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Viral Aggregation: Effects of Salts on the Aggregation of Poliovirus and Reovirus at Low pH

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As a first step toward the understanding of virus particle interactions in water, we have used the modified single particle analysis test to follow the aggregation of poliovirus and reovirus as induced by low pH in suspensions containing varying amounts of dissolved salts. Salts composed of mono-, di-, and trivalent cations and mono- and divalent anions were tested for their ability to reduce or increase the aggregation of these viruses in relation to that obtained by low pH alone. Mono- and divalent cations in concentrations covering those in natural waters were generally found to cause a decrease in aggregation, with the divalent cations having a much greater effectiveness than the monovalent cations. Trivalent ions (Al^{3+}), in micromolar concentrations, were found to cause aggregation over that at low pH alone. Anions, whether monovalent or divalent, had little ability to produce inhibition of viral aggregation, and thus the overall effects were due almost exclusively to the cation. This was true regardless of whether the overall charge on the virus particle was positive or negative, as determined by the relation between the isoelectric point and the pH at which the tests were carried out. Thus, whereas virus particles conform to classical colloid theory in many respects, there are specific exceptions which must be taken into account in the design of any experiment in which viral aggregation is a factor.

In the study of the disinfection of water supplies with antiviral agents such as chlorine, deviations from a strict first-order kinetic plot of inactivation versus time have frequently been ascribed to aggregation of the virus particles (2). Whereas aggregation is undoubtedly a significant but usually unmeasured factor in inactivation kinetics, it has only been recently that quantitative measurements of virus aggregation have been possible with the development of the centrifugal single particle analysis (SPA) test (9) and the virion aggregation test (36). Some studies of the influence or virus particle aggregation on the inactivation kinetics of chlorine have been published (4, 6, 34); however, these are mathematical models of inactivation in the presence of aggregates of virus and do not necessarily reflect the true situation in water and wastewater. Only recently have studies been made demonstrating the effects on single virus particles of antiviral agents suitable for use in water supplies, such as chlorine (R. Floyd, J. D. Johnson, and D. G. Sharp, submitted for publication) and bromine (8, 22, 23). These studies have usually been performed with suspensions of single particles because of the difficulty in obtaining a repeatable state of virus aggregation with which to do serial experiments.

In addition, the true state of aggregation of viruses in natural water has never been accurately measured because of the very low virus concentrations. It seems probable that those plaque-forming units isolated from water or wastewater (1) are due to aggregates of virus rather than to single particles. This possibility is based on the fact that aggregates of viruses are known to be much more resistant to the action of physical and chemical agents found under natural conditions, such as bromine (24), chlorine (Floyd et al., submitted for publication), and ultraviolet light (26). It is also based on the possible genetic interactions, such as multiplicity reactivation, recombination, or complementation, during the infection of a cell with more than one particle of the kinds of viruses found in water and wastewater. For example, multiplicity reactivation has been reported with several viruses (22); thus, the possibility remains that an aggregate of virus particles may be infectious even if all of the particles comprising the aggregate have been "hit" by an agent such as chlorine. Thus, the true state and activity of viruses in water is not known. It appears that the inactivation of viruses in natural waters may proceed by a thermal mechanism (18), but other inactivating agents are also involved in water (18).

Viruses adsorb to particulate matter in water (17, 35), but the extent of this adsorption in natural water is not known with any certainty. Therefore, because of the increased number of viruses appearing in those fresh waters used to provide drinking water (27) and because the concept of the water-recycling plant is a real possibility (16, 20), we feel that it is imperative that a full understanding of the behavior of viruses in water must be developed, especially as it concerns the interaction of viruses with themselves (aggregation) and with other waterborne particles (adsorption). For these reasons, we have undertaken a study of the aggregation of viruses with a view toward determining the mechanism of aggregation and the extrapolation, if possible, of this information to viruses in natural waters. This paper begins the study by examining some of the conditions which affect virus particle aggregation as induced or inhibited by the salt environment and the virus-salt relationship under strictly controlled laboratory conditions.

MATERIALS AND METHODS

Viruses and cell lines. Growth, purification, plaquing, and physical assay of the viruses used are described in previous publications (8, 21, 23, 25). The cell lines used have also been previously described (8, 24).

SPA test. The SPA test for the determination of amount of aggregation in a virus preparation (9) and its modification (10) have been described. Briefly, the modified SPA test is designed to determine the relative number of single virus particles in a suspension. Because each aggregate size has a characteristic sedimentation velocity, with singles being the slowest, then under precisely determined conditions aggregates can be centrifuged away from single particles, which in turn can be recovered and titrated. Throughout this paper, the term SPA test will refer to the modified test (10) only.

