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Viral Aggregation: Buffer Effects in the Aggregation of Poliovirus and Reovirus at Low and High pH

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The effects of the buffer employed in maintaining a given pH value were tested on the aggregation of two viruses, poliovirus and reovirus. Poliovirus was found to aggregate at pH values of 6 and below, but not at pH 7 or above, except in borate buffer. Reovirus aggregated at pH 4 and below, but was found to aggregate only in acetate or tris(hydroxymethyl)aminomethane-citrate buffers at pH 5. Other buffers tested for aggregation of reovirus at pH 5 (succinate, citrate, and phosphate-citrate) induced little aggregation. No significant aggregation was found for reovirus at pH 6 and above. For both viruses, the most effective aggregation was induced by buffers having a substantial monovalently charged anionic component, such as acetate at pH 5 and 6 or citrate at pH 3. Cationic buffers at low pH, such as glycine, were generally weaker in aggregating ability than anionic buffers at the same pH. These results, when correlated with the isoelectric point of the viruses (poliovirus at pH 8.2; reovirus at pH 3.9) indicated that both viruses aggregated strongly when their overall charge was positive, but only under certain circumstances when their overall charge was negative. Although reovirus aggregated massively at its isoelectric point, poliovirus remained dispersed at its isoelectric point. The conclusion can be drawn that those pH and buffer conditions which induce aggregation of one virus do not necessarily induce it in another.

Previous work on the aggregation of viruses (2, 4, 6) has established that the ionic composition of the medium plays a dominant role in determining the state of aggregation of the virus particles. Generally, viruses have been shown to remain dispersed in salt solutions of near physiological strength (0.14 M NaCl), but to aggregate in solutions of lowered ionic strength (2, 4). Viral aggregation also occurs in buffers at low pH (4-6). The addition of salts at low pH can modify the aggregation reaction, and the effects of a particular salt are strongly dependent upon two factors: (i) the cationic component of the salt as opposed to the anionic component, and (ii) the magnitude of the charge on the cationic component (6). Thus, Mg^{2+}, for example, is more effective in inhibiting aggregation of poliovirus at pH 3 in glycine buffer than is SO_4^{2-} or Na^+. These results, together with data from other laboratories (7, 8, 12-15), have suggested the presence of receptors on the surface of virus particles which are specific for divalent cations and which operate independently of the overall virion charge. Attachment of divalent cations to these sites on the viral surface has a marked effect upon the surface charge and ionic double layer of the particles and consequently strongly affects the state of aggregation (6).

These results demonstrate the importance of the ionic composition of the suspending medium in the control of viral aggregation, but they should not be taken to suggest that the anionic component is without effect. In fact, in the aggregation at low pH of a strongly positively charged virus particle, such as poliovirus at pH 3, aggregation is most probably due to compression of the ionic double layer of the particles primarily by the anionic component of the buffer. This paper establishes this mechanism of aggregation for several buffers at low pH and demonstrates that different buffers at similar pH and concentrations can have considerably different effects in viral aggregation.

MATERIALS AND METHODS

Viruses and cell lines. Poliovirus type 1, Mahoney strain, and reovirus type 3, Dearing strain, were grown in roller bottle cultures of HEp-2 and L cells, respectively. Plaquing was carried out in monolayer cultures of these cells as previously described (3, 11). The

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techniques for viral purification by Freon extraction and sucrose density gradient sedimentation in phosphate and tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffers have also been described (3, 11). Preparation of virus stocks for particle count was by the kinetic attachment method (10). The modified single-particle analysis (SPA) test (5) was used to quantitate viral aggregation.

