APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June, 1978, p. 1079-1083 Vol. 35, No. 6
0099-2240/78/0035-1079$02.00/0

Viral Aggregation: Quantitation and Kinetics of the Aggregation of Poliovirus and Reovirus

ROGER FLOYD AND D. G. SHARP*

Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received for publication 22 December 1977

The aggregation of poliovirus and reovirus was followed in buffers at various pH values by means of a single particle analysis (SPA) test. The SPA test used here was modified from the original test reported earlier to prevent disaggregation of virus clumps from invalidating the results. The modified SPA test demonstrated that the efficiency of aggregation, which is a measure of the percentage of collisions which are effective in producing an aggregate, may vary widely depending on the conditions in which the virus is placed. The modified SPA test was also used to demonstrate that the kinetic features of viral aggregation follow the classical laws of colloid particle aggregation, which in turn are solely dependent upon diffusion of the particles as caused by brownian motion.

In a previous paper (3) we reported that the state of aggregation of a virus suspension was strongly dependent on the ionic composition of the suspending medium. Although different viruses gave differing results, generally both reovirus and poliovirus were stable in saline solutions at or near physiological strength, and dilution into water at pH 7 or into buffers at low pH caused aggregation to occur. Aggregation in water at pH 7, however, was found to be reversible by the addition of salts. Furthermore, divalent or monovalent ions incorporated into the buffers at low pH were capable of preventing aggregation. In addition to these data, collected by electron microscopy, kinetic curves of aggregation were generated by the single particle analysis (SPA) test, which utilized the different sedimentation rates of different aggregate sizes in the ultracentrifuge to determine the relative proportion of single particles remaining in a virus suspension. These kinetic curves indicated that virus particle aggregation was similar to, but not identical to, theoretical curves of aggregation as calculated by the equations of von Smoluchowski (9, 10). We have determined that the discrepancy between the calculated and observed kinetic curves lay in the manner in which the test was performed, and we have modified the SPA test accordingly to produce results which now yield a closer fit to the predicted curve of aggregation.

These studies have been undertaken to produce a method which will provide quantitative data on the amount of aggregation in a virus suspension. As such, they are particularly appropriate to viral disinfection studies in which aggregation has been implicated in complications in the results (1). The modified SPA test described here allows such a quantitation, and the nature of the modification and the general characteristics of viral aggregation are described.

MATERIALS AND METHODS

Viruses and cell lines. The Mahoney strain of poliovirus type 1 and the Dearing strain of reovirus type 3 were grown in roller cultures of HEp-2 and L cells, respectively. Purification and plaquing techniques have been described (2, 8), although purified virus stocks were also prepared in sucrose gradients in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.2, as well as phosphate. Physical assays of poliovirus and reovirus by agar pseudoreplication and kinetic attachment methods have been previously described (6, 7). The SPA test has also been described (3). Briefly, an SPA test is an ultracentrifugal method of determining the concentration of single particles remaining in a viral suspension and is based on the difference in sedimentation velocity between single particles and aggregates. Conditions for the centrifugal run are selected such that aggregates are forced into the bottom half of the tube, leaving singles in the top half. The top half is then removed, plaque titrated, and compared to a control of known monodispersed virus.

Buffers. All buffers were made with American Chemical Society primary standard buffer salts or enzyme grade reagents whenever possible. All solutions were prepared in distilled, deionized water, then filtered through membrane filters (Millipore GS; 0.22-μm average porosity) for sterilization and to remove as much debris as possible to which virus particles might attach. Phosphate-buffered saline consisted of: NaCl, 0.14 M; KCl, 0.003 M; Na_2HPO_4-KH_2PO_4, 0.01 M, pH 7.4; $MgCl_2$, 0.5 mM; $CaCl_2$, 1.0 mM; and glucose, 0.1%.

