EPA-600/2-76-287
December 1976

Environmental Protection Technology Series

VIRUS PARTICLE AGGREGATION AND HALOGEN DISINFECTION OF WATER SUPPLIES

Municipal Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into five series. These five broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The five series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies

This report has been assigned to the ENVIRONMENTAL PROTECTION TECHNOLOGY series. This series describes research performed to develop and demonstrate instrumentation, equipment, and methodology to repair or prevent environmental degradation from point and non-point sources of pollution. This work provides the new or improved technology required for the control and treatment of pollution sources to meet environmental quality standards.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

EPA-600/2-76-287
December 1976

VIRUS PARTICLE AGGREGATION AND HALOGEN DISINFECTION OF WATER SUPPLIES

by

D. Gordon Sharp
University of North Carolina
Chapel Hill, North Carolina 27514

Grant No. R803771

Project Officer

John C. Hoff
Water Supply Research Division
Municipal Environmental Research Laboratory
Cincinnati, Ohio 45268

MUNICIPAL ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

DISCLAIMER

This report has been reviewed by the Municipal Environmental Research Laboratory, U. S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U. S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay beween its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

Using methods developed under a previous EPA Grant (R 802946) additional information on virus inactivation by halogens and the effects of virus aggregation on disinfection is provided. This information contributes to our understanding of disinfection and its use in water treatment processes.

Francis T. Mayo
Director
Municipal Environmental Research
Laboratory

ABSTRACT

Water suspensions have been produced that contain essentially all single virus particles. Both bromine and chlorine react very rapidly with these preparations. Reovirus is more sensitive to bromine than poliovirus by a factor of more than 10. Bromine destroys poliovirus a little faster than chlorine but both are very fast acting on single virions and special kinetic equipment had to be built to determine the kinetics. Tribromamine is about as active as HOBr on single polio virions in equal molar concentrations.

But both reo and polio viruses in aggregated form, as seen directly by the electron microscope, are much more resistant to bromine action than single particles. Aggregated seems to be the normal state of virus in water unless it is put there in dispersed form. Dilution of laboratory stock virus in water tends to aggregate it. Special methods were devised to detect this at high dilutions. There are natural, unbroken aggregates too that are difficult to disperse. Practical sterilization of water supplies will doubtless have to deal with these resistant aggregates, both natural and induced, rather than with single virus particles.

CONTENTS

	Page
Foreword	
Abstract	iv
List of Figures and Tables	vii
Acknowledgments	ix
Introduction	1
Conclusions	3
Recommendations	5
Materials and Methods	7
Experiments and Results	12
Reovirus	12
Poliovirus	19
Physical State of Viruses in Water	24
Reovirus	24
Poliovirus	28
Electron Microscope Observations	29
Dilutions of Poliovirus in Water	31
Natural Aggregates of Poliovirus	33
Discussion	39
Inactivation of Reovirus	39

CONTENTS (Continued)

	Page
Survival of Reovirus Through Clumping	40
Physical Stability of Virus in Water	44
References	47

FIGURES AND TABLES

Fig.

1. Apparatus for halogen treatment of virus in flowing water.

2. Inactivation of reovirus in water by bromine (HOBr) at 2C.

3. Frequency distribution of reovirus aggregates.

4. Bromine action on a known mixture of reovirus aggregates.

5. Reovirus aggregates in starting preparation of Fig. 6.

6. Sedimentation velocity spectrum of the reovirus of Fig. 5.

7. Partition of reovirus between singles and aggregates.

8. Poliovirus purified by freon extraction and velocity banding.

9. Count data from pictures of the fractions of Fig. 8.

10. Inactivation of poliovirus by bromine (HOBr) at 2C.

11. Inactivation rate of poliovirus vs. bromine concentration.

12. Inactivation of poliovirus by bromine (HOBr) at 10C.

13. Inactivation of poliovirus by bromine (HOBr) at 20C.

14. Inactivation of poliovirus by chlorine (HOCl) Agg. and Disp.

15. Inactivation rate of poliovirus vs. HOCl concentration.

16. Inactivation of poliovirus by Tribromamine.

17. Stock reovirus diluted 10 x then 20 x more with PBS.

18. Group size distribution in dilute stocks of Figs. 17 and 19.

19. Stock reovirus diluted 10 x with water then 20 x with PBS.

20. Typical purified stock suspensions of poliovirus.

FIGURES AND TABLES (Continued)

Fig.

21. Dilution of poliovirus (Fig. 20) 10 x with water.

22. Further dilution of poliovirus (Fig. 21) with PBS.

23. Poliovirus aggregates at pH 5 in 0.05 M acetate buffer.

24. Reovirus emerging from infected cells in large aggregates.

25. Aggregates of poliovirions found in dialyzed crude extracts.

26. Inactivation of crude poliovirus (Fig. 25) by bromine.

27. Sedimentation velocity spectra of poliovirus PFU.

28. Simple virion aggregation test (Red, White, and Blue).

29. Poisson approximation to the reovirus curve of Fig. 4.

30. Straight line approximation to the reovirus curve of Fig. 4.

Table

1. Purified poliovirus is randomly spread and mostly singles.

2. The physical state of poliovirus in water.

ACKNOWLEDGMENTS

This work was supported in part by Grant #R803771 from the U.S. Environmental Protection Agency and in part by the U.S. Army Medical Research and Development Command under contract DAMD17-17-A013. It has also been materially aided by Mr. Richard Boynton of the Sperry Rand Company of Durham, North Carolina, who provided the special motor windings for the magnetic mixing devices used in the Flash Flood Experiment.

The oil-driven OTD2 ultracentrifuge and the ARC-1 slow start device were gifts of the Ivan Sorvall company.

Most of the experiments were done by Roger Floyd and D. C. Young with the excellent technical assistance of Florence Stubbs, electron microscopist, who made the virion counts and aggregation analyses.

All of the devices for preparation of virus for kinetic attachment electron microscopic count and for the Flash Flood experiment were made by the author in his home machine shop.

INTRODUCTION

There have been many papers written about the survival of virus during exposure to disinfecting agents and most of them include, in the discussion of their results, some speculation regarding the possible influence of virus particle aggregation. This account differs from the others. It provides direct evidence of the kind and degree of aggregation that was present among the virions that were treated with bromine as well as the plaque forming unit (PFU) titer of the starters and the survivors. This physical evidence comes from electron microscopy (EM) of virus samples prepared by methods especially contrived to avoid the aggregation artifacts which are inherent in ordinary preparative techniques that have been designed primarily to show ultrastructure. Two methods for doing this have been devised here. Both of them are based upon the premise that the virus particles and clumps must become attached to the surface for observation while the surface is in contact with the whole body of the water suspension; they must not be deposited there during the time the water suspension of virus particles is drying because the attendant forces of surface tension exert strong aggregating effects on suspended particles. The first of these techniques, which works well with the larger viruses, including reovirus, involves sedimentation of the particles by centrifugation onto an agar receiving surface from which they can be transferred by pseudoreplica to the EM. The other involves kinetic attachment of the particles by Brownian bombardment of an aluminum coated collodion film which, after thorough washing, can be examined directly in the electron microscope. This method gives count and aggregation data on small viruses, such as polio, which do not yield to the sedimentation-pseudoreplica method.

Any series of experiments which inquire into the behavior of virus, aggregated or not, in the presence of disinfectants requires the preparation of a concentrated stock from which starting virus is taken for each experiment. Such a stock is usually kept frozen to preserve a high infectivity titer but in the present work it was necessary to avoid aggregation as well and we were unable to meet both requirements by freezing. Collection and storage of virus stock suspensions in concentrated sucrose solution has served both ends quite well. The preparation and handling of these virus stocks is a significant part of this project.

Observations on the physical state of aggregation of a virus in water require particle concentrations great enough so that EM pictures will show enough particles and groups of various sizes so that their numbers can be established with the necessary statistical significance. Also, they must be sufficiently purified so that there is no doubt about recognition of the particles, as virus, and no doubt about distinguishing the separate individuals in the

groups. This means about 10^8 per ml for reovirus (by sedimentation count) and about 10^{10} per ml for polio by the kinetic attachment (KA) method.[1,2] Such concentrations may seem very high, but actually the corresponding PFU titers are about 2×10^6 per ml for reo and 10^8 per ml for polio, both of which are well within the range normally experienced in unconcentrated lysates of infected cell cultures.

While EM provides quantitative data with a satisfying directness in experiments with virion aggregation, it can be applied only within the limited concentration range just described. In the stock concentrates as well as in the very dilute conditions likely to prevail in virus-polluted natural resource water, the physical state of the virus cannot be observed so directly. However, the relative rates of sedimentation of clumps and single virus particles are essentially the same no matter how many or few are present. Appropriate techniques have, therefore, been designed for zonal and swinging bucket rotors to detect and, to some extent, to measure virion aggregation under these extreme conditions. Centrifugation is better than filtration for this purpose because the results are more readily interpreted quantitatively.

Survival of both reo and polio viruses is shown in this work with bromine to be much longer for aggregates than for single virus particles. So it becomes important to know in experiments, as well as in the field, what state the virus is in. This work reveals some difficulties encountered when stock viruses were diluted in water for disinfection experiments. Sometimes they aggregated. Some of these aggregates were dispersed when diluted further with suitable solvent solutions and others were not. An effort has been made to define the conditions that lead to aggregation for the benefit of those who wish avoid them and the confusion caused by unexpected plaque titration results.

The virus particle aggregates that occur when monodisperse stock suspensions are diluted in water are not to be confused with natural aggregates that are present in virus preparations that have not been thoroughly dispersed during extraction from the infected cells. Evidence is provided that such natural aggregates may not disperse when suspended at high dilution in either laboratory distilled water or natural fresh water with or without normal particulate content.

Most of the experiments here dealing with virus inactivation have been done with bromine under conditions where the principal active agent was HOBr. A few were made with tribromamine and a few others with HOCl.

CONCLUSIONS

The general state of virus particles in water suspension is aggregated. Many viruses are produced as aggregates in infected cells and when we have examined suspensions of reovirus by zonal centrifugation or by quantitative EM, we have found a substantial fraction (often over 50%) sedimenting much faster than single particles, and in this fraction many aggregates can be seen by EM.

When aggregates are present in a reovirus suspension containing bromine, the disinfection rate of the mixture proceeds at a constantly decreasing rate which appears to be determined by the frequency of the different aggregate sizes in the mixture.

When aggregates are removed from the above mixture by taking advantage of their differences in sedimentation velocity, the rate of disinfection of the remainder by bromine becomes essentially a straight line. This is the basic reaction rate for single virions, and it is the only reliable basis for comparing the resistance of one virus with another with different disinfectants under different conditions.

When working with bromine and suspensions of single virions it is necessary to make very short time exposures. Even with poliovirus and 3 μM bromine, they must be as short as 5 sec and with reovirus, 1/2 sec or less. The continuous flow or "Flash Flood" apparatus that we have built has met this need and it promises to be of broad general value in such work.

Poliovirus suspensions of single particles are about 25 X more resistant to bromine than similar suspensions of reovirus, and there are some anomalies too. In a limited range of temperature and bromine concentration there is a slight delay in the onset of the disinfection process. Such delays have been seen by others but always case of what was presumed to be an aggregated virus. This delay must now be attributed to something else, possibly penetration of the virus capsid by the bromine. Poliovirus disinfection rates are proportional to bromine concentration at 10C and at 20C but at 2C they are not. Reasons for this are not apparent. This leads to a linear Arrhenius plot only at about 10 μM bromine concentration.

The disinfection of suspensions of single poliovirions by HOCl is generally first order with rate constants at 2 and 10C less than those for HOBr but under no conditions less than half. Experimental difficulties encountered at pH 6, where HOCl predominates, are due to colloidal instability. The virions tend to aggregate.

The rate of disinfection of poliovirus at 10C is roughly proportional to concentration of HOCl, but at 2C it behaves like HOBr in that the disinfection rate does not increase linearly with concentration.

We find in our preparation of both polio and reo viruses, two kinds of aggregates, natural and induced. The former are presumably unbroken parts of the cytoplasmic concentrations in which they were generated, and the latter occur in previously dispersed suspensions as a result of one or more of several destabilizing conditions.

Induced aggregates of reovirus are quite stable.

Induced aggregates of poliovirus break up quite easily.

Natural aggregates of both viruses are generally more stable than induced aggregates.

Dilution of either virus from monodispersed stock into distilled or natural resource water can induce aggregation; it depends upon how it is done. Often a single large dilution step will avoid aggregation.

Both reo and polio viruses aggregate in pH 5 buffer and usually in pH 6 buffer, especially at low ionic strength.

Natural aggregates of poliovirus are not dispersed when diluted several thousand fold in distilled or natural resource water.

Stock suspensions of single poliovirus particles do not become aggregated when diluted at one step (5000 fold) with distilled or natural lake water, but they do aggregate when water is used after treatment in the filter plant with lime and alum.