Buffer solutions. The reagents used, the composition of the buffers, and the pH ranges examined were as follows: 0.05 M potassium phosphate (KH_2PO_4) plus NaOH to pH 6 and 7; 0.05 M acetic acid (CH_3COOH) plus NaOH to pH 4 to 6; 0.05 M citric acid ($C_6H_8O_7$) plus NaOH to pH 3 to 6; 0.05 M succinic acid ($C_4H_6O_4$) plus NaOH to pH 3 to 6; 0.05 M glycine (NH_2CH_2COOH) plus HCl to pH 3 and 4, or plus NaOH to pH 9 to 11; 0.05 M boric acid (H_3BO_3) plus NaOH to pH 8 to 10; 0.05 M sodium barbital ($C_8H_{11}N_2O_3Na$) plus HCl to pH 7.5 to 9.5; 0.05 M Tris ($C_4H_{11}O_3N$) plus HCl to pH 7 to 9.

A buffer composed of Tris base and citric acid mixed in varying ratios to preselected pH values of 3 to 9 was prepared as shown in Table 1. The pH of 0.1 M Tris base was 10.4, and that of 0.04 M citric acid was 2.2. Preparation of the buffer in this manner insured that there were no cations of the alkaline and alkaline-earth families (such as Na^+ or Mg^{2+}) and no anions of the halogen family (such as Cl^-).

A second buffer of phosphate and carbonate was also prepared (Table 2) from 0.1 M KH_2PO_4 at a pH of 4.5 and from 0.05 M Na_2CO_3 at a pH of 11.2. This buffer was utilized in aggregation experiments at pH values from 7 to 10.

The phosphate-citrate buffer of McIlvaine (9) was prepared according to *Documenta Geigy* (1).

All buffers were prepared from American Chemical Society primary standard- or enzyme-grade reagents in distilled deionized water. Each stock buffer solution was filtered through a membrane filter (Millipore GS; 0.22-μm pore size) for sterilization and to remove as much debris as possible to which virus particles might attach.

Phosphate-buffered saline (PBS) contained 0.14 M NaCl, 0.003 M KCl, and 0.01 M Na_2HPO_4-KH_2PO_4, pH 7.4. Tris-buffered saline contained NaCl and KCl as in PBS, but 0.01 M Tris-hydrochloride, pH 7.4, was utilized as a buffer in place of phosphate. For plaquing of each virus, PBS was used for serial dilutions, and it contained, in addition, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% glucose.

TABLE 1. *Composition of Tris-citrate buffers*

pH	Amt of 0.1 M Tris base (ml/100 ml)	Amt of 0.04 M citric acid (ml/100 ml)	Amt of Tris base (ml/5 ml)	Amt of citric acid (ml/5 ml)
3	18.0	82.0	0.90	4.10
4	32.5	67.5	1.63	3.37
5	43.0	57.0	2.15	2.85
6	50.5	49.5	2.53	2.47
7	55.0	45.0	2.75	2.25
8	66.0	34.0	3.30	1.70
9	92.0	8.0	4.6	0.4

TABLE 2. *Composition of phosphate-carbonate buffers*

pH	Amt of 0.1 M KH_2PO_4 (ml/100 ml)	Amt of 0.05 M Na_2CO_3 (ml/100 ml)	Amt of KH_2PO_4 (ml/5 ml)	Amt of Na_2CO_3 (ml/5 ml)
6.0	86.75	13.25	4.34	0.66
6.5	70.5	29.5	3.525	1.475
7.0	51.0	49.0	2.55	2.45
7.5	40.5	59.5	2.025	2.975
8.0	36.0	64.0	1.8	3.2
9.0	32.0	68.0	1.6	3.4
9.5	27.0	73.0	1.35	3.65
10.0	20.0	80.0	1.0	4.0
10.5	11.0	89.0	0.55	4.45

