in the attachment. In view of the uniform distribution of the reovirus particles, a certain amount of attachment of reovirus to poliovirus aggregates would be expected by coincidence alone.

In phosphate buffer at pH 6.0, the control tubes showed aggregation of poliovirus but not of reovirus (7, 10). This yielded the same situation as in distilled water above. However, when these two viruses were mixed (Fig. 1C), there was a marked tendency for reovirus particles to attach to the aggregates of poliovirus. In some cases, the reovirus particles almost completely obscured the poliovirus aggregates, but generally poliovirions could be seen within the aggregates. In addition, there was an almost complete absence of poliovirus particles from the background around the aggregates. Thus, the particles of poliovirus appear to have aggregated and formed clumps of a size large enough that the reovirus particles could attach to them. There was, however, no tendency for small mixed aggregates of two or more particles to form. Apparently a certain minimum size of poliovirus aggregate was essential before reovirions would attach.

In acetate buffer at pH 5.0, the control tubes showed severe aggregation of both viruses (7, 8, 10). When the two viruses were mixed (Fig. 1D), large aggregates consisting of both particles were formed. There was also a virtual absence of single particles outside the massive aggregates. Under these conditions, when both viruses were diluted 1:20 into pH 5.0 buffer, the aggregation was so extensive that the large reovirus particles