Aggregated reovirus has survived for 300 seconds the same bromine treatment that destroyed single virions in 1 second. Aggregated poliovirus shows the same relative resistance of clumps over single particles. These facts show that the kinetics of halogen disinfection of virus in water is very much dependent on the physical state of virus in the water that is, in turn, a highly variable thing.

RECOMMENDATIONS

1. Continue experiments on chlorine disinfection of single polio and reo viruses, at least until a good comparison can be made with the bromine data now on hand.

2. Examine the survival of polio and reovirus aggregates, both natural and induced, when treated with chlorine and bromine.

3. Examine the effect of small-number aggregation on the halogen survival of poliovirus for comparison with similar data now in hand on reovirus. See if there is complementation in plaque formation. See if the data fit one of the schemes presented in this report or discussed by Clark[3] or by Wei and Chang[4].

4. Continue the effort to titrate poliovirus in such a way that induced aggregates can actually be presented to the cell monolayer.

5. Examine, in detail, the conditions that determine the physical state of the virus particles in water suspension with particular attention to natural resource waters, their pH, ion content and concentration etc. Look at divalent and travalent ions in particular.

6. Look for better ways to prepare and keep stocks of virus in both a dispersed and aggregated condition.

7. See if particles suspended in the water have any effect on virion aggregation, survival in the field or resistance to halogen action. Check bentonite and other substances that are frequently found in water to see how viruses interact with them.

8. Develop further the coordination between zonal centrifugation and EM, because they contribute independent data on virion aggregation, natural, induced and complexed with other particles. The centrifuge is blind but very sensitive to aggregation. The EM sees aggregates clearly but only in a very limited concentration range. The two together are a powerful analytical combination.

9. Examine carefully the possibilities for virion aggregation that occurrs when virus stock suspensions are employed in the dynamic flow apparatus for disinfection studies. The changes in concentration of virus, ions, sucrose, and pH that can occur can probably be influenced to some extent by optimum management, including quick mixing, choice of buffer etc.

It is of vital importance that the physical state of several of the enteric viruses be examined. The data gained so far about just two show major differences, and they differ from those gathered by others using bacteriophages. We need to see if polio, echo and coxsackie for example, are alike or different before any overall concepts can be formed about the behavior of enteric viruses in water.

10. Methods must be found for observing the natural aggregates of virus particles that are released by infected cells directly into water. It is likely to be these, rather than those induced from purified preparations, that will be the persistent survivors of disinfecting processes.

11. Virus isolated by change from low to high pH and back may be held or not by the filters because of electric charge alone but we believe induced and dispersed aggregates are involved as well. Inasmuch as this process is now the method of choice for concentrating virus from large volumes of water, it may be well to investigate this point before an incomplete concept of the mechanism congeals into textbook dogma.

12. As soon as we are able to produce by induced aggregation or select from a mixture of natural aggregates, a preparation of all pairs or containing only a narrow range of aggregate sizes, we want to get on with the measurement of plaquing efficiency vs. group size, particularly for survivors of halogen treatment.

13. Exploit as far as possible the improved resolving power now available in swinging bucket centrifuges because of the newly-developed slow-start and slow-stop techniques with density gradients.

14. Examine other sucrose gradient configurations and substances other than sucrose to improve the resolving power of aggregate spectra from the zonal centrifuge and also short-cut aggregation tests, such as the Half and Half as well as the Red, White, and Blue described later.

15. Employ each bit of new information on adhesion of virions to each other and to other things to best advantage in improving the technique of quantitative EM.

It is not expected that all of these areas can be pursued in depth, but certainly several of them should be.

MATERIALS AND METHODS

PREPARATION OF STOCK VIRUS FOR DISINFECTION EXPERIMENTS WITH DISPERSED AND AGGREGATED VIRIONS.

Reovirus type 3 (Dearing strain) was grown in and all plaque titrations were made on monolayer cultures of L cells, as previously described[5], except that cells were maintained in 200 ml milk dilution bottles and passaged into 32 oz prescription bottles for growth of virus. Virus was harvested at 16 to 18 h after infection at 10-20 PFU/cell rather than 20 to 24 h as previously noted.[5] In addition, the virus was extracted from the cell in 6 ml of phosphate buffered saline (PBS) without calcium and magnesium and 4 ml of Freon 113 for 2 min at one half speed in a Sorvall Omni-Mixer. The virus was placed on 20 → 40% w/w sucrose gradients in 0.05 M phosphate buffer at pH 7.2, and centrifuged at 25,000 RPM in the Beckman SW 27 rotor at 4C for 1 h. The virus, which was collected from the lower of two bands seen with a collimated beam of light, was allowed to remain in the sucrose at 4-6C and was not pelletized. The virus stored in this manner retains its infectivity for several weeks, and the state of physical aggregation is quite stable. There is no bacterial or fungal growth, and the sucrose does not produce any detectable bromine demand under the conditions of our experiments.

Poliovirus type I (Mahoney strain) was obtained from Dr. Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio, and was serially passaged in Human Epidermoid Carcinoma cells, HEp-2. The cells were grown in medium 199 containing 0.105% $NaHCO_3$ and 5% fetal calf serum (FCS). After the cells reached confluency, they were maintained on the same medium with 2% FCS.

Stocks of virus were produced in HEp-2 cells at 37C by infection at a multiplicity of 10-20 PFU/cell under a maintainence medium of 199 + 0.105% $NaHCO_3$ and 2% FCS. After 18-24 h when the cytopathic effect was 100%, the cells were frozen and thawed 3 times and the cell debris removed by low-speed centrifugation (∿800G) for 10 min. The supernatant fluids had titers of 4 x 10^{10} PFU/ml and were kept frozen at -70C.

Purified virus was produced in the same cells grown for 3 days in 32 oz prescription bottles inoculated with the above stock virus in Dulbecco's PBS containing 12.5 mM $MgCl_2$, at a multiplicity of ∿1000 PFU/cell. The virus was allowed to adsorb for 1 h at 37C, then 40 ml of maintainence medium containing 12.5 mM $MgCl_2$ were added to each bottle and the cells were further incubated at 37C for 11 h. The cells were chilled to 4C, and the supernatant fluid was decanted. The monolayers were washed 2 times with PBS and the cells of each bottle were scraped into 10 ml of PBS and pelletized at 250 G for 10 min. Cells remaining in the supernatant fluid were pelletized similarly and pooled with the scraped cells and pelletized again. The pellet of cells was

resuspended in 6 ml of PBS not containing Mg^{++} or Ca^{++}. Four ml of Freon 113 were added, and the cells were homogenized at one-half speed in a Sorvall Omni-Mixer for 2 min. The phases were separated at 800G for 10 min, and the upper aqueous phase was removed and held in an ice bath.

The Freon phase was re-extracted with a further 6 ml of PBS, and the phases were again separated. The aqueous phase was pooled with the previous one, and the Freon phase was again re-extracted with 6 ml of PBS. All aqueous phases were combined and made to a volume of 20 ml. Ten ml of this extract were placed on each of two 10 → 30% w/w sucrose gradients made in 0.05 M phosphate buffer, pH 7.2. The gradients were centrifuged at 25,000 RPM in the Beckman SW27 rotor at 4C for 135 min. Fractions of 2 ml each were collected from the tubes and examined by the kinetic attachment[2] for the presence of the virus. Twenty fractions were obtained from each tube[5], and the highest count of virus was most frequently found in fraction 14. All relevant fractions were pooled, and the virus was stored at refrigerator temperature without any attempt being made to remove the sucrose.

PLAQUE TITRATION

Plaque titrations of reovirus were performed on 3-day-old monolayer cultures of L cells[7] in tightly stoppered 1 oz prescription bottles under an overlay of 1% Difco agar containing Medium 199, 5% FCS, 0.245% $NaHCO_3$, and 0.003% neutral red. Plaques were counted after 6 days at 37C.

Plaque assays of poliovirus were performed on 4-day-old monolayers of HEP-2 cells in 1 oz prescription bottles under an overlay of 1% Difco agar containing medium 199 plus 5% FCS, 0.210% $NaHCO_3$, 0.003% neutral red, and 5 mM $MgCl_2$. The plaques were read after 3 days incubation at 37C.

PHYSICAL ASSAY (Particle counting and aggregation analysis)

For reovirus the physical assay by electron microscopy on most preparations was done by the sedimentation and agar pseudoreplica method.[2] This will be called the spin down or SD method. A few counts on reovirus were made by the kinetic attachment or KA method.[2] Poliovirus counts and aggregation analyses were done by the KA method.

BROMINE, GLASSWARE, AND WATER

Bromine measurement and preparation of glass apparatus and demand-free water were the same as previously reported[3] except that amperometric titrations were employed together with the method of Taylor[8], and all phosphate buffers were made with Fisher primary standard potassium salts.

ZONAL CENTRIFUGATION

Resolution of both crude and purified virus particle populations into sedimentation velocity spectra[9] was done with a BXIV titanium zonal rotor (provided by Dr. Norman Anderson[10] of the Oak Ridge National Laboratories) operating in a Sorvall OTD2, fluid-drive ultracentrifuge.

SEDIMENTATION VELOCITY PARTITION OF MIXTURES OF SINGLE VIRIONS AND AGGREGATES

The different sedimentation velocities of single virions and aggregates of various sizes have been utilized to provide fractions for plaque titration. Two types of experimental procedures have been employed for this, using the small plastic tubes that fit the Beckman SW50 or SW50.1 centrifuge rotors. The simpler involves partially sedimenting a homogeneous mixture of the virus sample. Aggregated preparations show a lower than normal fraction of infectivity remaining above the center line after spinning. The second variation, called the Red, White, and Blue, has been very useful in detection of virion aggregation. It involves placing a 2 ml layer of virus sample over a 2 1/2 ml layer of 20% sucrose solution containing trypan blue, over 1 ml of 50% sucrose solution containing neutral red and spinning enough to sediment about half of the PFUs from a well-dispersed preparation out of the top (white) into the center (blue) fraction. Test samples with high aggregation will leave most PFUs in the blue and red fractions.

Both of these techniques will be described in more detail, together with the particular experiments in which they were used.

APPARATUS AND PROCEDURE FOR EXPOSING VIRUS TO DISINFECTION BY BROMINE

Long-time experiments (exposure times of one or more minutes) were made by putting virus into beakers containing water with suitable buffer and bromine concentration and withdrawing samples for quenching and titrating at appropriate times. These experiments have been described in detail in our earlier publication.[5]

Short-time experiments involving exposure of virus to bromine for 1/2 to 20 seconds could not be made in the beakers. For them, a kinetic experiment was devised that would permit injection of virus into a fast-flowing stream of turbulent bromine water and sampling with instant quenching of residual bromine at several points down stream.[6]

The apparatus must provide enough volume of water to ensure stability of temperature, bromine concentration, and flow rate through the reaction siphon tube (Fig. 1) for several minutes. The reaction tube is 3/8 inch inside diameter and the bottle (20 l) height is adjustable so that <u>turbulent</u> flow at the desired rate can be achieved. Because turbulence is essential, the Reynolds Number has been kept equal to or greater than 3000 in all experiments. The reaction tube has 5 access ports covered by disposable serum bottle stoppers. Five 5 ml syringes with needles are inserted. The