RESULTS

Aggregation of poliovirus and reovirus at low pH. In previous work (4–6) we used the following buffers at pH values of ≤ 7 to induce aggregation of virus particles: at pH 7 and 6, 0.05 M phosphate; at pH 5 and 4, 0.05 M acetate; and at pH 3, 0.05 M glycine hydrochloride. The amount of aggregation produced was generally proportional to the hydrogen ion concentration (that is, the lower the pH, the greater the amount of aggregation). To quantitate the aggregation produced by each buffer on both poliovirus and reovirus, 4-h SPA experiments were performed on each virus in each of the buffers listed above. Briefly, the SPA test was conducted as follows. The virus was added to 5 ml of each buffer and to a control of PBS in cellulose nitrate centrifuge tubes (0.5 by 2 inches [1.27 by 5.08 cm]) to a final particle count of 10^9 particles per ml and allowed to remain at room temperature for 4 h. The tubes were then centrifuged at 30,000 rpm (poliovirus) or 15,000 rpm (reovirus) at 20°C for 20 min in a Beckman SW50.1 rotor in a Sorval OTD-2 ultracentrifuge, using the Reograd mode at the termination of the run. The top 2.3 ml of each tube was titrated, and the final titers were converted to $\log_{10}$ form and related to the control tube of PBS as the following ratio: $\log_{10}$ titer at pH < 7 minus $\log_{10}$ titer in PBS (Fig. 1). Relative to the PBS control, the aggregation of the two viruses presented some similarities and some important differences. Poliovirus was quite sensitive to the effects of phosphate buffer at pH 6, whereas reovirus was not (4, 6). Both viruses showed negligible aggregation at pH 7 in phosphate, however. Acetate buffers, whether at pH 5 or 4, caused marked aggregation of both viruses. Although pH 3 usually produced greater aggregation than pH 4, in the case of poliovirus aggregation in acetate buffer at pH 4 was greater than aggregation at pH 3 in glycine.

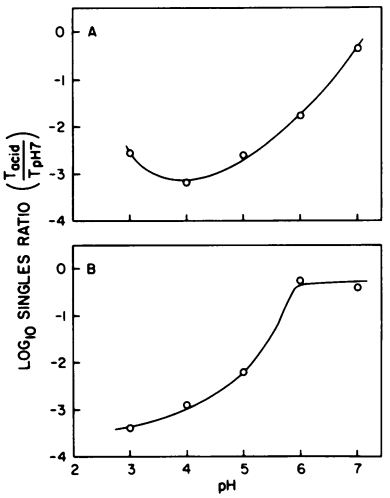

FIG. 1. *Aggregation of poliovirus (A) and reovirus (B) at low pH. The buffers used were 0.05 M phosphate at pH 7 and 6, 0.05 M acetate at pH 5 and 4, and 0.05 M glycine hydrochloride at pH 3. Aggregation was determined by the modified SPA test.*

Aggregation of poliovirus and reovirus in several buffers at pH 6 to 3. This variation in aggregation, which appeared to be dependent to a great extent on buffer type as well as pH, was examined in further detail in a series of SPA experiments with both viruses in several buffers at pH 6, 5, 4, and 3. The SPA tests were performed as described above, and the results are shown in Table 3 as the amount of aggregation relative to the control in either PBS or Tris-buffered saline. (Preliminary tests indicated that both PBS and Tris-buffered saline hold either virus in the essentially monodispersed state; thus, either is suitable as a reference control in SPA tests.)

The amount of aggregation at any given pH was clearly dependent on the buffer used. With poliovirus, citrate buffer at pH 6 (prepared by adjusting 0.05 M citric acid to pH 6 with NaOH) and McIlvaine phosphate-citrate (a mixture of 0.2 M Na_2HPO_4 and 0.1 M citric acid) (9) induced the least aggregation (−0.42 and −0.48 $\log_{10}$, respectively). Acetate buffer, on the other hand, induced −2.64 $\log_{10}$ of aggregation, which was the greatest amount of aggregation induced by any buffer at pH 6. Other buffers were intermediate between citrate and acetate. Acetate buffer, in fact, was a strong inducer of poliovirus aggregation at all pH levels where it was tested. Phosphate buffer at pH 6 containing 2 mM ethylenediaminetetraacetate induced aggregation similar to that produced in phosphate alone, indicating that the chelating activity of such buffers as citrate and succinate probably was not a factor in the induction of aggregation.