Isoelectrophoresis. This isoelectric points of poliovirus and reovirus were determined in an LKB 8101 Ampholine column of 110 ml with 1% ampholines. The sucrose gradient used as a stabilizer was 5 to 40%. The virus sample, 0.5×10^5 to 1.5×10^5 plaque-forming units, was added to the 5% sucrose-ampholine solution in the gradient maker before forming the gradient; this placed the virus throughout the entire ampholine column before establishing the electric field. The starting voltage and current were approximately 620 V and 11 mA, respectively, for a pH gradient of 3.5 to 10 and 670 V and 5.5 mA for a pH gradient of 7 to 9. As the current dropped over a period of 2.5 to 4 h, adjustments in the voltage were made at 15- to 30-min intervals so as to keep the total milliwattage (volts × milliamperes) constant. When the power supply (LKB type 3371C) reached maximum setting (ca. 1,200 V), the apparatus was allowed to remain at that level for 16 to 18 h more, during which time the milliamperage dropped to ~2.2 mA (2,490 mW). After shutdown, 2-ml fractions were collected from the bottom of the column by pumping water into the top of the column in a closed system to allow accurate control of the rate of fractionation. The pH gradient was read using a Corning model 109 digital pH meter, and a portion of each fraction was diluted 10-fold in phosphate-buffered saline (0.14 M NaCl–0.003 M KCl–0.01 M Na_2HPO_4-KH_2PO_4 [pH 7.4]–1.0 mM $CaCl_2$–0.5 mM $MgCl_2$–0.1% glucose) and titrated on cell monolayers in 1-ounce (0.03-liter) prescription bottles. The recovery of plaque-forming units was in the range of 75 to 100%. Both viruses were plaque purified before isoelectrophoresis.

Salts and buffers. All stock salt solutions were made in distilled, deionized water with A.C.S. reagent grade salts. Buffers were made with A.C.S. primary standard or enzyme grade salts insofar as possible. All solutions were filtered through membrane filters (Millipore GS; 0.22-μm average porosity) to sterilize and to remove debris to which virus particles might attach. The buffer used at pH 5 was 0.05 M acetic acid-NaOH, and that used at pH 3 was 0.05 M glycine-hydrochloride.

RESULTS

The basic underlying mechanism which governs the aggregation of virus particles and their adsorption to other particulate matter involves the nature of (i) the soluble ionic groups with the virus in suspension (such as Na^+, Cl^-, etc.), (ii) the charged groups on the surface of the virus particle (the isoelectric point of the virus is the single most important overall reflection of these groups), and (iii) the resulting ionic double layer (28, 29), which is a result of the interaction of the first two. The ionic double layer is quite markedly affected by the pH, ionic composition of the medium, and isoelectric point of the virus. Therefore, an examination of the effects of ionic species such as Na^+, Mg^{2+}, Cl^-, Al^{3+}, as well as others, on the aggregation of virus particles induced by low pH should provide some understanding of the nature of virus aggregation and adsorption.

Isoelectric point determination. To examine the effects of salts on the aggregation of viruses, it was essential to know the isoelectric point of each virus. Accordingly, the isoelectric points were determined in an LKB isoelectrophoretic apparatus as described above, and the results are shown in Fig. 1 and 2. In a pH gradient of 3.5 to 10, Mahoney poliovirus infectivity (Fig. 1A) was focused at a pH of 8.2 ± 0.1. There was also a small shoulder at the base of the main peak at a pH of 7.7, and it was thought that this shoulder might represent a second peak of infectivity similar to that reported by Mandel (14, 15) for poliovirus. However, when the same preparation of virus was subjected to electrophoresis on a pH gradient of 7 to 9 (Fig. 1B),

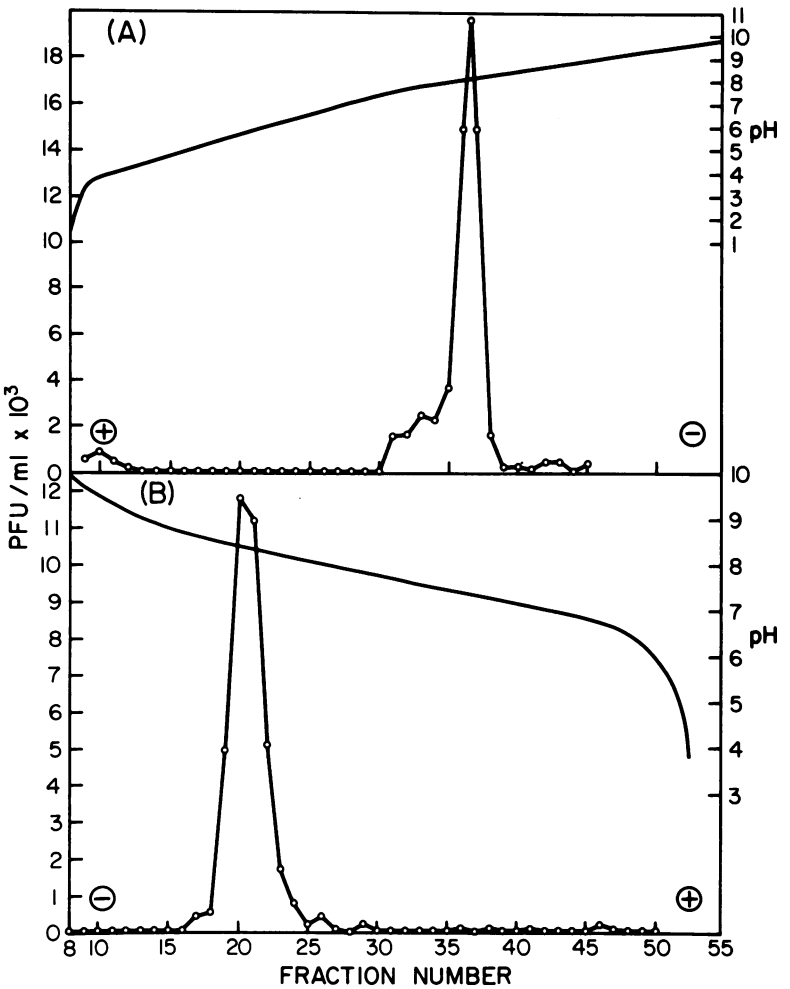

FIG. 1. *Isoelectric focusing of poliovirus I, Mahoney strain. (A) pH gradient of 3.5 to 10, 1% ampholines; (B) pH gradient of 7 to 9, 1% ampholines. Symbols: O——O, infectivity; ——, pH profile. Due to the nature of the ampholines and the pH gradients, the polarity of the gradient in (B) is reversed with respect to (A). The bottom of the column is at the left, and the top is at the right in both.*

only one main peak at pH 8.3 ± 0.1 was found, indicating the artificial nature of the lower shoulder found in the pH gradient of 3.5 to 10. This isoelectric point for the Mahoney strain is considerably different from the A forms at or near pH 7 as reported by Mandel (14, 15), although it is similar to the A form of a large (L) plaque varient of Mengo virus (5).