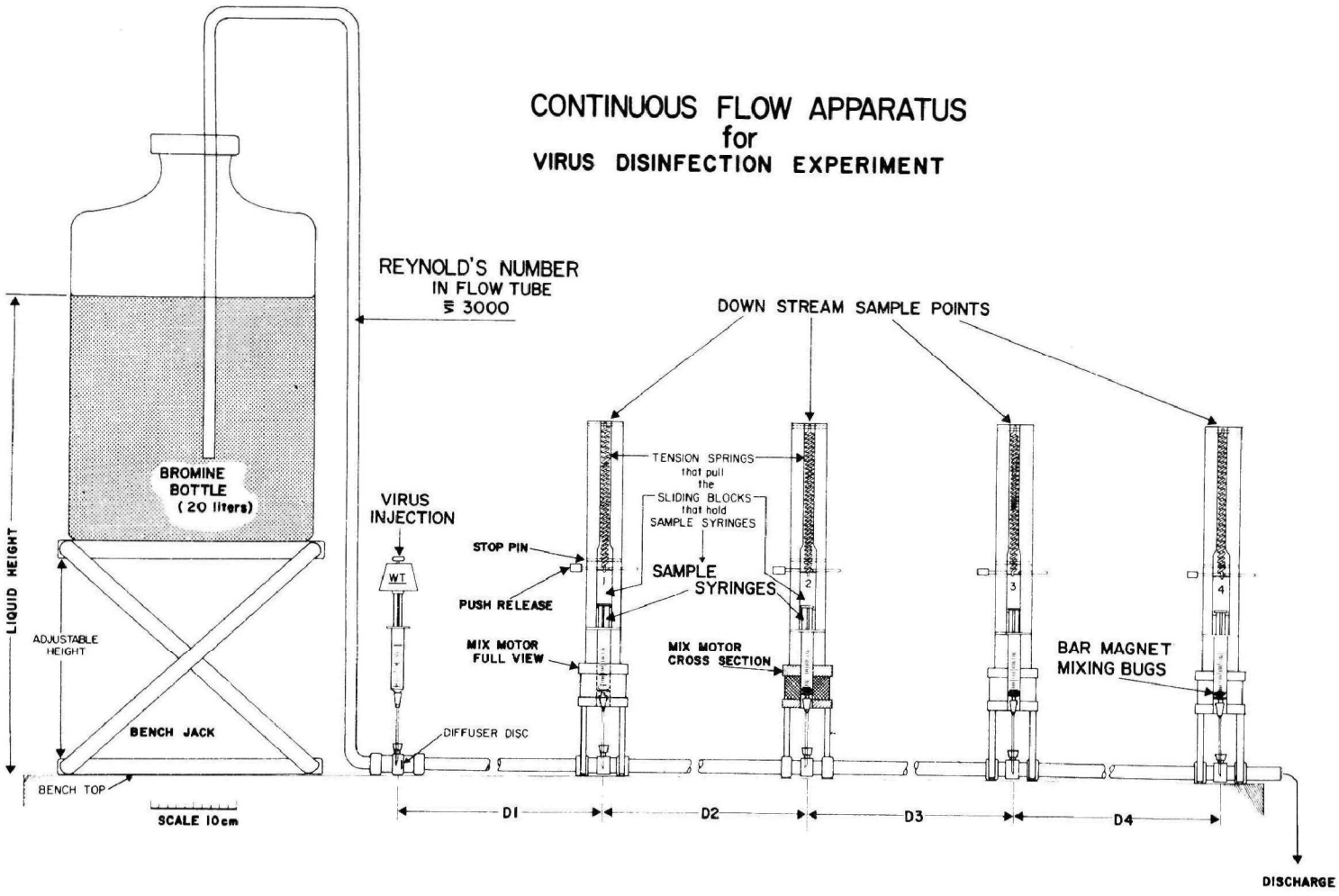


Fig. 1 Apparatus for injection into and recovery of virus from a turbulent stream of water containing a disinfecting agent, such as bromine, in the Flash Flood Experiment.

first of these (at the left) carries the virus inoculum (5 ml) which, when triggered, starts the weight-driven plunger which delivers the inoculum steadily for 5 sec, into the turbulent stream of buffered bromine water. There is a 6 mm diameter disk located on the axis of the tube, just downstream of the inoculum needle tip, to aid in mixing.

The time of transit from injection point to sampling point will be determined only by the flow rate and the distances D_1, D_2, etc. It is not in any way dependent on when the sample is taken as long as the sample is taken within the 5 sec interval during which the "polluted" water passes the sampling port in question.

Each of the 4 sampling syringes contains 1 ml of 2 mM sodium thiosulphate solution, 1 ml of air, and a small Teflon-coated magnetic stirring bar. The syringe plungers are held in metal blocks attached to helical springs, each under tension and restrained by a release pin that can be pushed at the desired time. When the spring is released, the syringe draws exactly 1 ml from the flowing stream. This is mixed with the thiosulphate solution to stop the reaction of the bromine on the virus. For virus-bromine exposure times of 1 sec or less, additional precautions were taken to ensure instant mixing. This is done by means of the magnetic stirring bar, which is made to rotate at 1800 RPM by 2-pole AC motor windings surrounding each sampling syringe. These are stator windings used in small electric servo motors kindly provided for us by the Sperry Rand Company of Durham, N. C.

Operation

Twenty liters of buffered chlorine-demand-free water are prepared and adjusted to the temperature required for the experiment. Enough bromine is added 17 h before the experiment to bring the concentration in the bottle to approximately the right value. Just before the experiment, more bromine is added to adjust the concentration to exactly the required level. Previous experiment has established the exact level of liquid in the bottle to produce the desired flow rate (20 to 40 ml/sec in different experiments). This level is marked for easy reference and all experiments start there. Beginning with the liquid at a higher level, the siphon is started and the flow continued for a few minutes to allow the tube to adjust to the temperature in the bottle and when the liquid level reaches the marked starting point, the virus injection is started. The stirring bars in all four sampling syringes are already running in their 1 ml volumes of thiosulphate solution, and it only remains to trigger each of the syringes as the flood passes and also to catch a sample of the virus-bromine mixture at the discharge end to determine its bromine concentration for comparison with that in the bottle. Neutralized bromine-virus mixtures from the sampling syringes and the virus from the inoculation syringe are then assayed for virus by the plaque method.

The dilution of virus as it enters the flowing stream is just the ratio of the rates of injection and stream flow. The injection syringe is timed by direct observation and stopwatch. The stream flow rate is easily obtained by timing the outflow of some conveniently measured volume, such as 500 ml.

Variation in flow rate with liquid level in the bottle is negligible during the experiment time, which has not been over 20 sec, because of the large (approx. 700 cm^2) surface area.

Tygon tubing is convenient for the flowing stream, but we have found that it absorbs substantial quantities of bromine. Glass is difficult to manage without catastrophic accidents. However, "High Density" polyethylene tubing has performed well. It is readily cut to desired lengths, and it exerts negligible bromine demand.

EXPERIMENTS AND RESULTS

REOVIRUS

Several experiments were made at 10C with samples taken at 4-sec intervals. These were made with virus prepared in the same way as described in our earlier publication [5], which involved pelletizing the purified virus to remove sucrose. These were done at 3, 5.8 and 5.9 μM bromine concentrations (Fig. 2), and they all show approximately the same level of persistent resistance previously reported in exposures of longer duration. They show also that the initial fast reaction is taking place during the first 4-sec interval. At this point we added the magnetic mixing devices to the sampling syringes and reduced the length of the flow tube to give samples at one- and later at 1/2-sec intervals. The temperature was reduced also, from 10 to 2C, to slow the reaction rate and still permit the use of bromine concentrations high enough to hold constant when confronted with expected bromine demand of the virus.

Three experiments were made at 2.8-3.0 μM bromine concentration with unpelletized virus. Two were done with 1-sec time intervals and one with the intervals reduced to 1/2 sec. The frequency of aggregates observed by EM

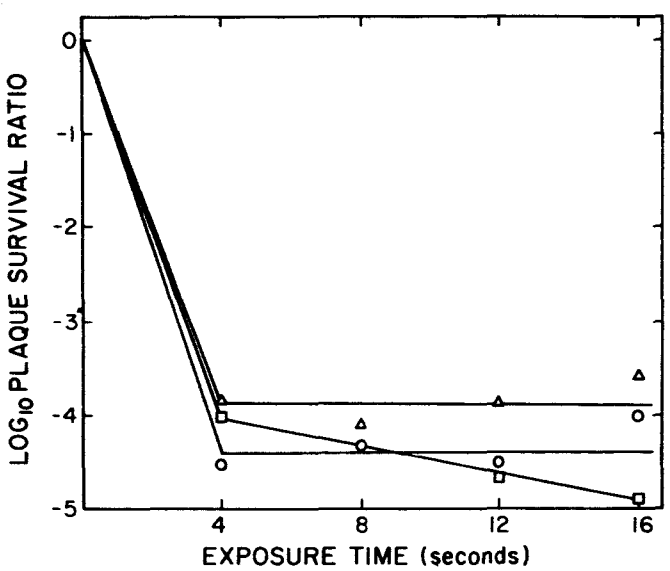
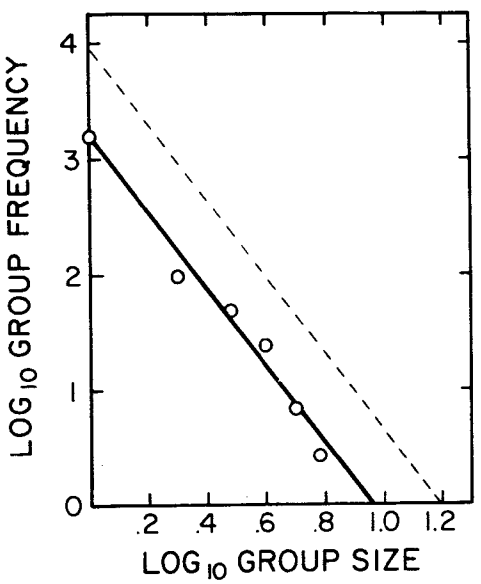


Fig. 2 Survival of reovirus infectivity in water containing 3 μM (triangles), 5.8 μM (circles), and 5.9 μM (squares) bromine at 2C and pH 7.0. The rapid phase of the reaction is done in less than 4 sec, and the slower phase has been shown to be caused by a few larger aggregates.

in this virus preparation is shown in the graph, Fig. 3. Of the whole particle population, 73% are singles, and the groups appear to be distributed in such a way that the log frequency of each group size is a linear function of the log of the number in that group. We[11] and others[12] have observed this same distribution with other viruses and also with polystyrene and acryl particles of comparable size. The inactivation of the virus by bromine in these three experiments is shown in the semilog plot of survival ratio vs time in Fig. 4. It is curved all the way. A single line has been drawn by inspection through all three sets of points. Correlation of this result with the aggregation distribution (Fig. 3) will be made later.

Zonal Centrifuge Experiments, Group 1

An effort was made to obtain a preparation of virus containing only single particles. This is needed to provide a frame of reference for comparing



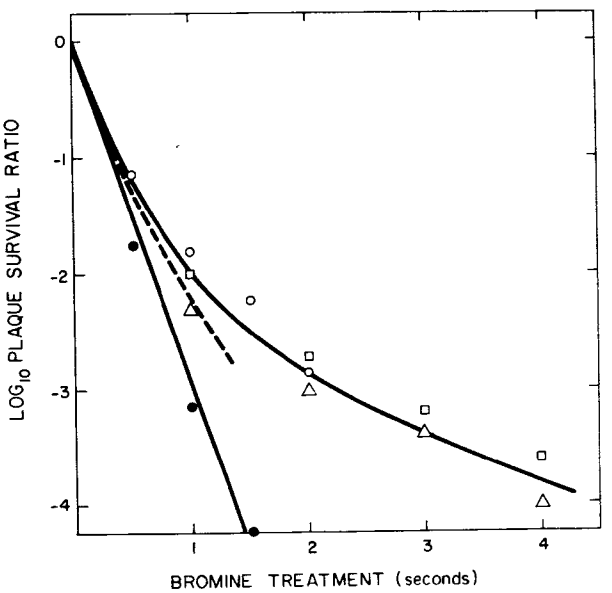


Fig. 3 (left) Frequency distirbution of aggregates observed by EM (circles). The dotted line indicates the same distribution for a more concentrated suspension containing a total of 10,000 particles.

Fig. 4 (right) Inactivation of reovirus during the first 4 sec of contact with 2.9 μM bromine in water at pH 7 and 2C. Three separate experiments are shown with different symbols. The frequency of aggregates of different sizes is that shown in Fig. 3. Filled circles show the nearly linear response of a preparation containing nearly all single virions. The broken line is a prediction for the survival of the same kind of particles as those of the straight line but aggregated as shown in Fig. 3 and subjected to degradation according to the multiple target concept as calculated by the Poisson limit.

the above results with aggregated virus. Fifteen ml of a purified virus preparation, aggregated as shown in Fig. 5 (circles), were layered over a 15-30% w/w sucrose gradient with the BXIV zonal centrifuge rotor running at 2000 RPM. The total number of virions put into the rotor was 10^{11} by EM count. After operating at 25,000 RPM for 30 min ($\Sigma\ \omega^2 t = 1.44 \times 10^{10}$) at 20C, the 640-ml volume of the rotor was completely displaced by 35% sucrose piston fluid and 25-ml fractions were collected in 1-oz bottles. The virus particle count on each was made by EM after dilution with 0.85% filtered sodium chloride solution. A dilution of at least 1/10 is required, otherwise the agar block that receives the sedimented virions in the pseudoreplica counting process will float to the surface because of residual sucrose. Particle counts are plotted against fraction numbers and a scale of radial distances is included on Fig. 6. Densities in the sucrose gradient were measured by direct pyknometer weighings.

Fractions 4 through 10 contained 69% of the recovered virus. In the peak fraction (#9) there were 93% single particles. Six percent consisted of pairs and the remaining one percent comprised triplets and groups of four (see bottom line of the graph Fig. 5). Thirty-one percent of the total recovered virus made a broad secondary peak with maximum count in bottle #13; 63% of the particles contained in it were in pairs. The total count of all the fractions indicated 110% of the 10^{11} particles that were put in. Apparently there was no detectable loss in the partition process, and calculations from the EM frequency distribution chart (upper line of Fig. 4) predict that 71% of the in-going population was single particles, a figure in excellent agreement with that observed in this actual sedimentation velocity spectrum from the zonal centrifuge.