The average amount of aggregation at each pH value increased as successively lower pH levels were tested. This increase may be a reflection of the activity of the anion of the buffer in the compression of the ionic double layer of the virus particles leading to aggregation.

Reovirus aggregation at low pH presented results somewhat different from those of poliovirus. At pH 6, reovirus aggregation was minimal, especially with phosphate buffer, with or without ethylenediaminetetraacetate, consistent with earlier results (4). Citrate buffer was also a weak inducer of reovirus aggregation, whereas acetate yielded the greatest (−0.51 $\log_{10}$) amount of aggregation. Acetate, however, was not consistently among the greatest inducers of reovirus aggregation, although it was generally above the

TABLE 3. *Aggregation of poliovirus and reovirus in various buffers at pH 6, 5, 4, and 3, as determined by a 4-h SPA test*[a]

pH	Buffer type[b]	Poliovirus aggregation[c]	Reovirus aggregation[c]
6	Phosphate	−1.73	−0.13
	Acetate	−2.64	−0.51
	Citrate	−0.42	−0.14
	Succinate	−1.86	−0.20
	Phosphate + 2 mM EDTA[d]	−1.64	−0.09
	Phosphate-citrate	−0.48	−0.18
	Tris-citrate	−1.48	−0.42
	Average at pH 6	−1.46	−0.24
5	Acetate	−3.65	−2.74
	Citrate	−2.50	−0.37
	Succinate	−3.59	−0.89
	Phosphate-citrate	−1.36	−0.47
	Tris-citrate	−1.52	−1.15
	Average at pH 5	−2.52	−1.12
4	Acetate	−4.47	−2.53
	Glycine	−1.27	−1.79
	Citrate	−2.40	−3.16
	Succinate	−2.15	−3.48
	Phosphate-citrate	−3.39	−2.93
	Tris-citrate	−2.24	−1.68
	Average at pH 4	−2.65	−2.59
3	Glycine	−2.63	−3.12
	Citrate	−2.85	−3.53
	Succinate	−3.66	−2.52
	Phosphate-citrate	−3.93	−1.47
	Tris-citrate	−3.19	−2.67
	Average at pH 3	−3.25	−2.66

[a] Poliovirus at 1.8×10^9 particles per ml; reovirus at 1.2×10^9 particles per ml.

[b] All buffers were at 0.05 M except phosphate-citrate and Tris-citrate which were mixtures of two components.

[c] $\log_{10}$ titer in acid minus $\log_{10}$ titer in PBS or Tris-buffered saline.

[d] EDTA, Ethylenediaminetetraacetate.

average value. At the lower pH values (pH 4 and 3) buffers such as succinate and citrate, as well as Tris-acetate, were excellent inducers of aggregation. Phosphate-citrate was generally weak at all levels tested.

In contrast to poliovirus, the average values of reovirus aggregation reached a minimum of -2.9 $\log_{10}$ at pH 4 and was not increased at pH 3. This may be a reflection of the fact that the isoelectric point of this strain of reovirus is at pH 3.9, and suggests that reovirus aggregates maximally at or near its isoelectric point.

Aggregation of poliovirus and reovirus in phosphate-citrate and Tris-citrate buffers. The preceding results strongly suggest an effect on the virus of the buffer system at any given pH value, but most buffers are limited in pH range and can buffer effectively only through 2 to 3 pH units. To gain a better understanding of the relationship between the aggregation at any pH value and that at another value, and between different types of buffers, several SPA experiments were performed with phosphate-citrate and Tris-citrate buffers and both poliovirus and reovirus. These two wide-range buffers were used since they were capable of producing buffered pH values from 7 to 3. The results of these experiments are shown in Fig. 2 and 3. Figure 2 shows the aggregation of poliovirus and reovirus in phosphate-citrate buffer. Both curves were sigmoid in character due to the much greater aggregation at pH 4 and 3 than at pH 7 to 5. Poliovirus aggregation at pH 6 was negligible compared with that in phosphate alone