RESULTS

Quantitative aggregation of poliovirus. In our previous publication (3) we showed that the aggregation of poliovirus and reovirus could be quantitated by the use of the SPA test and that the kinetic curve showing the rate of aggregation could be divided into two phases: (i) an initial rapid aggregation phase and (ii) a level phase during which little or no further aggregation took place. This type of kinetic curve was found to be at variance with the theoretical rate curves of aggregation as defined by von Smoluchowski (9, 10) who determined that aggregation should be a continuing process, not marked by a period during which no aggregation takes place. Furthermore, we also found that aggregation of reovirus at pH 3 was characterized by greater aggregation at lower virus concentrations than at higher concentrations. This characteristic is not accounted for by the von Smoluchowski calculations which predict lesser aggregation for lower particle counts. Poliovirus never exhibited this apparent reversal of predicted behavior. However, when experiments were undertaken to examine in detail the first phase of the kinetic aggregation curve, it became apparent that the final amount of aggregation was relatively constant from experiment to experiment regardless of the length of time between samples. For example, with 7×10^9 poliovirus particles per ml at pH 3, the titers of the aggregated supernatants after centrifugation were about 0.4 $\log_{10}$ below that of the control at pH 7, regardless of whether samples were taken at 1-min intervals or 4-min intervals. We considered the most probable explanation for such behavior to be disaggregation of the clumps formed at low pH. This could most likely happen when the reaction sample, 0.5 ml in a 5-ml centrifuge tube, was diluted to 5 ml immediately before the centrifugation. Therefore, it was necessary to devise a test which would examine this possibility without a dilution step. The test finally used was conducted as follows. (i) A 5-ml amount of 0.05 M glycine-hydrochloride buffer, pH 3.0, was added to five cellulose nitrate centrifuge tubes (0.5 by 2 inches [1.27 by 5.08 cm]), and 5 ml of 0.05 M phosphate buffer, pH 7, was added to a sixth tube. (ii) At hourly intervals, 5 μl of a poliovirus preparation was added to each of the pH 3 tubes (final particle count, 7.5×10^8 particles per ml), and when virus was added to the fifth pH 3 tube, virus was also added to the pH 7 tube. (iii) After 1 h more, all six tubes were centrifuged as previously described for poliovirus (3). (iv) After centrifugation the top 2.3 ml of each tube was carefully removed and neutralized with 0.1 M glycine-NaOH buffer, pH 11.0, and titrated on HEp-2 cells.

The curve of aggregation obtained without the dilution step is shown in Fig. 1A. Included is a curve drawn through the points obtained from the von Smoluchowski equation (9, 10) at the same virus concentration as the experimental test. Although the aggregation of the virus took place at a slower rate than predicted, the general shape of the curve was identical to that of the von Smoluchowski curve and was distinct from the kinetic curves previously described (3) as there was no phase during which aggregation was not taking place. This demonstrates that the unusual results previously described (3) were due to the disaggregation on dilution of some or all the clumps already formed and underscores the fact that aggregation is a continuing phenomenon under the influence of the brownian motion of the particles.

Calculation of aggregation and efficiency. Because the rate of aggregation of the

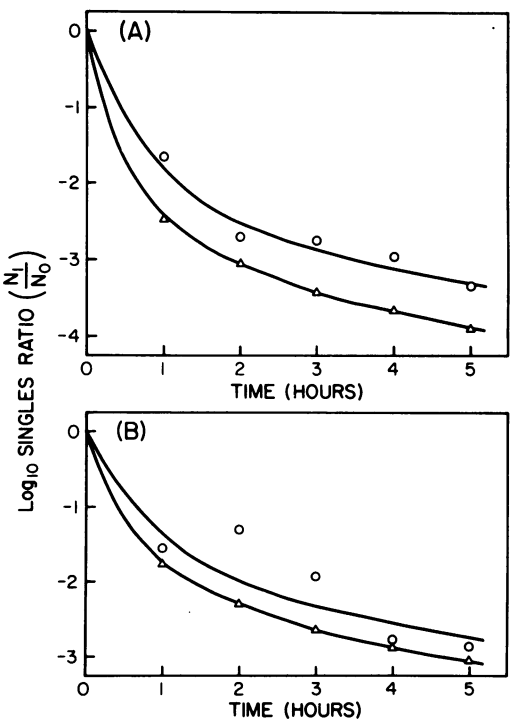

FIG. 1. *Aggregation of (A) poliovirus in 0.05 M glycine-hydrochloride, pH 3.0, at 25°C, and (B) reovirus in 0.05 M acetate, pH 4.0, at 24°C. ○, Observed data; △, calculated from the von Smoluchowski equations. Modified SPA test as described in the text.*

poliovirus particles was slower than the calculated rate, it is possible to calculate an efficiency factor which will indicate the percentage of collisions effective in producing an aggregate. The general formula (9, 10) for calculating the amount of aggregation, assuming 100% efficiency, is as follows:

$$N_k = \frac{(N_0)\left(\frac{t}{t_{1/4}}\right)^{k-1}}{\left(1+\frac{t}{t_{1/4}}\right)^{k+1}} \quad (1)$$

where N_k is the number of aggregates of size k, N_0 is the initial number of particles (assumed to be all single particles), t is the time in seconds, and $t_{1/4}$ is the time for aggregation to proceed to a point such that exactly one-quarter of the single particles are remaining. (Von Smoluchowski used "T" to refer to this time point [9, 10], which is also the same as the time for the total number of aggregates plus single particles to reach one-half of the initial number, N_0. We used "$t_{1/4}$" here in reference to single particles only and to prevent confusion with T = temperature.) The value $t_{1/4}$ is calculated according to the following equation:

$$t_{1/4} = \frac{3\eta}{4N_0kT} \quad (2)$$

where η is the absolute viscosity of the medium in poise, k is Boltzmann's constant (1.38×10^{-16} erg/degree), and T is the absolute temperature. When $k = 1$, that is, single particles, $N_k = N_1$, equation (1) is reduced to the following:

$$N_1 = \frac{N_0}{\left(1+\frac{t}{t_{1/4}}\right)^2} \quad (3)$$

and the number of single particles remaining at any time t can be calculated.

The efficiency of aggregation can be calculated by the use of the factor ϵ according to the equation:

$$\epsilon = \sqrt{\frac{\text{Calculated titer}}{\text{Observed titer}}} \quad (4)$$

and can be related to the aggregation equations above by insertion into equation 3 (as demonstrated by Hartman [4]) as follows:

$$N_{1_{(obs)}} = \frac{N_0}{\left(1+\frac{\epsilon t}{t_{1/4}}\right)^2} \quad (5)$$

With respect to the factor ϵ, we suggest two definitions for its use: (i) the efficiency factor ϵ^2, which gives that portion of collisions which are effective in producing a permanent aggregate (expressed in terms of percent); and (ii) the time reduction factor ϵ, which, when applied as in equation 5, alters the result to closely approximate the amount of aggregation actually observed. Table 1A gives the numerical data relating to the curves plotted in Fig. 1A. Under the conditions employed in this experiment (η = 0.009 poise; t = 298°K) the average efficiency was 25% ($\epsilon^2 = 0.2587$), indicating that one out of four collisions of single particles was effective in producing a permanent aggregate.

TABLE 1. *Efficiency and time reduction factors for curves shown in Fig. 1*

Virus	Time (min)	Observed		Calculated		ϵ^2 Efficiency (%)	ϵ Time reduction
		Titer[a]	Log_{10} reduction[b]	Titer[a]	Log_{10} reduction[b]		
A. Poliovirus	0	7.5×10^8		7.5×10^8			
	60	1.64×10^7	−1.66	2.46×10^6	−2.484	15	0.387
	120	1.64×10^6	−2.66	6.52×10^5	−3.061	39.8	0.63
	180	1.36×10^6	−2.742	2.96×10^5	−3.404	21.76	0.466
	240	8.43×10^5	−2.949	1.68×10^5	−3.650	19.9	0.446
	300	3.28×10^5	−3.359	1.08×10^5	−3.841	32.9	0.574
Avg value						25.87	0.5006
B. Reovirus	0	3×10^8		3×10^8			
	60	8.49×10^6	−1.548	5.37×10^6	−1.747	63	0.795
	120	1.57×10^7	−1.281	1.54×10^6	−2.29	9.7	0.313
	180	3.54×10^6	−1.928	7.19×10^5	−2.62	20.3	0.451
	240	4.53×10^5	−2.821	4.15×10^5	−2.859	91.6	0.957
	300	4.25×10^5	−2.849	2.7×10^5	−3.046	63.5	0.797
Avg value						49.62	0.663

[a] Particles per milliliter.

[b] Log_{10} titer at time t minus log_{10} titer at zero time.