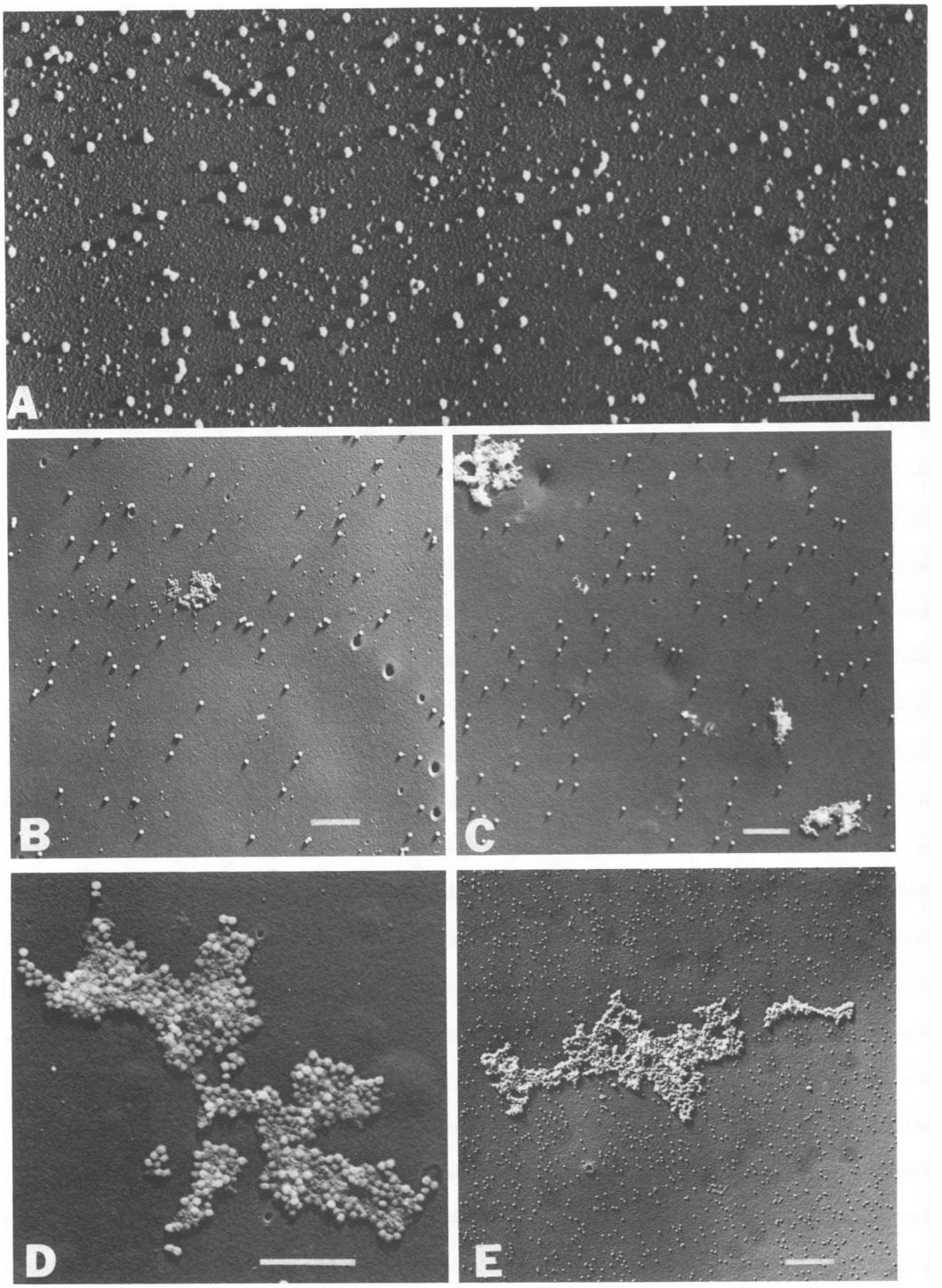


FIG. 1. *Aggregation of mixed suspensions of poliovirus and reovirus. Each virus was diluted 1:20 into (A) 0.1 M phosphate buffer, pH 7.2; (B) distilled water; (C) 0.1 M phosphate buffer, pH 6.0; (D) 0.1 M acetate buffer, pH 5.0 [the reovirus dilution in (D) is 1:100 rather than 1:20]; and (E) 0.1 M glycine-hydrochloride, pH 3.0, containing 0.5 M $MgCl_2$. In each figure the bar represents 800 nm.*

completely obscured the smaller poliovirus particles, and demonstration of mixed aggregation was difficult. To overcome this problem, an experiment was performed in which the reovirus stock (at $5 \times 10^{11}$ particles per ml) was added to a 1:20 dilution of poliovirus such that the final dilutions of reovirus were 1:40, 1:100, and 1:200, In all cases, massive aggregation was obtained, but at a reovirus dilution of 1:100 (final reovirus count, $5 \times 10^9$ particles per ml) the relative number of reovirus particles was low enough to allow observation of the poliovirus particles within the mixed aggregates. It is this dilution that is shown in Fig. 1D.

At pH 3.0 and 4.0 in glycine-hydrochloride and acetate buffers, respectively, large mixed aggregates of the two viruses were formed, as at pH 5.0 above.

The preceding results indicated that under conditions of lowered pH, particles of reovirus will tend to adhere to aggregates of poliovirus, even under circumstances where reovirus aggregation is not taking place. It was of interest, therefore, to examine the reverse possibility, that of poliovirus attachment to aggregates of reovirus. To examine this question, both viruses were diluted 1:20 into 0.1 M glycine-hydrochloride buffer at pH 3.0 containing 0.5 M $MgCl_2$. Under these conditions, the control tubes showed that reovirus was severely aggregated, but poliovirus was well dispersed. This is consistent with previous results (7, 8). A micrograph of typical results from the tubes of mixed viruses is shown in Fig. 1E. There was marked aggregation of the reovirus particles and a uniform background of dispersed poliovirus particles. There was no evidence of any specific attachment of poliovirus particles to the aggregates of reovirus.

The demonstration of the formation of mixed aggregates of poliovirus and reovirus at low pH raises the question of the stability of such aggregates, that is, the ability of the aggregates to maintain integrity under changing conditions of pH and ionic strength. To examine this question, mixed aggregates of poliovirus and reovirus were formed at pH 3 in duplicate tubes in a Tris-citrate buffer (10) in a final volume of 1 ml. As above, the poliovirus particle count was $5 \times 10^{10}$/ml (a 1:20 dilution), and the reovirus count was $10^{10}$/ml (a 1:50 dilution was used in this experiment). Duplicate control tubes of each virus alone were also established. After 2 h at room temperature, one test and one of each of the control tubes were neutralized by the addition of 0.41 ml of a a stock of 0.2 M Tris-hydrochloride, pH 10.2. The other test and control tubes were allowed to remain at pH 3. All suspensions were then examined by the kinetic attachment method (22) for aggregation.