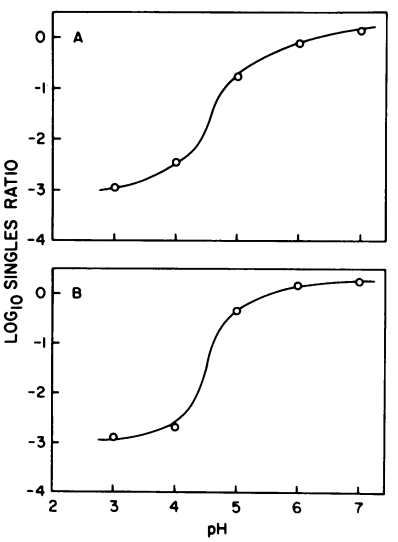

FIG. 2. *Aggregation of poliovirus (A) and reovirus (B) in McIlvaine phosphate-citrate buffer. Aggregation was determined by the modified SPA test.*

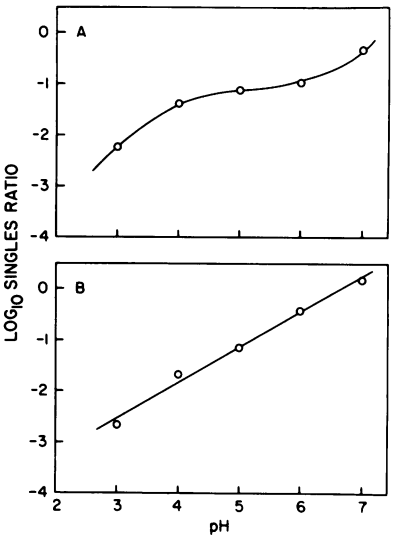

FIG. 3. *Aggregation of poliovirus (A) and reovirus (B) in Tris-citrate buffer prepared as described in the text. Aggregation was determined by the modified SPA test.*

(compare with Fig. 1A and Table 3), as was aggregation at pH 5 compared with that in acetate. The curve for reovirus (Fig. 2B) was similar to that in Fig. 1B with respect to pH 6, but aggregation in phosphate-citrate at pH 5 was much less than in acetate at pH 5.

Aggregation in Tris-citrate buffer (Fig. 3) was somewhat different than that in phosphate-citrate or the more usual buffers (Fig. 1). The curve of poliovirus aggregation in Tris-citrate was also sigmoid in character, but differed from the curve in phosphate-citrate due to the significant amount of aggregation at all pH values less than 7. This is also reflected in Table 3. Reovirus aggregation, on the other hand, demonstrated a linear plot with respect to aggregation versus pH in Tris-citrate. A least-squares analysis of the plot revealed a decrease of 0.7 $\log_{10}$ per pH unit, which resulted in a 5-fold decrease in the number of particles remaining in the top of the centrifuge tube for each 10-fold increase in hydrogen ion concentration.

Aggregation of poliovirus and reovirus in alkaline pH buffers. In contrast to the aggregation in acid buffers, poliovirus and reovirus showed no tendency to aggregate in buffers in the alkaline range, with the exception of prominent aggregation of both viruses in borate buffer. Table 4 shows all of the buffers and pH levels tested for aggregation potential on poliovirus and reovirus. Further tests on borate-induced aggregation of both viruses have revealed a complex form of aggregation which is at variance with the known fundamentals of aggrega-

TABLE 4. *Neutral and alkaline pH buffers tested for aggregation of poliovirus and reovirus by a 4-h SPA test*

Buffer[a]	pH range[b]	Aggregation of:	
		Poliovirus	Reovirus
Boric Acid-NaOH	8–10	Yes	Yes
Barbital-HCl	7.5–9.5	No	No
Glycine-NaOH	9–11	No (to pH 10.5)[c]	No (to pH 10.5)[c]
Tris-hydrochloride	7–9	No	No
Tris-citrate	7–9.5	No	No
Phosphate-carbonate	7–9	No	No

[a] Concentrations were 0.05 M, except for Tris-citrate and phosphate-carbonate.