On a gradient of pH 3.5 to 10, the isoelectric point of reovirus III, Dearing strain, was found to be pH 3.9 ± 0.1, and no other peaks of infectivity were found (Fig. 2).

Effects of salts on aggregation of poliovirus and reovirus. The examination of the effects of salts on the aggregation of poliovirus and reovirus was performed in conjunction with the isoelectric focusing data. Because the isoelectric point of reovirus was at pH 3.9, these particles would possess a net positive charge at pH values below this level, and a net negative charge at pH values above it. Furthermore, in buffers at pH 3 and 5, both reovirus and poliovirus aggregate markedly (9). It was possible, therefore, to study the effects of salts on the surface of the reovirion under two different overall charge configurations and to compare these effects with those on poliovirus, which is very strongly positively charged at both of these low pH values. For these reasons, the pH values of 5 and 3 were chosen for the work on salts and aggregation of both viruses. Subsequent work on the aggregation of poliovirus and reovirus at

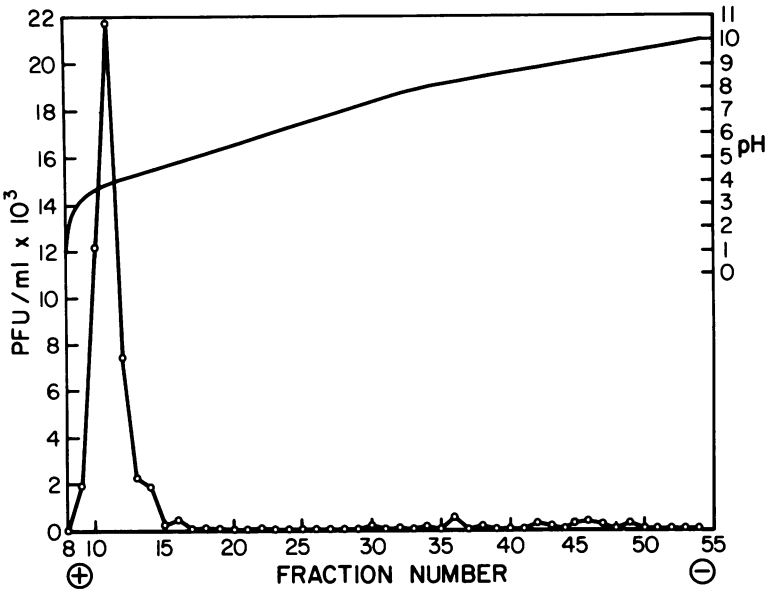

FIG. 2. *Isoelectric focusing of reovirus III, Dearing strain. pH gradient of 3.5 to 10, 1% ampholines. Bottom of the column is at the left; top is at the right. Symbols: O——O, infectivity; ——, pH profile.*

high pH values (>7) will be reported later.

The examination of effects of salts on aggregation of each virus utilized the following SPA test: six cellulose nitrate tubes (0.5 by 2 inches [1.27 by 5.08 cm]) were filled with 5 ml of the buffer under study, five of the tubes containing increasing amounts of salt and the sixth tube with low pH buffer only. At zero time the virus was added in microliter amounts to a final concentration of 0.5×10^9 to 1.0×10^9 particles per ml, and all tubes were covered and incubated at room temperature (~24°C) for 4 h. All tubes were then placed in the SW50.1 rotor and centrifuged as previously described (9). The top 2.3 ml was carefully removed and neutralized to ~pH 7.2 by using a phenol red end point with 0.2 M NaOH, pH 11.7. This neutralization served two purposes: (i) because it was usually necessary to plate out the undiluted material to obtain countable plaques, a pH at or near 7.2 was essential, and (ii) the addition of the glycine at pH 11.7 allowed for a check by back-titration of the pH of those tubes containing large concentrations of salts. In all cases, the pH in the salt-containing tubes was identical to that in the pH control tube.

After the neutralization, the suspensions were plaque titrated, and the final titers were recorded in $\log_{10}$ relation to the control tube without salts at low pH, used as the zero reference value. With this method of plotting the data, positive values represent an increase in single particles and, hence, a decrease in aggregation; conversely, negative values represent a decrease in single particles in the top 2.3 ml after centrifugation. In the absence of salt, the aggregation of both viruses at pH 5 was −2.0 to −2.5 $\log_{10}$, whereas that in pH 3 buffer was −2.5 to −3.0 $\log_{10}$. This data has not been entered on the figures because it varied somewhat from test to test, but it does indicate the average amount of aggregation obtained at low pH alone.