Quantitative aggregation of reovirus. Figure 1B shows the results of a similar test with reovirus aggregated at pH 4.0 in 0.05 M acetate buffer. Again, the aggregation was generally parallel to the calculated values, but not as rapid. Table 1B gives the numerical data pertaining to the curves in Fig. 1B. The overall efficiency was close to 50%, indicating that one out of every two collisions was effective in producing an aggregate. The points defining the observed data curve in Fig. 1B are much more widely spread than those in Fig. 1A. This is characteristic of aggregation experiments and results from two main causes: (i) slight differences in efficiencies within each tube and (ii) variation among tubes resulting from mechanical factors in the handling of the tubes, buckets, rotor, pipettes, etc. The second factor probably contributes more to variation within an experiment due to the lack of any stabilizing material, such as a density gradient, in the virus suspension.

Efficiency of aggregation under different conditions. Table 2 shows several values for the efficiency (ϵ^2) of aggregation when each of the two viruses were placed in various buffers at different pH values. The ϵ^2 values were calculated according to equation 4 at the times given in the table. The total amount of aggregation which took place under each condition was dependent on time, temperature, viscosity, and viral particle concentration, but the efficiencies which are calculated for each set of conditions can be seen to vary from a low of 0.38 to almost 90%. In the case of poliovirus in borate buffer at pH 9, the time value taken was only 1.5 h because aggregation was so rapid as to preclude taking samples at much longer times. However, in this particular case, repeat experiments at similar or slightly lower particle concentrations in borate buffer at pH 9.0 confirmed the efficiency of $\geqq$90% at longer time values.

TABLE 2. *Efficiencies of aggregation (ϵ^2) for poliovirus and reovirus in buffers at various pH values*

Virus	Buffer (0.05 M) and pH	Time (h)	Efficiency of aggregation (ϵ^2) (%)
Poliovirus	Borate, pH 9.0	1.5	87.9
	Glycine-hydrochloride, pH 3.0	4.0	19.9
	Acetate, pH 5.0	4.0	3.03
	Phosphate, pH 6.0	4.0	0.4
Reovirus	Acetate, pH 4.0	4.0	63
	Glycine-hydrochloride, pH 3.0	4.0	2.5
	Acetate, pH 5.0	4.0	0.38

DISCUSSION

In the study of viral aggregation, we have found it necessary to have a reliable method by which the amount of aggregation produced under a given set of conditions can be measured and quantitated before any meaningful studies can be carried out. The modified SPA test described in this paper fulfills this criterion and has made it possible to measure the amount of aggregation that can occur in a virus preparation.

The basic SPA test, as described previously (3), utilizes the difference in ultracentrifugal sedimentation rate between single particles and aggregates of various sizes. Because all aggregates in any coagulating system must arise from single particles, a measurement of the number of single particles remaining after a given time can be used as a measure of the amount of aggregation in the virus suspension. The modified SPA test as described herein provides such a measurement but differs from the previously described test (3) in that no dilution is used to slow down the continuing aggregation. In the modified SPA test, the ultracentrifugal force is the mechanism by which the aggregation is stopped so that reliable plaque titrations can be carried out. Because the centrifugal force removes the aggregates from the upper half of the tube, at the termination of the run the remaining single particles are in a much reduced concentration, a situation which effectively prevents further aggregation. Also, because plaque titrations are performed in phosphate-buffered saline at pH 7.4 containing 0.14 M saline, any remaining aggregates are broken up (3), and plaque titrations are therefore an accurate reflection of the single particle concentration. We feel that the accuracy of the test as it is now performed justifies the name single particle analysis, SPA.

The SPA test has been used in this paper to obtain a measure of the amount of aggregation of a given concentration of virus under various conditions and has been presented in the form of kinetic-time curves of aggregation. These curves demonstrate three important results. First, viral aggregation proceeds according to, and can be described by, the classical laws of aggregation of spherical particles as formulated by von Smoluchowski (9, 10) (equations 2 and 3). Although the efficiency factor may vary from one set of conditions to another, the aggregation which takes place with these viruses does so via the same basic mechanism as that assumed by von Smoluchowski for colloidal particles in general, that is, diffusion of the particles under the influence of brownian motion. This is apparently

the only force causing movement of the particles in suspension.

Second, the results in this paper demonstrate that the efficiency of aggregation, which is a measure of the percentage of collisions which result in a permanent aggregate being formed, can vary considerably. We have observed the highest overall efficiency in borate buffer at pH 9.0 with poliovirus. (These data were not reported in our earlier paper [3] because the original SPA test was used and disaggregation probably prevented an accurate titration.) Other efficiencies at lower values can and do occur, as shown in Table 2.