[b] Tested in 0.5-pH unit steps within the range specified.

[c] Viruses were inactivated at pH 11.0, and aggregation could not be determined.

tion of virus particles as induced by low-pH buffers (5), and will not be treated here. It should be noted, however, that, although borate buffer is widely utilized in the pH range 8 to 10, purified virus preparations may be induced to aggregate severely in borate suspension, whereas unpurified virus of comparable plaque-forming units per milliliter does not aggregate. More complete data on borate-induced aggregation will be presented in a subsequent paper.

DISCUSSION

It is clear from the data presented here that the buffer employed in maintaining a given pH of a virus suspension can have a considerable effect on the amount of aggregation the virus may undergo. Quite obviously, in any experiment on aggregation or where aggregation is an important factor, the nature of the buffer will have to be considered as one factor in the design of the experiment. In most instances, as shown in Fig. 2 and 3 and Table 3, we found that below pH 7 the greater the hydrogen ion concentration, the greater the aggregation that took place. This was not always true, however, especially when different buffers were used to maintain different pH values. Thus, the proper method of studying buffer effects is through the use of one buffer to maintain all pH levels to be tested. This is most easily accomplished by wide-range mixed component buffers, such as phosphate-citrate (9) or the Tris-citrate buffer described here. In the case of these buffers, the same components are present at all pH values tested. Although the concentrations and ionic forms of the components vary (as do the ionic strengths to a slight extent), the importance of these buffers lies in the fact that there is no change in buffer type from one pH value to another. Thus, a more uniform picture of the effects of pH on the viral aggregation is obtained.

In quantitating the amount of aggregation of both viruses, whether by single-component or mixed buffers, it appears that those conditions which cause aggregation of one virus may cause more or less aggregation or none at all in the other. The most conspicuous example is the difference in aggregation at pH 6 (Table 3). Whereas poliovirus was aggregated to an average of -1.46 $\log_{10}$, reovirus yielded an average of only -0.24 $\log_{10}$. Other differences are also apparent in the data. There was a considerable difference in amount of aggregation in citrate, succinate, and phosphate-citrate buffers at pH 5, and furthermore, at lower pH values there were other differences between specific buffers, although they were not as pronounced. In general, the two viruses reacted differently to the aggregating conditions, and this can be most easily seen in the curves of Fig. 1 to 3, which show the aggregation with different buffers. The isoelectric point of Mahoney poliovirus has been found to be at pH 8.2, and that of reovirus, Dearing strain, is at pH 3.9 (6). Since the isoelectric point is a measure of the pH at which the net electrical potential on the virus particle is neutral, it can be considered, at least in a general manner, as an indicator of the relationship between the virus particle and the ions which constitute the buffer. Although the values for the isoelectric points of the viruses were determined in the absence of salts (using amphoteric Ampholines) (6) and they are influenced by the ionic strength and composition of the buffer solution, the values obtained in this way are still a useful guide.

Poliovirus, with an isoelectric point of 8.2, has an overall positive charge on the particles in buffers of acid medium, where the most significant aggregation took place. Under these conditions, it would be expected that the anionic component of the buffer would be very important in aggregation and, further, that the amount of aggregation produced should be in relation to the magnitude of the charge on the anion. Qualitatively this was true for poliovirus; however, the most aggregation was induced by univalent anions rather than multivalent anions. Acetate, at any pH tested, was the greatest producer of poliovirus aggregation, whereas citrate, a tribasic buffer, produced much less at pH 6. Acetate, with a pK at 4.76, is monovalently ionized at pH 5 and 6 and has a substantial nonionized

component at pH 4. Citrate, with pK's at 3.13, 4.76, and 6.4, is 28% trivalently ionized at pH 6 and 72% divalent. However, as lower pH values were tested, the amount of monovalent citrate increased so that at pH 3, citrate was 57.5% monovalent. At pH 3 and 4, citrate was near average in induction of poliovirus aggregation. Other buffers tested (Table 3) appear to fit the same pattern. Phosphate, with pK's of 2.1 and 7.2, contains a substantial monovalent component in the range of pH 3 to 6 and, when used in combination with citrate, produced extensive aggregation.