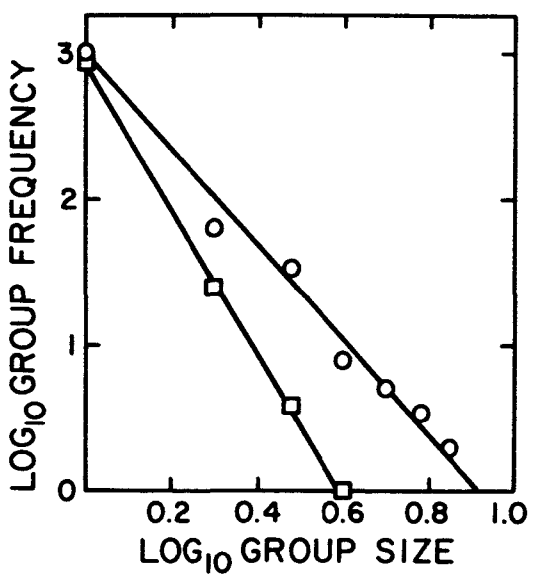


Fig. 5 Frequency distribution of aggregates observed in starting virus preparation for zonal centrifuge experiment #1 (circles). The squares show how the distribution was altered by removal of most of the aggregates from the region of the principal peak (Fig. 6).

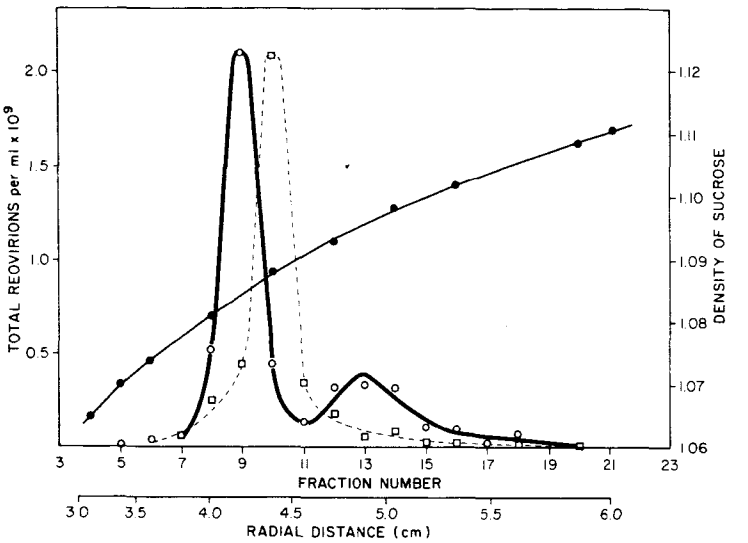


Fig. 6. Sedimentation velocity spectrum obtained in the zonal centrifuge of spontaneously aggregated reovirus (solid line). Ordinates are particle counts made by electron microscopy. Sixty-nine percent of the virus is in the major peak, which has 93% single particles and a few remaining small aggregates as shown in Fig. 5. The secondary peak contained mostly pairs and triplets in Fraction 13. The dotted line shows a similar spectrum of crude (unpurified) reovirus showing that the original state of freeze-thaw-release virus contains very few small aggregates. Large aggregates have sedimented to the rotor rim.

Zonal Centrifuge Experiments, Group 2

Inasmuch as zonal selection provides a method of obtaining very high percentage single reovirus particle suspensions we prepared a fresh quantity of purified virus containing about 50 x more virus than that of the above zonal fractionation. This preparation yielded the velocity spectrum with a singles peak like that of Fig. 6, but the secondary peak constituting 31% of the first population examined now contained aggregates in the same size range but relatively few (less than 10%) of the total. Apparently freshly prepared freon-extracted virus has very few aggregated particles. In the peak fraction (#10) there were 87% singles. It is possible that at this high concentration (2.5×10^{10} virions/ml) some spontaneous aggregation took place before preparations could be made for EM. A part of this fraction was frozen immediately to preserve both infectivity and physical dispersion for subsequent bromine inactivation experiments.

Zonal Centrifuge Experiments, Group 3

One more zonal velocity spectrum experiment was made with crude freeze-thaw lysate of infected L cells without even low-speed clarification. This crude preparation contained the virus from the same number of infected cells as

that providing virus for the experiment just described. Again, a single particle peak was observed as in Fig. 6, and again there was very little virus in the region where aggregates of 2 to 8 were seen in substantial numbers in the first experiment. This unexpected result drew attention to the fact that the total quantity of virus recovered from the crude starting material was only about 1/5 of that usually recovered by freon extraction of an equal number of infected cells. It would appear that an excellent fraction of single particles can be obtained in this way with no previous purification, and that there are very few small aggregates in such a preparation but that the major part of the virus must have been in large aggregates or combined with larger cell debris which sedimented beyond the sampling range and reached the rim of the zonal rotor.

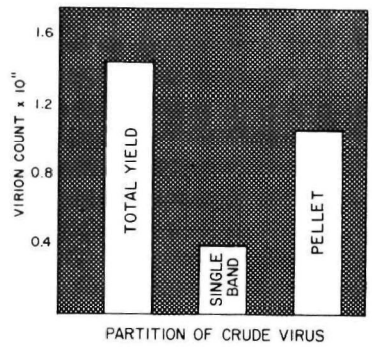
Bromine Inactivation of "Singles" fraction from velocity spectrum:

The singles peak fraction from zonal velocity spectrum (Group 2 above) was frozen at -40C in 1-ml vials to preserve infectivity and state of dispersion. Subsequently thawed samples had not lost infectivity and the relative number of single particles and groups in the size range of 2 to 8 had not changed, but bromine inactivation showed that changes had, nevertheless, taken place. After a rapid start, the reaction slowed and appeared to reach a resistant level at about 10^{-3} survival after 2 sec at 3 μM and 2C. If these surviving PFUs are aggregates, they must have "grown" from single particles during the short time interval before freezing or in the thawing process. This has lead us to doubt the value of freezing stock virus preparations to prevent the state of dispersion.

Five ml of the same virus were treated with bromine in the same way, except that it was centrifuged 17 min at 20,000 RPM at 20C in a Beckman SW50 rotor. Only the top 4 ml were used in the bromine experiment. All large aggregates should have been removed from this preparation. The resulting rapid linear decline in PFUs (Fig. 4) provides excellent confirmation.

Partition of crude reovirus between singles and large aggregates by means of a sucrose gradient in the swinging bucket centrifuge rotor.

Zonal sedimentation velocity spectra of crude reovirus (Group 3 above) yields a prominent sharp band of single virions and relatively few small aggregates, but most of the virus seems to have sedimented much faster than either. An experiment was done in the large (37-ml) buckets of the Beckman SW-27 centrifuge rotor to determine, if possible, the quantity and physical state of virions and the plaque titer of the two major components of this particle population, the singles band and the pelletized material. To do this, a 15-30% sucrose gradient was established and 10 ml of the freeze-thaw lysate (the same as that used in experiment 3) was layered over it. After spinning at 25,000 RPM (mean centrifugal field 81,000 XG) for 1 h, the supernatant fluid above the visible singles band was discarded. Ten ml, including the singles band, were collected and then the pelletized material was resuspended in the remainder of the supernatant fluid by pumping with a pipette. These two fractions will be called Band 1 and Pellet 1.



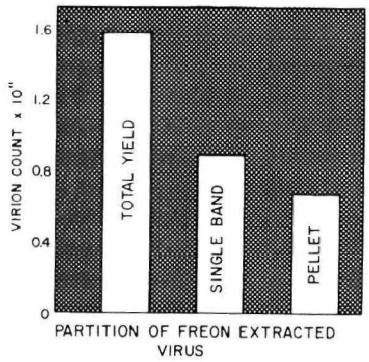


Fig. 7 The total yeild of reovirus extracted from a given number of infected cells is about the same whether it is done by 3 freeze-thaw cycles (top panel) or by freon extraction (bottom panel), but only 27% of it appears as a band of single particles when the crude virus is sedimented in a 15-30% w/w sucrose gradient. The rest is in the pellet. For freon-extracted virus the partition is 53% to 47%. Fig. 6 shows that there are very few small aggregates present.

An equal quantity of the crude virus preparation was extracted with freon and layered over a similar sucrose gradient, centrifuged, and harvested in the same manner, Band 2 and Pellet 2.

Half of the resuspended Pellet 1 material was extracted with freon, made up to the same volume (10 ml), and banded and harvested like the previous ones, Band 3 and Pellet 3.

A part of fractions B1 and P1 were treated 30 sec with 20-KHz acoustic waves from the micro tip of a Branson Sonifier Model LS-75. The sample volumes treated were 5 ml each, and they were immersed in ice water during treatment. The maximum temperature attained during treatment was 15C. There were 8 samples in all prepared for virion count and observation of aggregation in the electron microscope and for plaque titration. The results of physical assay are shown in Fig. 7. Particle count of pelletized fractions from freon-extracted preparations contained a large amount of cellular debris, which made the virions difficult to count. Nevertheless the total particle yield (sum of counts from pellet and band) was approximately the same. Freon extraction yielded 57% of the particles in the band, but with the crude virus only 28% was in the band. Freon extraction of virus from the pelletized fraction of the crude virus experiment (P1) yielded 67% of the particles in the band. Apparently the freon extraction process is quite efficient for extraction of reovirus in monodisperse form from the infected cells.

Plaque titration of banded and pelletized fractions from several identical partition experiments have shown 1/7 of the freon-extracted virus to be in the pellet and 6/7 in the band. The partition of PFUs in crude preparations has been erratic. The pelletized fraction always has much more than the band, and once there were 50 X as many PFUs in the pellet as there were in the band. Treatment of banded and pelletized fractions with 20-KHz acoustic waves has not made any substantial change in particle count but in this case it reduced the plaque titer of the pelletized fraction by a factor of 10. The same treatment has made no change in the titer of the well-dispersed virus in the banded fraction.

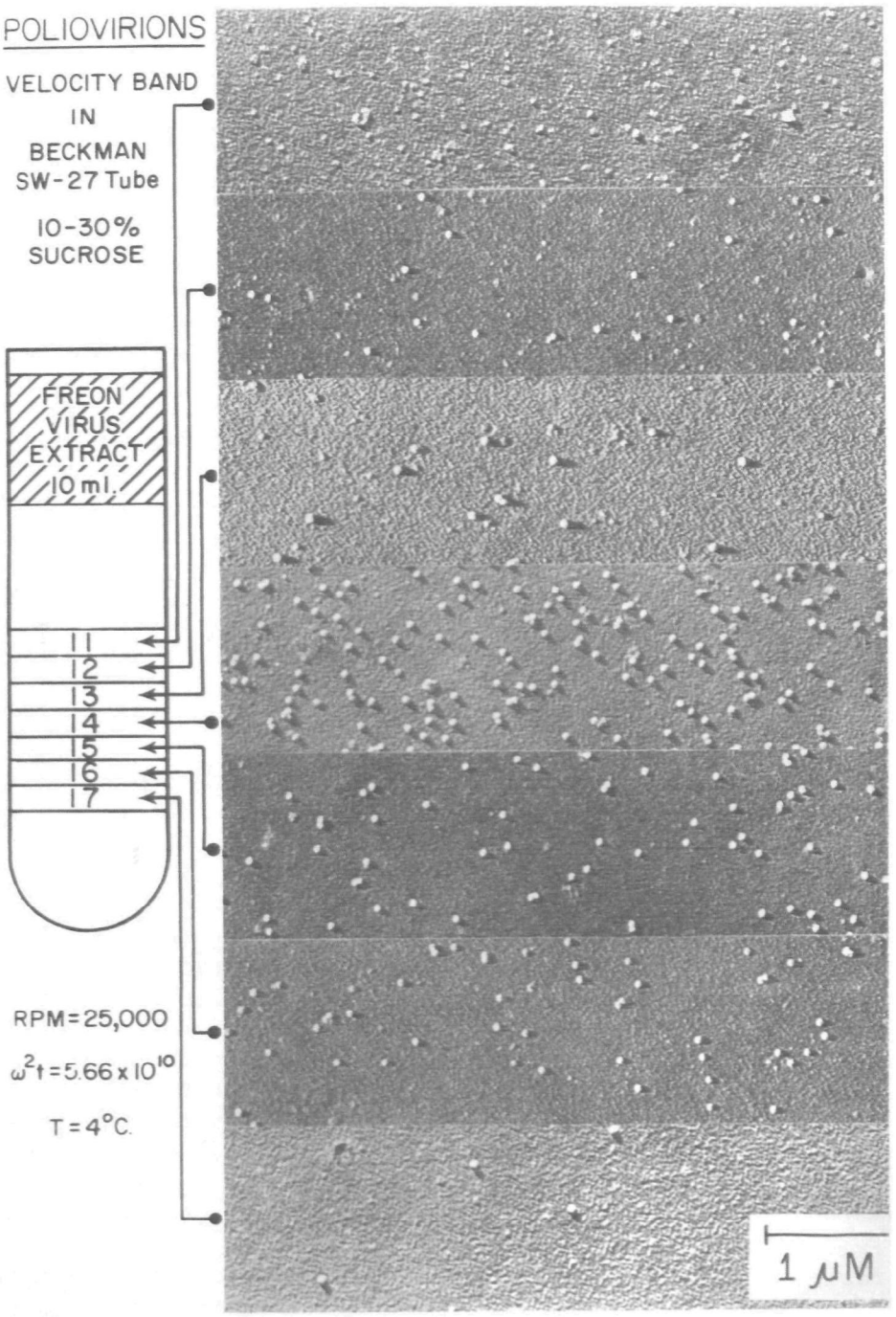


Fig. 8 Poliovirus velocity banded in a sucrose density gradient after freon extraction from infected HEp-2 cells.