Figure 3 shows the effects of $MgCl_2$ on the aggregation of poliovirus and reovirus at pH 5 in acetate buffer and pH 3 in glycine buffer. At pH 5 (Fig. 3A) there was an initial enhancement of aggregation of poliovirus at a low concentration of 0.02 M $MgCl_2$, but as the salt concentration was increased, aggregation was markedly inhibited to values as high as 1.0 M $MgCl_2$. Reovirus, on the other hand, showed no initial enhancement even at $MgCl_2$ concentrations of 0.005 M, but as the concentration of $MgCl_2$ was increased, aggregation was gradually inhibited. Although a smooth curve has been drawn through the points to approximate the best fit, it is important to note the rather wide distribution of the points used as the basis for drawing a curve. This variance results from a slight difference in the efficiency of aggregation from experiment to experiment. Within each experiment, however, variation is minimal. The net result is that the importance of each of the curves presented in this manner lies in the general shape of the curve, not in the absolute values of each of the points.

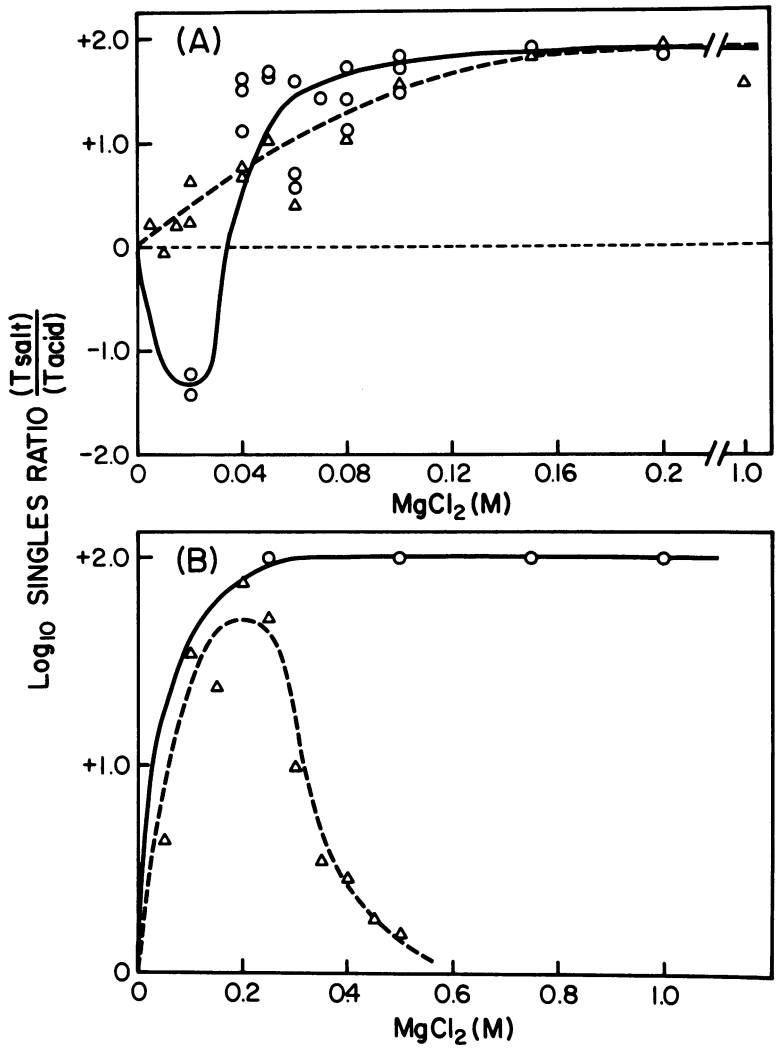

FIG. 3. *Aggregation profiles of poliovirus and reovirus in the presence of $MgCl_2$. (A) pH 5. (B) pH 3. Symbols: O——O, poliovirus; Δ- - -Δ, reovirus; -----, level of aggregation in the absence of $MgCl_2$.*

In Fig. 3B, the aggregation of poliovirus at pH 3 in $MgCl_2$ has been taken from electron microscope data. This method is less quantitative, but it is possible to demonstrate that <1% of the particles is in aggregates, hence this has been plotted as +2.0 $\log_{10}$. Reovirus showed the same inhibition of aggregation at low $MgCl_2$ concentrations as did poliovirus, but reaggregated when the $MgCl_2$ concentration was raised above 0.4 M $MgCl_2$. No further inhibition was found at higher concentrations of $MgCl_2$. All reovirus data was generated by the SPA test.

The effects of $CaCl_2$ on virus aggregation at low pH are shown in Fig. 4. The effects on poliovirus (Fig. 4A and B) were similar to $MgCl_2$, except that the initial enhancement of aggregation at pH 5 in 0.02 M $CaCl_2$ was not as marked as with $MgCl_2$. Reovirus at pH 5 (Fig. 4A) showed an enhancement of aggregation in the presence of 0.02 to 0.04 M $CaCl_2$ not shown with $MgCl_2$, but at higher concentrations aggregation was inhibited markedly. At pH 3, (Fig. 4B) reovirus showed the same peak of inhibition at 0.2 to 0.25 M $CaCl_2$ as with $MgCl_2$, and rapidly reaggregated at higher $CaCl_2$ concentrations.