The amine-containing buffers, Tris (combined with citrate) and glycine, contain the cationic $-NH_3^+$ group, especially at low pH. The Tris molecule has one pK at 8.1 and hence is ionized at all pH levels shown in Table 3. This may tend to partially neutralize the anionic citrate molecule and may explain the substantial aggregation at pH 6, for example. Glycine produced little poliovirus aggregation at both pH 4 and 3, being of the same overall charge as the virus. Aggregation under these circumstances was probably influenced more by the chloride anion (of the HCl used to establish the pH of the buffer).

With respect to reovirus, the isoelectric point at pH 3.9 indicates a difference in the overall sign of the potential on the viral particle within the pH range of 6 to 4, compared with poliovirus. At pH values above 3.9 the reovirion would carry an overall negative potential and at pH 3.0 would be positive. Thus, the effects of buffer anions would be expected to be considerably different from the effects on poliovirus, and this is borne out in the examination of Table 3 and Fig. 1 and 3. At pH 6 (Table 3 and Fig. 1) reovirus remained almost completely dispersed, in contrast to the aggregation of poliovirus. (It should be noted that reovirus preparations are never as fully dispersed as are poliovirus preparations; there is always a small proportion of small aggregates of two and three particles. Any measure of reovirus aggregation is a measure of aggregation over and above these few small clumps normally present [4, 11].) At pH 5, only two buffers, acetate and Tris-citrate, caused significant aggregation of the particles. At pH 4 and 3, all buffers tested produced aggregation, but there were important differences in aggregating ability of each of these buffers. At all pH values tested, phosphate-citrate was weak in inducing reovirus aggregation, as was glycine at pH 4. Acetate, on the other hand, produced aggregation at all pH values tested and was even able to induce -0.5 $\log_{10}$ aggregation at pH 6. Tris-citrate, except at pH 6, was also very effective in reovirus aggregation (Fig. 3 and Table 3). The data suggest that the effects of the negative anion are minimal upon a particle which carries a strong overall negative potential as reovirus does at pH 6; even at pH 5 aggregation occurred only with the buffer containing the cationic $-NH_3^+$ group, in addition to acetate. However, when the particle was placed at pH 4, near its isoelectric point or below it, the effects of the buffer anion on reovirus were similar to those on poliovirus, and severe aggregation occurred.

In the pH region of 7 to 10.5, both poliovirus and reovirus showed no tendency to aggregate, except in borate buffer and then only with purified, not with crude, viruses (see above). In the pH region of 8 to 10.5, both viruses would maintain an overall negative potential, and, as with reovirus at pH 6, the anionic component of the buffer had little effect on the virus particles. Generalizations do not appear readily from these results, but one does stand out clearly; those conditions which induce aggregation of one virus do not necessarily do so in another and different buffers do not duplicate aggregating conditions, even though they may maintain the same pH. These things are particularly significant in the study of inactivation of viruses in water by chlorine.

A water solution of chlorine may, for example, act on virus as HOCl or as OCl^- or a mixture of these, depending on ionic strength and the pH of the range 6 to 10. If the relative potency of these two forms of chlorine for destroying virus in water is ever to be clearly revealed, it must be done under conditions where aggregation of the virions will not be present to influence the reaction rate in any of the experiments involved. Experiments with aggregated virus at pH 6 compared with dispersed virus at pH 10 will certainly not give the relative effectiveness of HOCl versus OCl^-.

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