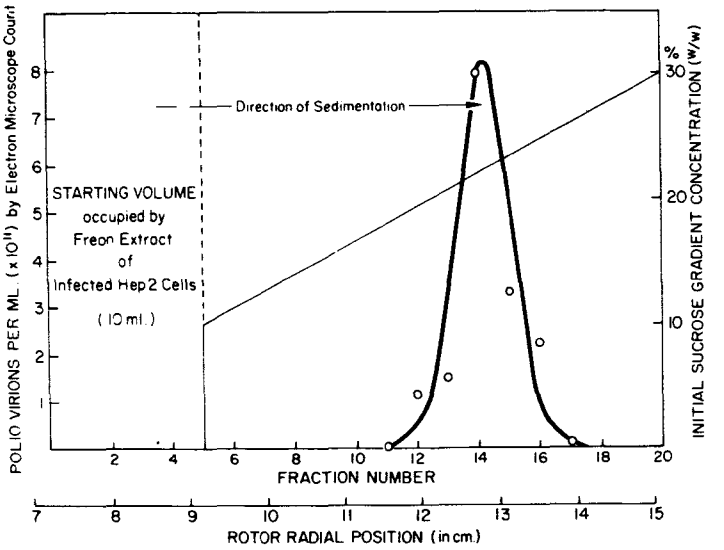


Fig. 9 Graphical display of poliovirus particle count by EM, taken from the fractions and pictures shown in Fig. 8.

POLIOVIRUS

The virus used in these experiments was extracted from the infected HEp2 cells with freon and velocity-banded in a sucrose gradient, and the particle content of several successive fractions from such a gradient is shown in Fig. 8. The concentration of particles calculated from counts from such pictures is plotted against radial position in Fig. 9. Many particles smaller than virus, probably ribosomes, can be seen in fractions 11 and 12 just above the virus peak. These were not counted. At no point below the main virus peak was there any secondary peak in the region where pairs, triplets, etc., would have been expected had they been present in substantial numbers. It appears from this that the freon extraction process has yielded a virus preparation with a very high proportion of single particles.

The strip pictures of Fig. 8 were taken at high particle concentration, presenting enough particles so the reader may judge their relative number and degree of purity. Other pictures made with more dilute preparations were used to determine the true degree of aggregation, free of the coincidence effect of one particle falling upon another by accident. Calculation of the number of such accidental pairs is made in the discussion. They indicate that the starting virus for these experiments contains at least 95% single particles.

Inactivation by bromine at 2C and pH 7

Six experiments were made at 2C, three of which are shown graphically in Fig. 10. All were characterized by an initial linear phase, constant decline in

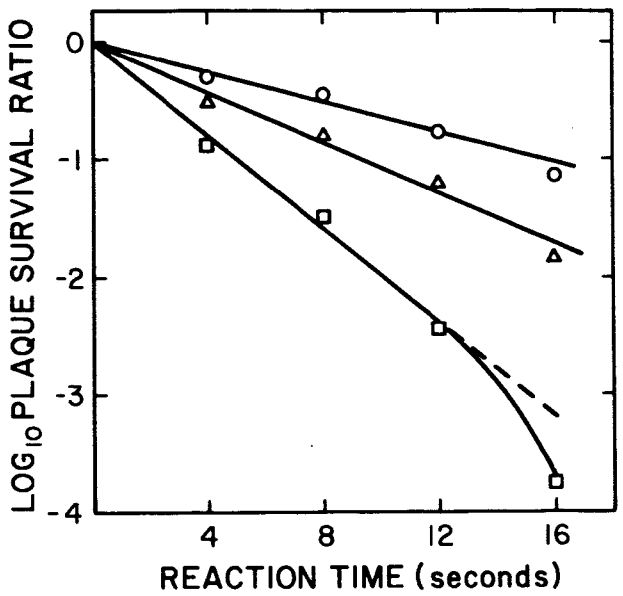


Fig. 10 Inactivation of poliovirus at 3 different concentrations of bromine at pH 7 and 2C; top line 0.6 μM, middle line 2.2 μM, and bottom line 22 μM.

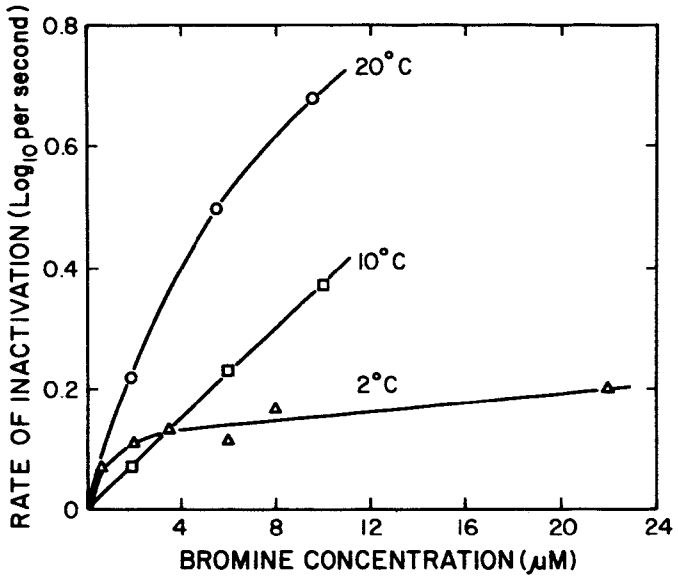


Fig. 11 Inactivation rates (Slopes in Logs/second taken from Fig. 10, 12, and 13) for poliovirus at pH 7 as function of temperature and bromine concentration.

log PFU survivor ratio per unit time of exposure. None of these reactions showed any tendency toward delay during the first time interval which was 4 sec. The slopes of all the reactions ranging from 0.6 to 22 μM bromine concentration have been plotted (Fig. 11) where it can be seen that increasing bromine concentration does not produce a linear increase in reaction rate. The increase becomes progressively less with increasing bromine concentration, indicating a progressive decrease in efficiency. Similar results have been calculated from the data of Weidenkopf[16] as previously published.[6]

In several inactivation experiments at bromine concentrations of 3.5 μM or greater, there has been a slight increase in the reaction rate after a substantial period of linearity (see the 22 μM line of Fig. 10). We have not been able to produce this effect regularly. Slopes for Fig. 11 were taken from the linear part of each survival graph.

Inactivation by bromine at 10C and pH 7

At 10C the inactivation rate was constant at 1.9 μM bromine concentration (Fig. 12). There is no indication, in this experiment, that there is any time delay before inactivation begins. However, at higher bromine concentrations the linear part of the graph begins only after the first 4-sec time interval (Fig. 12). It seems unlikely that the initial rate at the higher concentrations could be less than that of the straight line for 1.9 μM bromine so the lower curves have been drawn tangent to it at zero time.

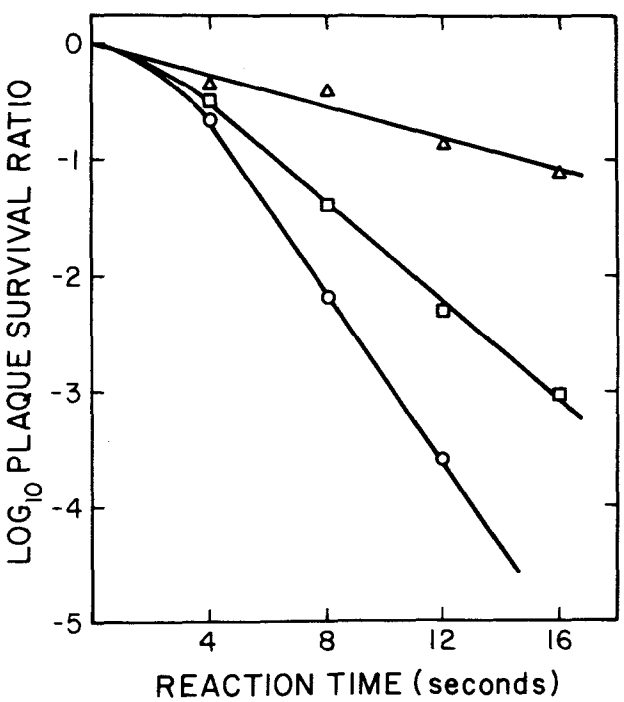


Fig. 12 Inactivation of poliovirus at 3 concentrations of bromine at pH 7 and 10C, top line 1.9 μM, middle line 5.9 μM and bottom line 10 μM.

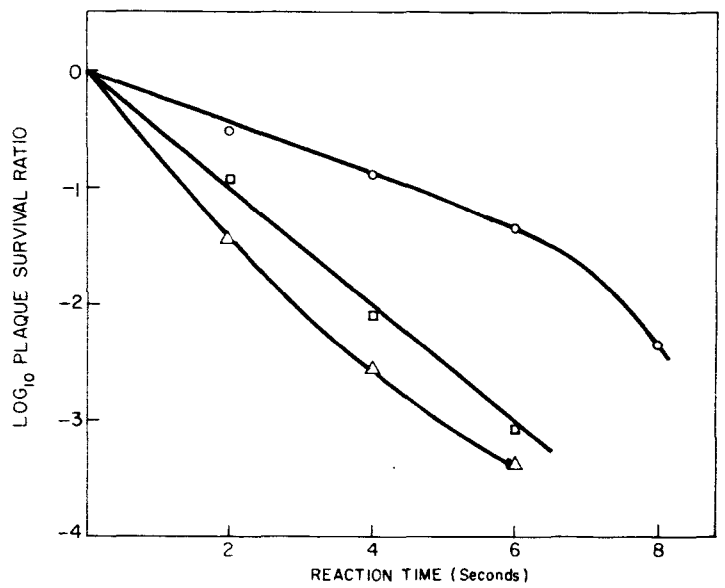


Fig. 13 Inactivation of poliovirus at 3 concentrations of bromine at pH 7 and 20C; top line 1.9 μM, middle line 5.9 μM, and bottom line 10 μM.

The reaction rates, slopes of the linear parts of the three kinetic experiments, are shown (Fig. 11) to be a linear function of bromine concentration with an intercept indicating zero reaction for zero bromine.

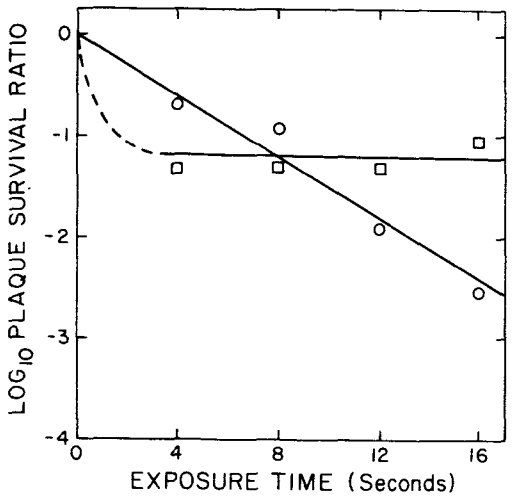
Inactivation by bromine at 20C and pH 7

At 20C the inactivation of the virus proceeds faster at all bromine concentrations that it did at 10C (Fig. 13). There is some indication in the graph for the 9.5 μM experiment that aggregation is showing its effect at survival levels below 10^{-3}. Reaction rates were determined from the straight part of each line.

Inactivation by chlorine

Published rates of inactivation of poliovirus by chlorine have been approximately one fifth of the rates we have found for suspensions of single poliovirus particles in bromine. We suspected that a part of this difference was due to the presence of aggregates in virus preparations used in earlier work so we made some chlorine experiments using the same apparatus and technique that were used for bromine, except that the pH was reduced to 6 in order that the major active agent would be HOCl for direct comparison with the HOBr of the earlier experiments. The buffer was the same (Phosphate), and its concentration in the flowing stream was 0.01 M.