Figure 5 shows the effects of $AlCl_3$ on the aggregation of poliovirus and reovirus. Aggregation in the presence of the trivalent cation Al^{3+} suffers even more from the wide variation in values between experiments than those with divalent cations, so it has been necessary in some cases to use average values rather than individ-

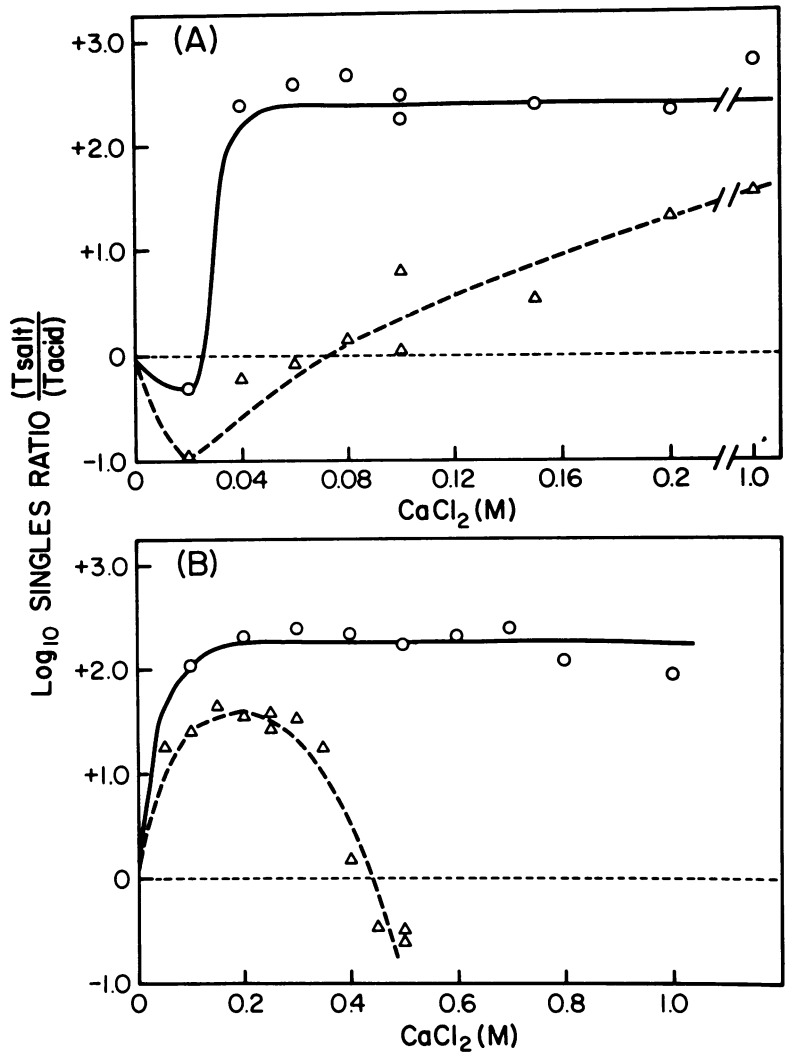

FIG. 4. *Aggregation profiles of poliovirus and reovirus in the presence of $CaCl_2$. (A) pH 5. (B) pH 3. Symbols: O——O, poliovirus; △- - -△, reovirus; -----, level of aggregation in the absence of $CaCl_2$.*

ual experiments in the plotting of $AlCl_3$ data; this is especially true at pH 3. Generally, aggregation in the presence of $AlCl_3$ was greater than in the absence of salt, although reovirus aggregation seemed to be slightly inhibited at pH 3 Poliovirus consistently showed a marked increase in aggregation at very low levels of $AlCl_3$, between 0.001 to 0.01 mM; thereafter, aggregation was generally about −0.35 to −0.75 $\log_{10}$. Reovirus aggregation was extreme at pH 5 in the higher concentrations tested (>0.05 mM); this was probably due to attachment of particles to the insoluble $(AlOH)_x^{2+}$ floc formed at this pH. Such a floc is very much reduced at pH 3, where Al^{3+} ions are considerably more soluble, and the effects on the reovirion produced an inhibition of aggregation.

Cation effects on the aggregation of both viruses. The effects of specific salts as demonstrated in the previous section strongly suggested that the positively charged cation played a more important role in disruption or enhancement of aggregation than did the negatively charged anion. This was suggested primarily by the much greater effects of the aluminum ion Al^{3+} than the divalent Mg^{2+} or Ca^{2+}. Furthermore, monovalent ions as Na^+ in the form of NaCl required concentrations greater than 1 M to prevent aggregation at pH 3 with both poliovirus and reovirus, but lower concentrations of 0.2 to 0.6 M to prevent aggregation of these viruses at pH 5 (9). To examine this effect fur-