The three control tubes allowed to remain at pH 3 showed severe aggregation of each virus individually, as well as the formation of massive mixed aggregates similar to those shown in Fig. 1D. However, when the suspensions were neutralized by the addition of Tris-hydrochloride, all of the mixed aggregates were dispersed, leaving suspensions of almost all single particles, with occasional clumps of two and larger size aggregates. Figure 2 shows the state of aggregation of mixed aggregates after neutralization. No evidence was found of any remaining mixed aggregates, regardless of size. To obtain more quantitative data on the dispersion of aggregates

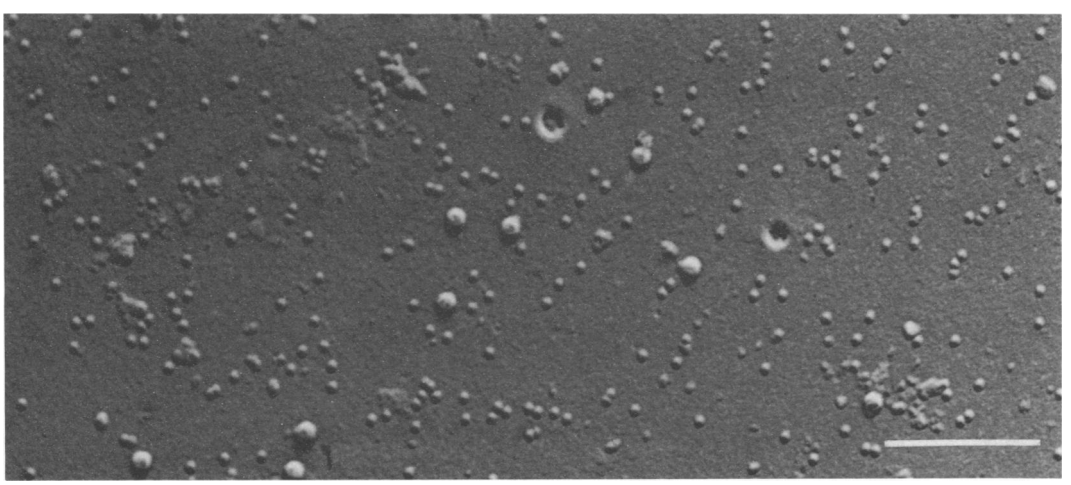

FIG. 2. *Dispersion of mixed aggregates of poliovirus and reovirus. The two viruses were aggregated at pH 3 in Tris-citrate buffer and then neutralized by the addition of further Tris base. The bar represents 800 nm.*

after neutralization, both entire negatives of which Fig. 1A and 2 are a part were printed, and complete aggregation analyses of poliovirus particles were carried out; these are shown in Table 1. Before any low-pH treatment, the single particles in the stock poliovirus suspension accounted for almost 95% of the total. The rest was virtually all accounted for by doubles. After low-pH aggregation and neutralization, the percentage of single particles had fallen to 78%, and the proportion of particles within doubles had risen to 17%. The proportion of particles within triples was also markedly enhanced, from 0.16 to 3.56%. A few aggregates of larger sizes were also found, which were not seen before aggregation. Thus, complete dispersion was not achieved. As a means of comparison, a similar analysis of the aggregation of the complete negative of reovirus before low-pH treatment is also given in Table 1 and demonstrates that reovirus was not completely dispersed initially. This is consistent with previous results (7).

## DISCUSSION

The experimental results in this paper have been presented to attempt to answer the question of whether mixed aggregates of virus particles can possibly occur in freshwater supplies which may be used for human consumption, in wastewater, or in the concentrates of water or wastewater which are prepared for laboratory examination. The data demonstrate that mixed aggregates can be induced to form under certain special laboratory conditions; however, whether such mixed aggregates are in fact important in drinking water supplies or in wastewater cannot be answered by the data presented here for at least two reasons. First, one virus used in these experiments, reovirus, is not frequently isolated from samples of water or wastewater, although the proper conditions for its isolation probably have not been fully explored. Reovirus was used here because of its size difference as compared to poliovirus (72-nm diameter for reovirus, 24 nm for poliovirus). This allowed for ease of identification of the two viruses in the electron microscope. Probably more meaningful results would have been obtained if two enteroviruses had been used; however, no means presently exist for the distinction of two viruses of similar size and morphology by electron microscopy, with the possible exception of immunoelectron microscopy or ferritin-labeling. However, neither of these methods possesses the accuracy which is available with the use of two viruses of unequal size. Other more sophisticated means, such as radiolabeling combined with density gradient centrifugation, might possibly yield significant results, but since it has been established that simply diluting an aggregated preparation of virus can change the amount of aggregation (9), considerable care must be exercised in developing a method to demonstrate aggregation of virus particles in mixed suspensions.