Seventeen inactivation experiments were performed with chlorine concentrations ranging from 1 to 40 μM and temperature of 2C and 10C. Then eight more experiments of the same type were made without any chlorine. The



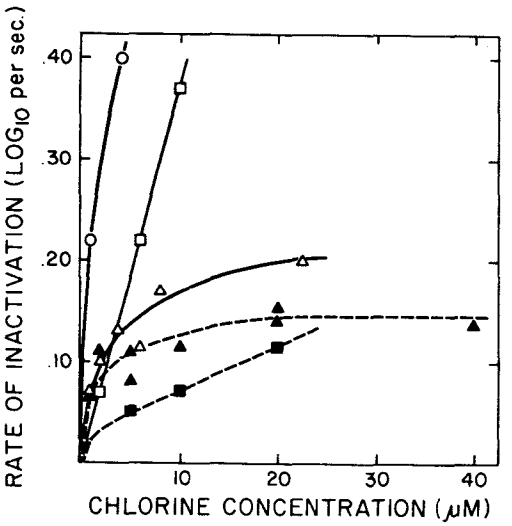


Fig. 14 (left) Inactivation of poliovirus by HOCl at pH 6 sometimes proceeds at a constant rate (circles) and sometimes it appears to start quickly then stop (squares).

Fig. 15 (right) Inactivation rate of poliovirus at 2C and at 10C as a function of HOCl concentration.

results were, at first, very confusing. Some of the experiments showed strictly linear semi-log disinfection behavior like that shown on Fig. 14 and others were characterized by a quick initial drop in titer followed by an interval of very little change (also shown on Fib. 14). When the slopes of all the experimental lines that were straight are plotted together as a function of HOCl concentration (Fig. 15), the relationship does not appear to be a simple one. At 2C the rate increases from about 0.08 logs/sec at 1 μM to double that value at 20 μM but there was no further increase in disinfection rate at 40 μM HOCl. The line looks as though it would intersect the vertical axis before reaching zero chlorine concentration. Inasmuch as this is not possible, the decline in rate below 1 μM HOCl must be very fast indeed. This in indicated by the dotted line on the graph.

At 10C the increase in disinfection rate with concentration of HOCl is linear from 5 to 20 μM but the line lies below that for 2C; the reaction is definitely slower in this concentration range, although it appears that it might rise above the 2C line if extended to higher concentrations. Something like this was encountered in the bromine experiments with poliovirus (also reproduced on Fig. 15 for comparison), but the reason for it is still obscure.

In an effort to explain the curved line of Fig. 14, a series of eight experiments was made without chlorine in the flowing stream. In the previous chlorine experiments the stock virus was diluted with 0.001 M phosphate buffer (pH 6) just before injection into the flowing stream of chlorine-water containing 0.01 M phosphate buffer at pH 6. Suspecting that dilution of the virus concentrate with reduction in both sucrose and salt concentration and

change in pH may have caused the virus to aggregate, we made dilutions from stock in chlorine-demand-free (CDF) water and reduced the buffer concentration in the stream from 0.01 to 0.002 M. Experiments run at 2, 10 and 25C all give titration curves like that of Fig. 14 with different degrees of initial drop, ranging from 0.3 to 1.4 log units. When the stock virus was diluted with 0.14 M NaCl solution and injected into 0.020 M buffer at pH 6 in the stream, there was no drop in titer other than that expected from dilution at the point of injection. Also, when the pH of the streams was raised from 6 to 7, there was no drop in titer with the passage of the virus through the apparatus. These experiments removed the possibility that virus was being inactivated by residual chlorine or chlorine compounds in the water after dechlorination, and they strongly suggested that curvature in some of the semi-log plots of survival titer may have been caused by aggregation of the virus during the experiments. As a result of this, a study was made of all experimental data taken with both reo and poliovirus during the past 3 years to learn what conditions, likely to be encountered in water experiments, would promote virion aggregation. Findings of this study and results of new experiments that it suggested are presented below in Physical State of Viruses in Water.

Inactivation by Tribromamine[13]

Nitrogen tribromide was generated at pH 4.5 by pouring together and stirring equal volumes of buffered ammonium chloride and standard bromine solution. The final molar ratio (NH_3:Br_2) was 1:3 in the test reactor. Identical ratio solutions were scanned in the UV on a Cary 14 Spectrophotometer against a buffered ammonium chloride reference to check the purity of the NBr_3 formed at 258 and 323 nm.

Reaction rates with polio virus at 4C were quite similar to those for HOBr at the same molar concentration, temperature, and pH. No evidence of curvature appears in the semi-log plot of survival ratio against treatment time, and the dependence of reaction rate on tribromamine concentration (Fig. 16) is quite similar to that of HOBr (Fig. 11).

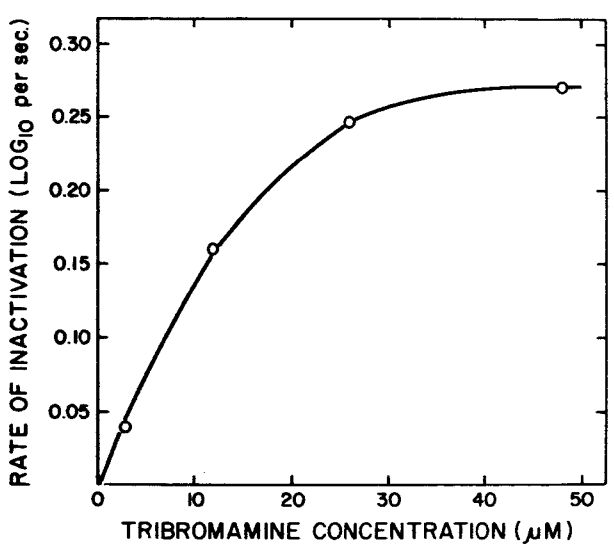


Fig. 16 Inactivation of poliovirus by tribromamine.

THE PHYSICAL STATE OF VIRUSES IN WATER.

Reovirus

An example of many concentrates prepared by freon extraction and

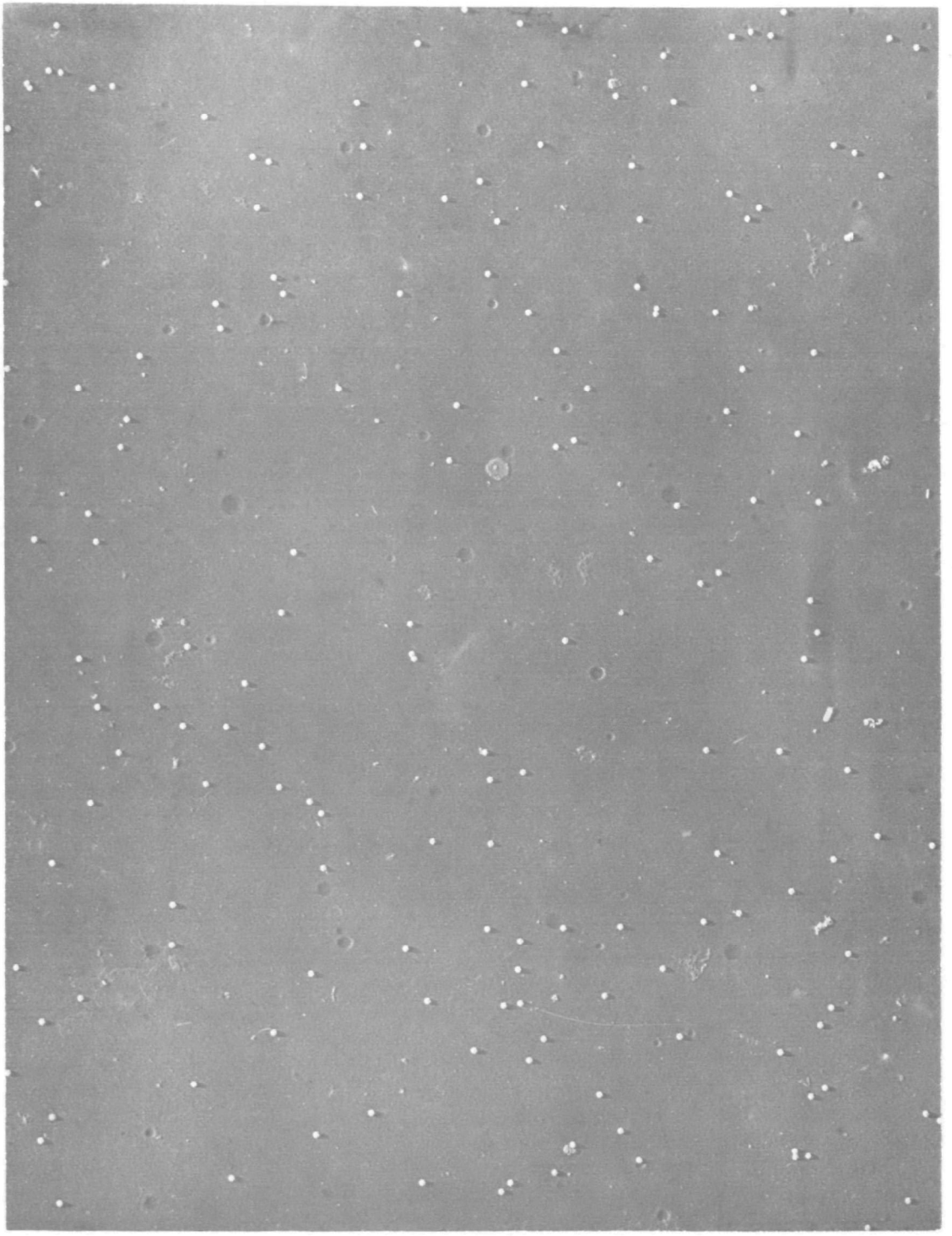


Fig. 17 A stock reovirus preparation diluted 10 x then 20 x with PBS. The virions are well dispersed.

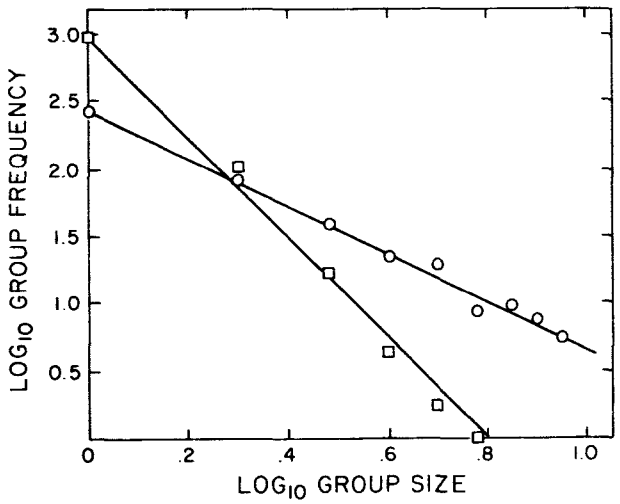


Fig. 18 The distribution of group sizes counted from 5 pictures like Fig. 17 and from 5 more pictures aggregated like Fig. 19.

velocity banding in sucrose containing 0.05 m phosphate buffer pH 7.0 is shown in EM picture Fig. 17. The stock was diluted in two steps, 10 x then after 3 h, 20 x more with PBS and sedimented for agar pseudoreplica counts by EM. The number of single particles, pairs, etc., per thousand counted is plotted as squares on the frequency chart Fig. 18. The degree of aggregation is very nearly the same in every preparation. Single particles are 79% of the total, which was 1.5 x 10^{10} virions per ml.

Quite a different picture is obtained when this stock is diluted the first 10 x step with water and then 3 hours later the second 20 x step with PBS as above. The water dilution reduces the sucrose to about 3%, the buffer concentration to 5 mM, and the virion concentration to 1.5 x 10^{9}/ml. These conditions regularly produce the aggregation seen on Fig. 19, and it is apparent that the aggregates were 1) not present in the stock concentrate and 2) they were not dissolved when PBS was added in the final 20 x dilutions. Their frequency is plotted as circles on Fig. 18, where the decreased slope shows the change in state of dispersion.

The physical state of the virus in the undiluted concentrate cannot be seen by EM, but pictures of virus deposited by Brownian bombardment of an aluminized collodion film in contact with a 5 x PBS dilution of the concentrate look just like Fig. 17 and the frequency distribution of group sizes from such a set of pictures is like the squares of Fig. 18. This preparation was diluted 100 X in one step with 5 mM phosphate buffer at pH 7 and sedimented for agar pseudoreplica count and group frequency counts. The plot of frequency distribution was indistinguishable from that of the 5 x dilution above. Presumably 100 x dilution did not change the relative frequency of the aggregates. They neither increased nor broke up.