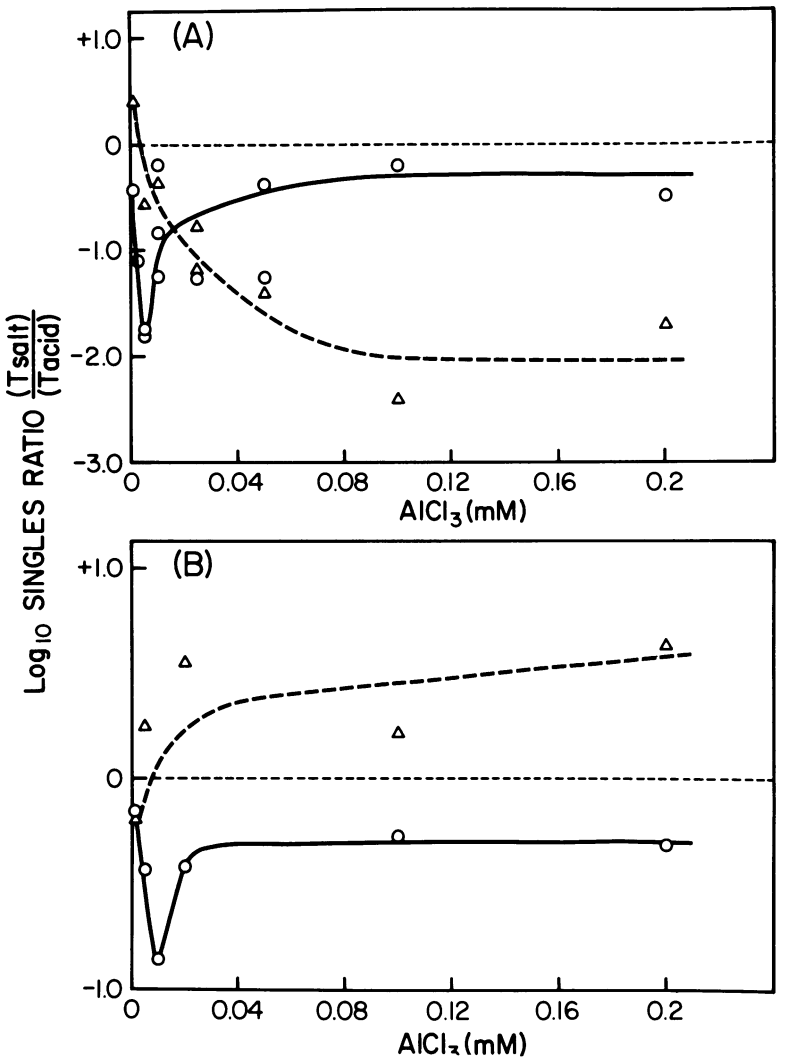

FIG. 5. *Aggregation profiles of poliovirus and reovirus in the presence of $AlCl_3$. (A) pH 5. (B) pH 3. Symbols: ○——○, poliovirus; △- - -△, reovirus; ·····, level of aggregation in the absence of $AlCl_3$.*

ther, several experiments were performed on reovirus at pH 5 and 3 and poliovirus at pH 3 with monovalent and divalent cations and anions. The results of this study are shown in Fig. 6 and 7. At pH 5 (Fig. 6A), the reovirion carried a net negative charge, and inhibition of aggregation was obtained by significantly lower concentrations of the divalent Mg^{2+} ion than the monovalent Na^+ ion, regardless of whether the cations were in the sulfate or chloride form. At pH 3 reovirus carried a net positive charge, but Fig. 6B shows that at this pH inhibition of aggregation was again more sensitive to the divalent Mg^{2+} than to the divalent SO_4^{2-}. Magnesium sulfate produced the typical paraboloid curve of inhibition-aggregation at pH 3 (compared with $MgCl_2$ in Fig. 3B and $CaCl_2$ in Fig. 4B), whereas Na_2SO_4 at the same concentrations caused a slight but measurable increase in aggregation, about 0.3 $\log_{10}$, and had no effect on disruption of aggregates even when carried out to 0.5 M. Sodium chloride produced a slight increase in single particles at pH 3, but the effects fell off after 0.5 M, and no further effect was noted when the NaCl concentration was increased to 1.0 M.

Figure 7 shows similar results with poliovirus at pH 3. At this pH, poliovirus is strongly positively charged, yet the Mg^{2+} cation, either in the form of $MgSO_4$ (Fig. 7) or $MgCl_2$ (Fig. 3) was

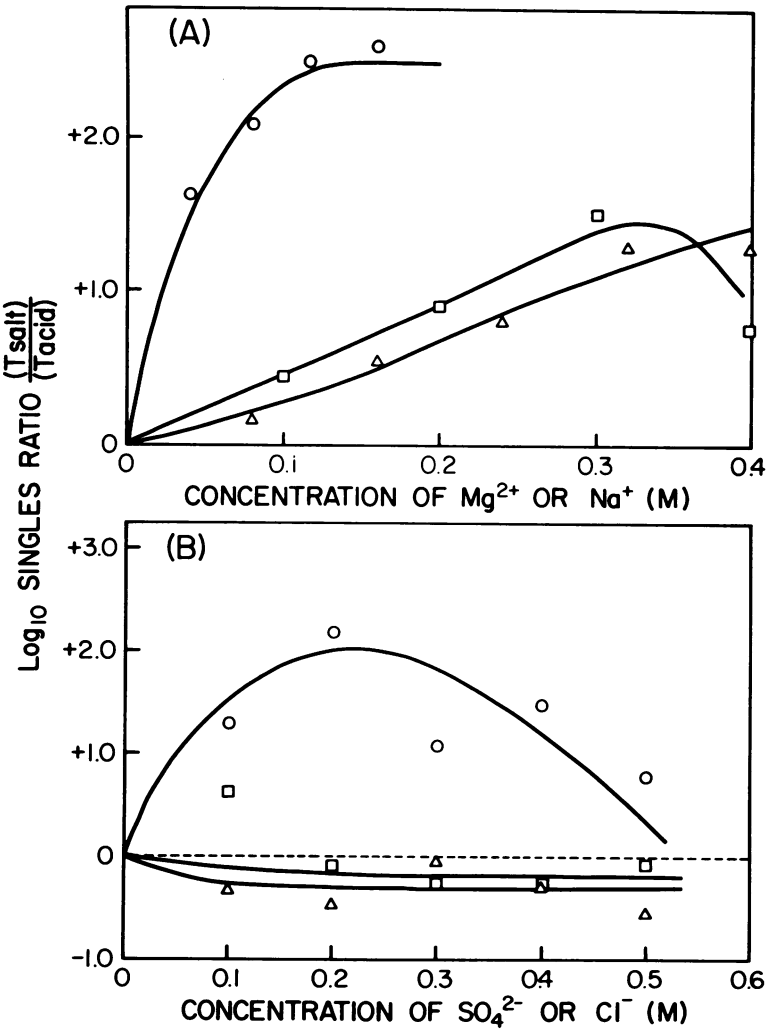

FIG. 6. *Comparison of the effects of monovalent and divalent cations and anions on the aggregation of reovirus at low pH. (A) pH 5. (B) pH 3. Symbols: ○, $MgSO_4$; △, Na_2SO_4; □, NaCl.*