Third, the SPA test can be used under a wide variety of conditions. Because the efficiency of aggregation of virus particles is usually within the range 0 to 90%, the SPA test can be used to investigate the effect of various conditions on virus aggregation. Any one of the curves is completely described when the efficiency factor ϵ^2 is known, and hence it will be useful as a means of characterizing, quantitatively, the physical state of a virus suspension when it is subjected to, for example, disinfecting agents under laboratory or field conditions. It can also be used to compare results with different viruses. Although we have used only buffers at various pH values to induce aggregation, other effects, such as ionic composition of the medium, can also be investigated, as will be shown in a subsequent paper.

The SPA test as described here is designed to be used as a test of the amount of aggregation in a virus preparation relative to a known control. The control is usually a monodispersed virus preparation, but is not limited to it. The monodispersity of the control must be checked by other means, usually electron microscopy. The SPA test can, however, be used as an absolute measure of aggregation, and for low particle concentrations ($<10^9$/ml) it may be the only method available. This use is based on the fact that at the end of a centrifuge run, the top half of the tube contains single particles only (3), but the particles are present only in one-third of the top half. Thus, the titer of a noncentrifuged preparation will be 3× that of a centrifuged suspension of single particles.

It should also be noted that the von Smoluchowski theory does not itself provide any reason why one virus should have an ϵ^2 factor different from another. In fact, physical values of virus particles such as mass, diameter, sedimentation coefficient, diffusion constant, and so forth do not appear in the equations. This is due to the fact that whereas larger particles have a greater surface contact area than smaller particles, they also have a correspondingly slower diffusion constant. These two factors are inversely proportional to one another and hence cancel each other exactly (5). Thus, the actual rate of aggregation is dependent only on temperature, viscosity, initial number of particles, and efficiency.

ACKNOWLEDGMENTS

Financial support for the work reported in this paper came from the Environmental Protection Agency grant R804635020.

We thank J. Donald Johnson for much helpful advice and criticism during the progress of this work and for critically reading the manuscript.

LITERATURE CITED

1. **Berg, G., R. M. Clark, D. Berman, and S. L. Chang.** 1967. Aberrations in survival curves, p. 235–240. *In* G. Berg (ed.), Transmission of viruses by the water route. Interscience Publishers, Inc., New York.
2. **Floyd, R., J. D. Johnson, and D. G. Sharp.** 1976. Inactivation by bromine of single poliovirus particles in water. Appl. Environ. Microbiol. **31**:298–303.
3. **Floyd, R., and D. G. Sharp.** 1977. Aggregation of poliovirus and reovirus by dilution in water. Appl. Environ. Microbiol. **33**:159–167.
4. **Hartman, R. J.** 1947. Colloid chemistry, p. 270. Houghton Mifflin Co., Cambridge.
5. **Overbeek, J. T. G.** 1952. Kinetics of flocculation, p. 282. *In* H. R. Kruyt (ed.), Colloid science, vol. I. Elsevier, Amsterdam.
6. **Sharp, D. G.** 1960. Sedimentation counting of particles via electron microscopy, p. 542–548. *In* W. Bargmann, G. Möllenstedt, H. Niehrs, D. Peters, E. Ruska, and C. Wolpers (ed.), Proceedings of the Fourth International Conference on Electron Microscopy. Springer-Verlag, Berlin.
7. **Sharp, D. G.** 1974. Physical assay of purified viruses, particularly the small ones, p. 264–265. *In* C. J. Arceneaux (ed.), Proceedings of the 32nd Annual Meeting of the Electron Microscopy Society of America. Claitor's Publishing Div., Baton Rouge, La.
8. **Sharp, D. G., R. Floyd, and J. D. Johnson.** 1976. Initial fast reaction of bromine on reovirus in turbulent flowing water. Appl. Environ. Microbiol. **31**:173–181.
9. **Von Smoluchowski, M.** 1916. Drei Vorträge über Diffusion, brownische Molecularbewegung und Koagulation von Kolloidtielchen. Phys. Z. **17**:557–585.
10. **Von Smoluchowski, M.** 1917. Versuch einer mathematischen Theorie der koagulationskinetic Kolloide. Z. Phys. Chem. (Leipzig) **92**:129–168.