Sedimentation velocity analyses of freon-extracted concentrates have shown faster moving peaks corresponding to pairs and other small groups,[8] but the presence and size of such peaks seems to be related to storage time (4-6C). Freshly prepared virus has not shown any sedimenting peak where pairs should be. Equally sharp single peaks have been seen when the sucrose gradient contained 0.85% NaCl without buffer, 0.01 M phosphate buffer at pH 7 or no salts at all. But dilution of concentrates with no salt at all have given irregular results. Dilution of such a concentrate containing 1.5 X 10^{10} virions per ml with 9 volumes of PBS produced aggregates on 15 min of

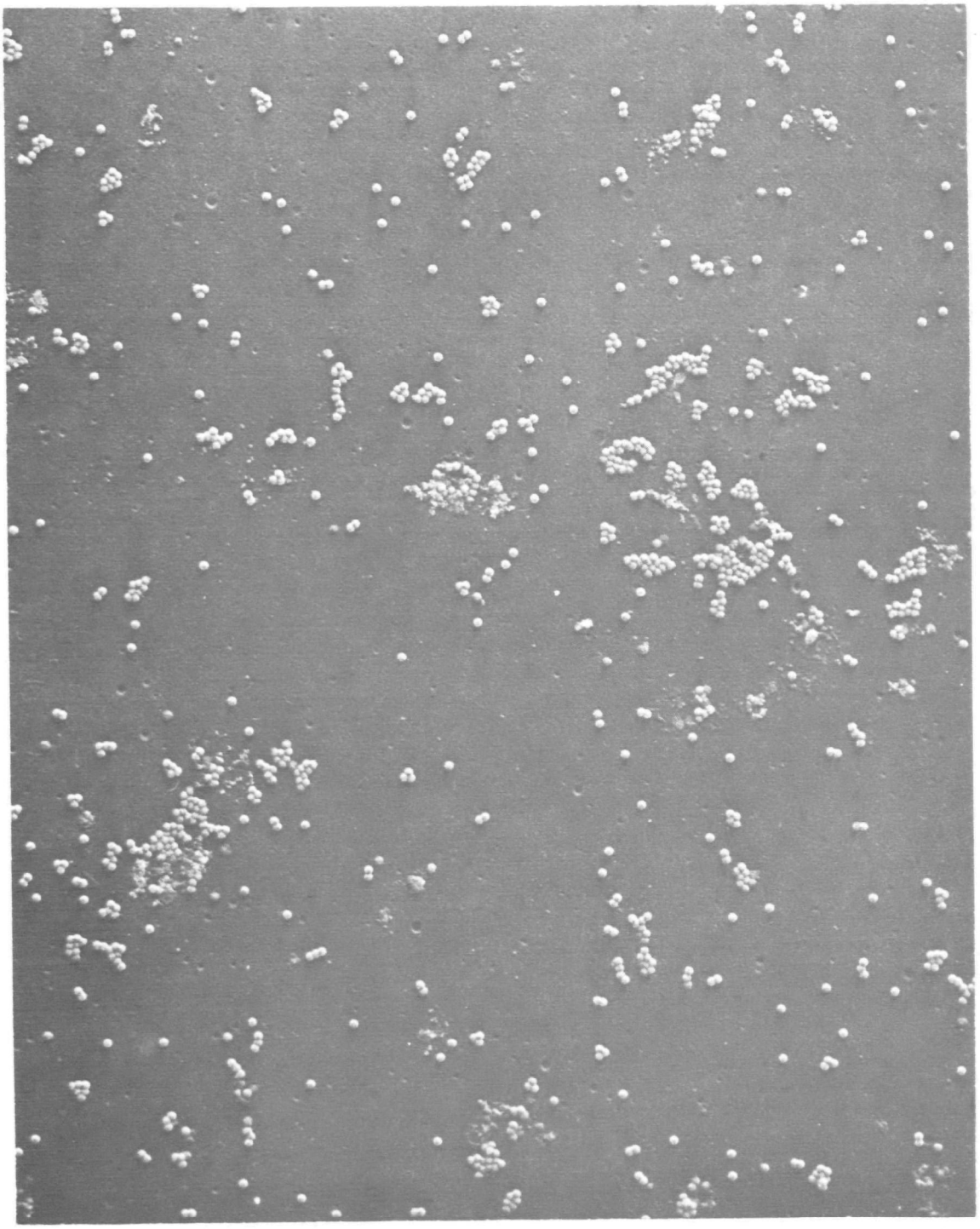


Fig. 19 A stock reovirus preparation diluted 10 x with water then 20 x with PBS. The virions are aggregated. Group frequencies are shown on Fig. 18.

standing that were not dispersed by 15-fold further dilution with PBS, but when the two dilution steps were made without delay or if the 150 x dilution were made in one step, there was no aggregation.

The water-banded virus (sucrose gradient without salt) had only a single peak that we proceeded to use as a source of single particles for a bromine inactivation experiment, but we found after the experiment that the virus had aggregated when it was diluted into the 0.01 M phosphate buffer at pH 7 Fig. 19. It is not possible to say just how much of the aggregation we saw was present during the 4 sec of the bromine action, but the semilog plot of the surviving virus titer was curved like Fig. 4, very different from that obtained subsequently when we were able to produce suspensions of singles only. So it is almost certain that this curve was caused by aggregation of the virions either just before or during the disinfection experiment, even though the starting virus showed no aggregation.

Other independent evidence of aggregation of reovirus particles, particularly when they are diluted from sucrose-banded concentrates into water, comes from partition experiments in a swinging bucket centrifuge tube. If a centrifuge tube is filled with a diluted suspension of virus and spun at such a speed and time as to just sediment all the single virions out of the top quarter of the tube, and the rotor is carefully stopped and the top half of the supernatant fluid is collected, mixed and titrated, there should be 1/2 of the original titer remaining if all the particles in the tube were singles. If there was aggregation present in the suspension, the remaining PFU must be less than 1/2. While this test will not give a strictly quantitative measure of the aggregation, it has the advantage of providing evidence for or against aggregation at virion concentrations much less that those required for electron microscopy. This Half and Half experiment was tried on a stock of poliovirus containing 3×10^{10} virions per ml in 0.05 M phosphate buffer at pH 7 in 33% sucrose. This stock virus was diluted 10 x with water and with PBS. After standing for 3 h at room temperature, both dilutions were diluted another factor of 10 with the same diluent as before. Both were titrated and the PBS dilution revealed 5.7×10^{6} PFU/ml, while the water dilution made 3.2×10^{6} plaques per ml. This small reduction in titer might be evidence of aggregation of the virions in the water. Both dilutions of virus were then subjected to the sedimentation velocity partition experiment described above. The saline dilution left 56% of the PFUs behind in the top half of the supernatant fluid, while the water dilution left only 21%. Apparently many of the PFUs in the water dilution sedimented faster than the single virions, which have been shown to be the chief component of the saline dilution.

Poliovirus

The physical state of poliovirus after freon extraction and velocity-banding in a density gradient of sucrose has not been determined as well as that for reovirus. Poliovirus cannot be prepared for EM counting and aggregation analysis by the agar pseudoreplica method. Only the KA method has been successful in producing EM pictures for aggregation analysis, therefore only virion concentrations 10^{10} and above can be examined directly.

When poliovirus is extracted from infected cells with freon and velocity-banded on a sucrose gradient at 5×10^{11} virions per ml, the suspension does not scatter enough light to be visible to the unaided eye. This water-clear virus suspension contains .05 M phosphate buffer at pH 7 and 22% sucrose. Dilution with PBS by a factor of 5 or more permits kinetic attachment of single virions and aggregates to aluminized films for EM. Four pictures from a 10 x PBS dilution of virus were divided into 5 equal areas each and counts of single particles, pairs, triplets, etc., were made on all 20 areas. A part of one such picture is shown (Fig. 20), and the group frequency data are shown in Table 1, where it can be seen that the mean singles count per area was 124 with a standard error σ=15 and a mean frequency of pairs was observed to be 3.4T of the total count. Inasmuch as 124 particles would "exclude" 6% of the picture area, we estimate an average of 7 pairs per picture would have been produced by one single particle accidentally falling upon another.[14] The expected accidental frequency of triplets and groups of four has not been calculated, but it appears from the number of pairs observed that the actual amount of aggregation in this polio stock suspension is very near zero, even though the total particle concentration is 6×10^{11} per ml. These pictures were made 19 days after this stock solution was prepared. Another observation made 81 days after preparation made from plates averaging 1104 total virions showed an average of 86 pairs per picture. Since 64 were expected by coincidence, there seems to have been an irreversible pair production of 22 per 1104 or about 2% in this time.

EM Observations

As with reovirus, dilution of poliovirus stock with water (without buffer or neutral salt) tends to aggregate the virions, but the nature of the aggregation is very different from that seen with reovirus. In Fig. 21 is shown a 10 x water dilution of the same stock virus from which the salt dilution gave Fig. 20. The polio aggregates are fewer, contain many more virions than reo aggregates, and do not spread out flat when deposited on the aluminized film for EM. They appear to be more tightly bound than the reo aggregates that spread out and rarely appear to pile up. Still, the polio aggregates formed by water dilution are readily broken up by addition of salt to the suspension. A 2 x further dilution of the virus of Fig. 21 with PBS dispersed the aggregates quite completely (Fig. 22).

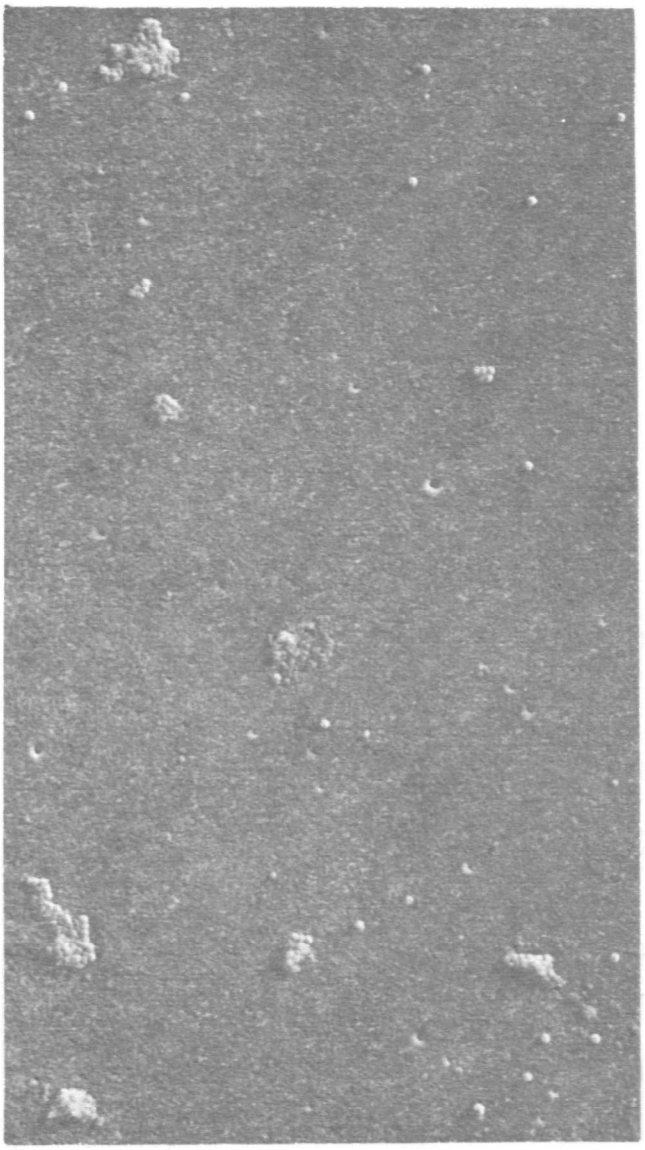
At pH 7 in .05 M phosphate or tris buffer, the virus is physically stable (as it is in .05 M borate buffer at pH 8 and at pH 9), but at pH 6 and .05 M phosphate buffer, aggregates form as they do also at pH 5 in .05 M acetate buffer (Fig. 23). There is some evidence already that these isoelectric aggregates are dispersed when the pH is increased to or above the neutral range.

We have not observed any conditions under which any virus which aggregates when diluted with a given diluent solution will deaggregate on further dilution with the same diluent. In the present work, we were limited in search of such data by the lack of sensitivity of the KA method. Nevertheless, it is a matter of considerable importance to know what happens to

TABLE 1. EXAMPLE OF NUMERICAL DATA FROM 20 PICTURE AREAS TAKEN WITH POLIOVIRUS PREPARATIONS LIKE THOSE ON FIG. 20.

Group Size	Group Frequency	Standard Error σ	Total Virions Counted
1	124	15	2480
2	4.5	2.1	89
3	0.35		7
4	0.05		1

Fig. 20 Typical poliovirus preparation for EM count by the kinetic attachment method. The virions are well dispersed.