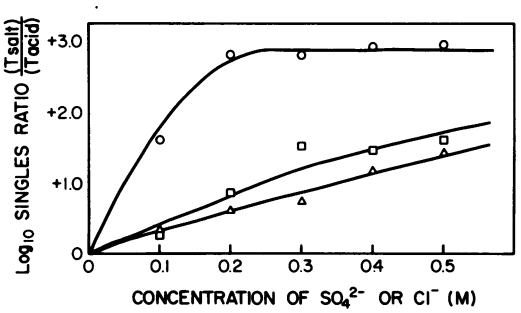

FIG. 7. *Comparison of the effects of monovalent and divalent cations and anions on the aggregation of poliovirus at pH 3. Symbols: ○, $MgSO_4$; △, Na_2SO_4; □, NaCl.*

more effective in inhibiting aggregation than was the SO_4^{2-} anion, in the form of Na_2SO_4.

DISCUSSION

The demonstration of aggregation of virus particles under conditions in which the efficiency of aggregation (ϵ^2) (10) is less than 100% has allowed for the examination of the effects of the ionic environment on the aggregation of these particles. Thus, aggregation in the presence of particular ions may occur at a greater or lesser rate than in the presence of the low pH buffer alone. Under these circumstances it has been possible to gain a rather detailed idea of the nature of the ionic double layer surrounding the virus particles (28, 29).

With respect to divalent cations, the overall effect on both poliovirus and reovirus is an inhibition of aggregation at low pH. This effect is due primarily to the attachment of the divalent cations to the electron-rich sites on the surface of the particles. These electron-rich sites appear to include primarily the terminal carboxyl groups of the amino acids glutamic and aspartic (19), but may also include the nitrogen-containing end groups of lysine and arginine, as well as clusters of amino acids, and to a lesser extent may include the hydroxyl groups of threonine and serine.

At pH 5, with both $MgCl_2$ and $CaCl_2$, poliovirus showed an initial increase in aggregation at 0.02 M, but at higher concentrations aggregations was markedly inhibited. Probably the initial aggregating effect was due to either a bridging between particles by the divalent salts or the inward collapse of the ionic double layer due to the neutralizing effect of chloride ion on the positively charged particle. The second effect, that of inhibition of aggregation at divalent cation concentrations above 0.02 M, was undoubtedly due to the attachment of the Mg^{2+} or Ca^{2+} to the surface of the particles, causing them to become even more strongly positive and, hence, causing the ionic double layer to extend further into the solution. Thus, aggregation would be inhibited.

At pH 3, the same inhibition of aggregation was seen, minus the initial aggregation over that in the absence of cations. This inhibition demonstrates that although the virus particles may possess a strong positive charge, there are certain sites on the surface of the particles which do interact with divalently charged cations, at least with Mg^{2+} and Ca^{2+}.

In contrast to poliovirus, reovirus was negatively charged at pH 5. The addition of small amounts of divalent cation would be expected to cause a neutralization of the negative charge and a concomitant increase in aggregation, and this was seen with $CaCl_2$, although not with $MgCl_2$. The reason for the failure to see this initial effect with the Mg^{2+} cation may have been due to the formation of Mg-acetate complexes with the acetate buffer used at this pH. The overall effect was again one of inhibition of aggregation as increasing amounts of divalent cation were added. This was due to the change in overall charge of the virion from negative to positive by the presence of the Mg^{2+} or Ca^{2+} on the virion surface.

At pH 3, reovirus was positively charged. Here, the initial effect was one of inhibition of aggregation as the divalent cation concentration increased to 0.2 M. Greater concentrations caused a marked aggregation, however. The initial effect, as with poliovirus, can most likely be attributed to a positive virus becoming even more positive, but the reason for the second effect, the reaggregation, was probably due to the chloride in the medium causing a compression of the ionic double layer. Why this should happen with reovirus and not with poliovirus is unknown. A reaggregation effect may have been masked with reovirus at pH 5 by formation of soluble complexes of magnesium and calcium acetate. This would have the effect of sequestering Mg^{2+} or Ca^{2+} and preventing these ions from interacting with the viral surface.

To gain some information on the effects of the anions on the virus particles, several experiments as shown in Fig. 6 and 7 were carried out. Because reovirus at pH 3 and poliovirus at both pH 5 and 3 possess an overall positive charge, the effects of the anion were expected to be significant. With poliovirus at pH 3 (Fig. 7), there was a slight difference in the ability of Na_2SO_4 as compared with NaCl to inhibit aggregation. The divalent SO_4^{2-} was expected to cause a greater compression of the ionic double layer than the monovalent Cl^-, and although this was seen the difference is small and probably not significant. There was a marked difference, however, in the ability of $MgSO_4$ to inhibit aggregation (Fig. 7) compared with Na_2SO_4.