TABLE 1. *Comparison of state of aggregation of poliovirus and reovirus at pH 7 and after mixed aggregation and neutralization*

| Size of group containing given no. of particles | Poliovirus | | | | Reovirus (pH 7) | |
|---|---|---|---|---|---|---|
| | pH 7 | | After aggregation and neutralization | | | |
| | No. counted | % of total[a] | No. counted | % of total[a] | No. counted | % of total[a] |
| 1 | 1,724 | 94.46 | 921 | 78.05 | 665 | 70.00 |
| 2 | 47 | 5.15 | 101 | 17.12 | 110 | 23.16 |
| 3 | 1 | 0.16 | 14 | 3.56 | 17 | 5.37 |
| 4 | 1 | 0.22 | 1 | 0.34 | 2 | 0.84 |
| 5 | —[b] | | 1 | 0.42 | — | |
| 6 | — | | 1 | 0.51 | 1 | 0.63 |
| >6 | — | | 4 | | 1 | |

[a] Percent of total refers to the percentage of all particles within aggregates of the total number of particles.
[b] —, This size aggregate was not observed.

A second reason we cannot fully answer the question of whether mixed aggregates occur in natural water is the low concentration of virus particles in water. Because viral plaque-forming units are so low in natural water (24), it is impossible at the present time to measure the state of viral aggregation in natural water. Although the concentration of virus particles in wastewater is higher than that in freshwater, the concentrations are still too low to make direct measurements of aggregation meaningful or worthwhile, especially in the presence of other particulate matter and soluble organic material.

Generally, the results presented here tend to suggest that mixed aggregation in water or wastewater is not likely or significant in a natural situation. They suggest, however, that mixed aggregates are of significance in laboratory situations where the concentration of virus particles in a sample of concentrated water or wastewater may approach $10^5$ to $10^6$ particles per ml. This arises from the fact that since the formation of a permanent aggregate of virus particles is dependent upon the collision between two virions, then the overall rate of collisions will be a second order reaction, and hence the rates of collision and aggregation will depend upon the square of the particle concentration. In practical terms, this means that if the virus concentration in a sample of water is increased, say, $10^4$-fold, the rate of collision of these particles will be

increased $10^8$-fold. If the particles are then subjected to aggregating conditions such as low pH or low ionic strength at pH 7, considerable aggregation may occur. This may include some mixed aggregation as well. The results presented in this paper demonstrate that the conditions which promote mixed aggregation are not the same as those that promote aggregation of pure viral suspensions. Poliovirus and reovirus formed mixed aggregates only at low pH; in water at or near pH 7.0, mixed aggregation did not occur. This was true even though poliovirus aggregated by itself. It was necessary to reduce the pH to at least 6.0 to induce reovirus to become attached to the poliovirus aggregates.

The most likely possibility for the formation of mixed aggregates would be during the process of concentrating viruses for the examination of the viral content of water or wastewater under conditions wherein one or more low-pH steps are used. Probably the most frequently used concentration procedure fulfilling the above criterion is the use of filters to adsorb viruses from large volumes of water. With this method, concentrations of up to $10^4$- to $10^5$-fold can be obtained (24). In the usual procedure, filter concentration of virus particles involves two steps. Particles are induced to attach to membrane (25–27) or depth filters (14, 18), either at neutral or low pH (1, 14, 25), in the presence or absence of salts (14, 25, 26). Elution from these filters involves raising the pH to alkaline levels either in the presence or absence of organic material (1, 14, 25), and a second adsorption step is carried out in those cases where organic eluants are not used. This is followed by a second elution at high pH which is quickly neutralized (18, 25). Mixed aggregation could conceivably take place within the acid conditions used to enhance virus adsorption to the filter, especially during the second adsorption step when the particles have already been concentrated to a considerable degree. Although the results presented here show that upon neutralization of a suspension of mixed aggregates of poliovirus and reovirus, all mixed aggregates were dispersed, the same cannot necessarily be said of mixed aggregates of two more closely related viruses. For example, Fig. 2 and Table 1 show that although mixed aggregates of poliovirus and reovirus were dispersed after neutralization, some small poliovirus aggregates remained. The question arises, if a mixed aggregate were composed of, for instance, virulent type I poliovirions and avirulent vaccine strain type I poliovirions, would this aggregate break up completely under similar circumstances? If not, the presence of small mixed aggregates might lead to an incorrect value for either the virulent or vaccine strain of viruses in a sample of water or wastewater. Such a dual infection of cells with a mixed aggregate may be one possible explanation for the isolation of $d^+T^-$ type II poliovirus strains from sewage (13) since infection of a cell with a mixed aggregate could lead to intramolecular recombination (4, 19) within the genomes of the infecting particles and the resulting appearance of an unusual virus strain.

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