With reovirus at pH 3 (Fig. 6B), the same general effects were noted with $MgSO_4$, Na_2SO_4, and NaCl. Again, the ability of the sodium salts, whether SO_4^{2-} or Cl^-, to disrupt aggregation was minimal; the $MgSO_4$ salt was considerably more effective and showed the typical paraboloid aggregation curve similar to that of $MgCl_2$ and $CaCl_2$. The peak of inhibition was at 0.2 M $MgSO_4$ and reached the reference level (as determined in the absence of salt) at ~0.5 M $MgSO_4$. It was expected, however, that $MgSO_4$ would be far more effective in allowing aggregation than $MgCl_2$ (Fig. 3) because the SO_4^{2-} anion should have been more effective in compressing the ionic double layer. The fact that this was not the case, plus the lack of any significant effect of SO_4^{--} over Cl^- in the case of poliovirus (Fig. 7), suggests that the cation, most prominently the divalent cation, plays a far more important role in aggregation phenomena with viruses than might have been thought from the classical colloid chemistry (28, 29).

The data in Fig. 6A with reovirus at pH 5 also showed a much more pronounced effect in inhibition of aggregation by Mg^{2+} (cf. Fig. 3A) over that of Na^+ salts. Under these circumstances, however, reovirus possessed a net negative charge, and thus the data fit the classical

Schulze-Hardy rule due to the more prominent effect of the positively charged ion on the negative virion.

These data suggested to us that poliovirus and reovirus possessed specific sites on their surface for the uptake of divalent cations and that these sites were operative at a wide range of pH values, regardless of the isoelectric point of the virus and regardless of the presence of mono- or divalent anions in solution. The data, in fact, demonstrated that the anion had very little effect on the virus particle, considerably less than expected.

The possibility of divalent cations complexing with poliovirus and reovirus has been reported earlier. Enteroviruses in general have the property of being stabilized to heat by Mg^{2+} or Ca^{2+} at 1 M concentration (30–32). Furthermore, $MgCl_2$ stabilizes poliovirus against inactivation by urea, but labilizes the virus in the presence of guanidine (11, 12). Divalent cations also have differing effects on reovirus; the virus can be activated by heating or inactivated by freezing in the presence of 2 M $MgCl_2$ (33), which suggests specific binding of the Mg^{2+} ion to the particle in such a manner as to induce stability to heat and lability to cold. Wallis et al. (33) also noticed that at pH 4 reovirus could not be activated by heat. This pH value is very close to the isoelectric point of our strain of reovirus (type III, Dearing).

In addition to the above reports on specific effects of divalent cations on viruses, it is generally well known that divalent cations have been found capable of aiding the attachment of poliovirus to host cells (13), and this is further evidence that there are specific receptors for the divalent ions on the particles. This may, in fact, explain the necessity for such cation receptors, allowing the virus to adsorb to host cells under a wide variety of conditions in a natural infection (3).

The effects of the trivalent Al^{3+} ion on poliovirus and reovirus as shown in Fig. 5 are considerably different from those of the divalent cations. Generally, the effect is aggregation. At pH 3, a large part of the aluminum is in the form Al^{3+}, although there are some aluminum hydroxide polymers of $(AlOH)_x^{2+}$. Reovirus at pH 3 (Fig. 5B) seems to be affected by the Al^{3+} form attaching to the surface of the particle, causing an increase in the ionic double layer and consequent inhibition of aggregation. Poliovirus appears to be affected principally by the polymeric form of $(AlOH)_x^{2+}$ species which will tend to cause bridging between two particles but will also tend to cause entrapment within a floc or precipitate of aluminum hydroxide. At pH 5, considerably more of the floc is present over that of the Al^{3+} form, and both poliovirus and reovirus appear either to be trapped in this solid form or to be aggregated by the polymeric form of the hydroxide. The concentrations of aluminum ion required to obtain these effects are on the order of 10^{-3}-fold of those of divalent cations used in this study. This is due partly to the insoluble nature of the aluminum salts at pH values above ~pH 2, and partly to the trivalent nature of the ion, which, according to the Schulze-Hardy rule, should have an effect at a much lower concentration than a divalent cation.

The general picture of viral aggregation as presented in this paper and the two previous (9, 10) is that whereas viruses conform in most instances to classical laws of colloidal aggregation and interactions, there are specific exceptions to these laws. These exceptions are at least three in number. First, as shown in a previous paper (9), poliovirus and reovirus aggregated when diluted into distilled water, which had the effect of lowering the ionic strength of the solution. This has also been shown for preparations of influenza virus vaccines (7). Colloids in general, however, are stable against aggregation in water and aggregate when salts are added. Second, the effects of the anion on virus particle aggregation has not always been found to obey classical laws of colloidal theory. Whereas the aggregation at low pH is due almost exclusively to the anion of the buffer compressing the ionic double layer, the inhibition of aggregation by salts appears to be due to the influence of the cation regardless of the overall charge on the virus particle. The third specific exception is concerned with the difference between viruses. Only reovirus showed any reaggregation in the presence of high (>0.3 M) concentrations of divalent salts at pH 3. Although this was consistent with general colloid theory based on the activity of the anion against the positively charged particle, it was unusual to find that this effect did not occur with poliovirus. Thus, it must be emphasized, the conditions which induce aggregation of one virus cannot necessarily be used to induce it in another.

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