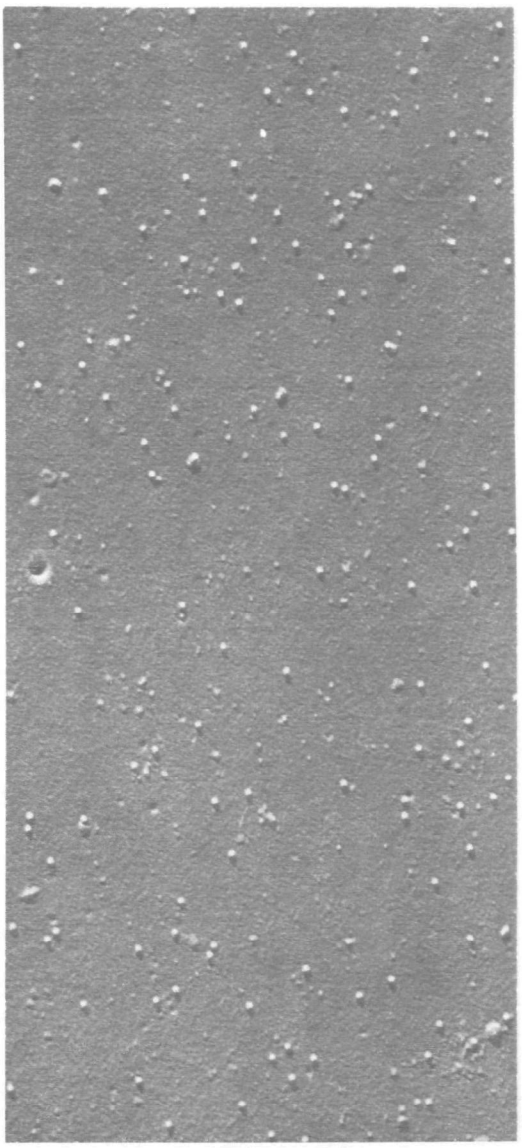


Fig. 21 (left) Water dilution (10 x) of poliovirus stock suspension aggregates most of the virions.

Fig. 22 (right) The aggregated poliovirus of Fig. 21 is dispersed by dilution with PBS.

such aggregates as are formed when stock viruses are diluted 5 to 10 x in water, when they may be diluted much further with water.

Dilutions of poliovirus in water - beyond the level visible in the EM.

It was determined by experiment that 20 min at 30,000 RPM would sediment about 50% of well-dispersed virus out of the top half of an SW 50.1 centrifuge tube at 20C. When such a tube was filled with a 10 x water dilution of

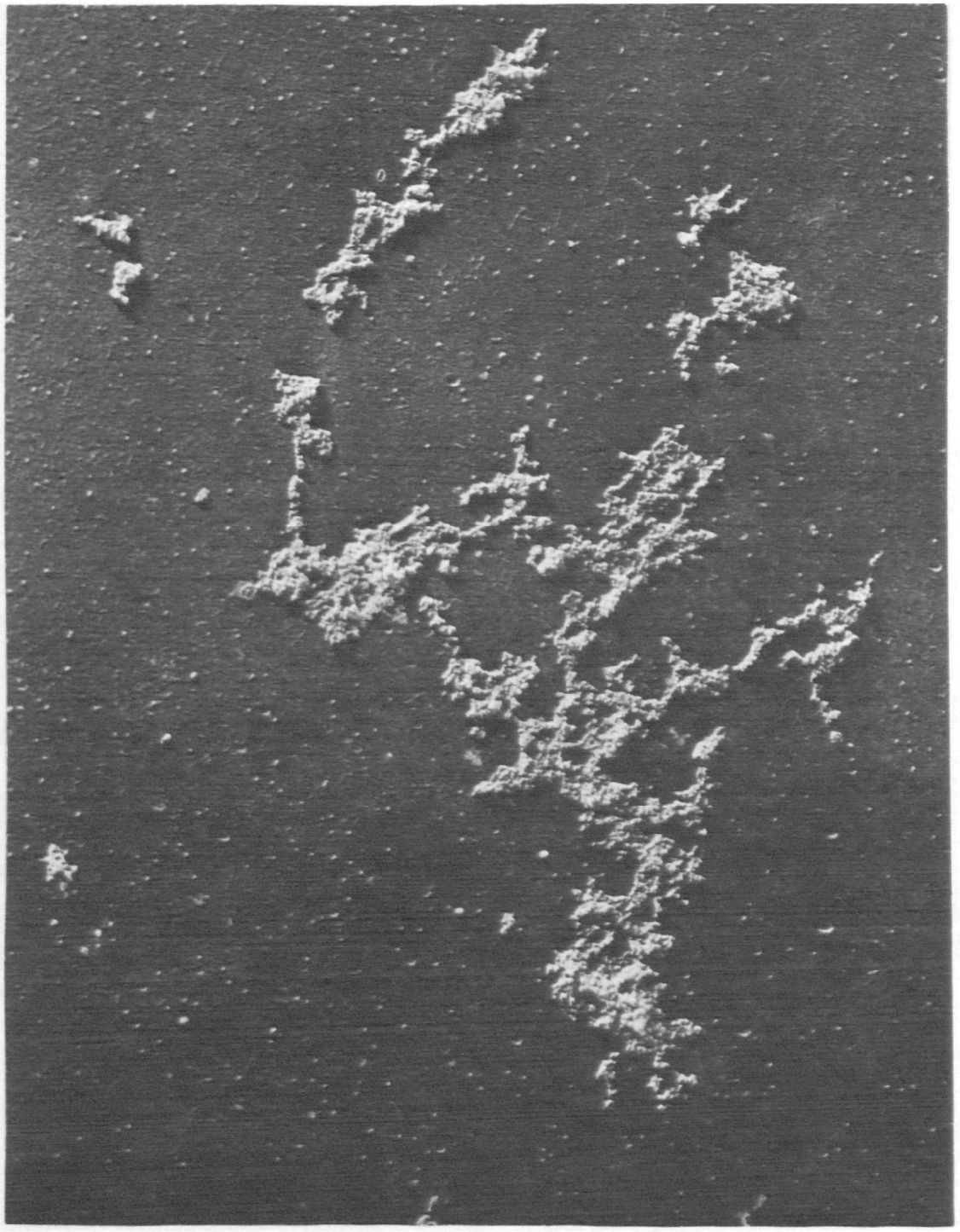


Fig. 23 Poliovirus aggregates in .05 M Acetate buffer at pH 5.

stock virus, allowed to stand for 1 h at 25C, and then centrifuged (using slow start and coast stop) 4% of the original PFU remained in the top half of the test tube. When this same water-dilution was diluted 2 x further with PBS and centrifuged as before, 48% of the PFUs were found in the top half of the tube. This experiment, performed at about 2 x 10^8 PFUs/ml (2 x 10^{10} virions/ml), shows that the aggregates were present in the initial water dilution but that they were essentially dispersed by further dilution in PBS.

When 10 x PBS dilutions of virus were diluted with more PBS to ca 10^3 PFUs/ml. 58% of the original PFU remained in the top half of the centrifuge tube after partition, showing good dispersion of the virions. Water dilutions of the original 10 x water dilution left much less virus in the supernatant region, ranging from 14% down to 1.3%. Apparently the aggregates that were formed by the first 10 x water dilution and seen at that level of concentration by EM do persist when diluted 100,000 times further.

Because dilutions for plaque titration are normally made with PBS, it was not surprising to find that 10 x water dilutions of stock poliovirus usually gave the same plaque titers as 10 x salt dilutions, even though the former must have been severely aggregated at the start of the dilution series for titration. When dilutions of the two were made with 1/4 molar sucrose solution and monolayer cells were washed with the same before receiving the virus inoculum, the titer of 10 x water dilutions (aggregated virus) was usually lower than that of the salt dilutions but never less than half, and sometimes the two titers were the same. This is the only attempt so far to dilute the virus and put it on the cell monolayer in aggregated form.

Stepwise dilution of stock virus by factors of 10 in water have produced stable aggregates, expecially if there is some time delay after the first step. However, dilution of the same stock virus by a one-step factor of 100 with water has sometimes produced suspensions in which no aggregation could be detected by sedimentation partition.

Natural Aggregates of Poliovirus Particles

The reovirus can be photographed directly in the EM as it emerges from infected L cells (Fig. 24). With poliovirus, evidence for original aggregation is not quite so direct, but cytoplasmic crystals of poliovirus are shown in the textbooks, and we have dialyzed crude freeze-thaw lysates of infected HEp2 cells over Amicon XM300 filter membranes and managed to get clear pictures of large polio particle aggregates (Fig. 25).

These aggregates are generally different from the induced aggregates just described. They are usually not dispersed by any of the conditions that easily disperse the induced aggregates. They are probably present along with induced aggregates in such preparations as the dialyzed one mentioned above. In fact, Fig. 25 might be showing both. There are groups showing evidence of two dimensional symmetry which may be reassembled from singles, and there are other groups enmeshed with what appears to be the cellular material in which they were formed.

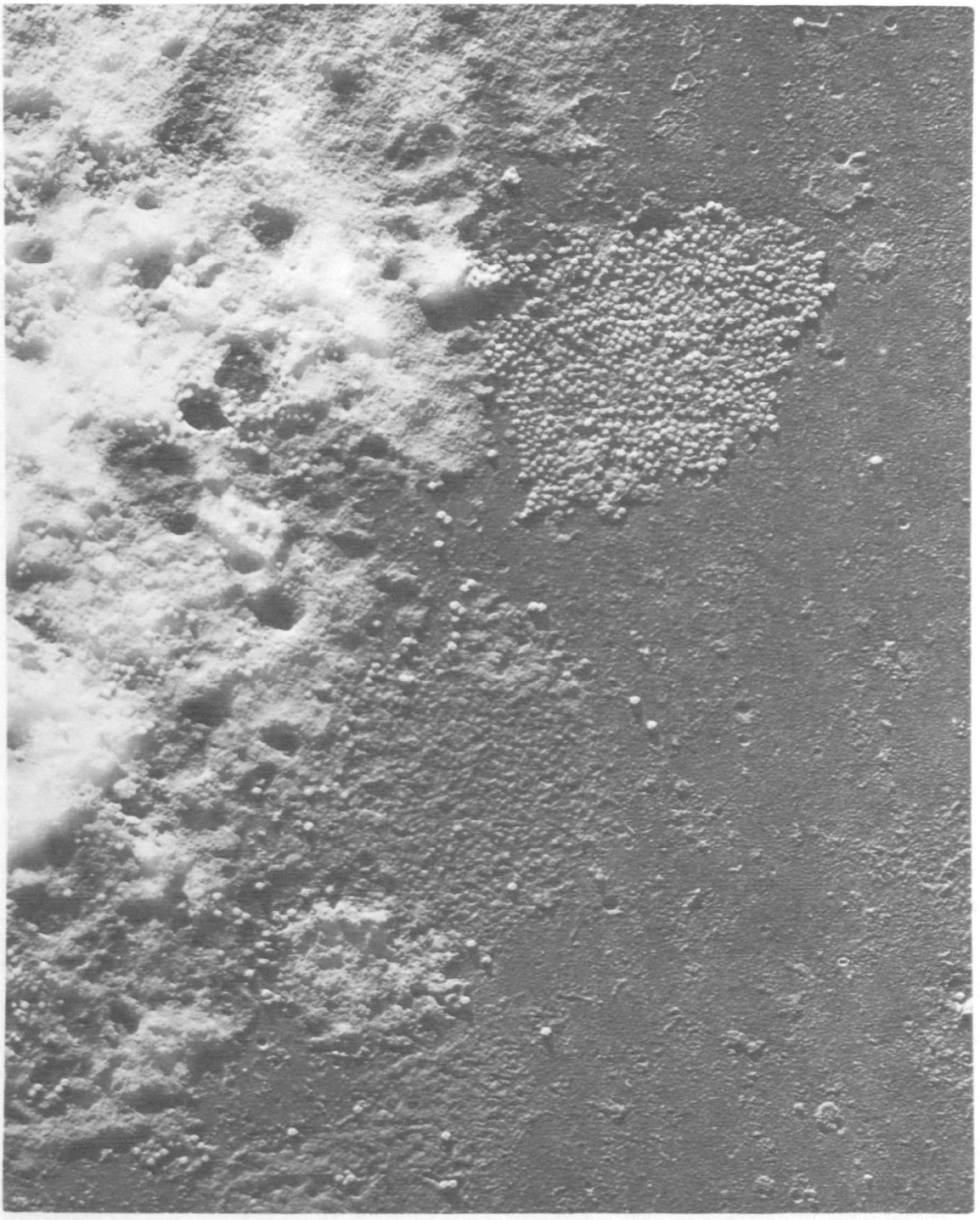


Fig. 24 Reovirus within and emerging from the periphery of an infected L cell. Such large clumps are very common.

Fig. 25 Poliovirus, dialyzed crude preparation showing aggregates of virions enmeshed with cellular debris and other aggregates that appear to have reassembled after dispersion.

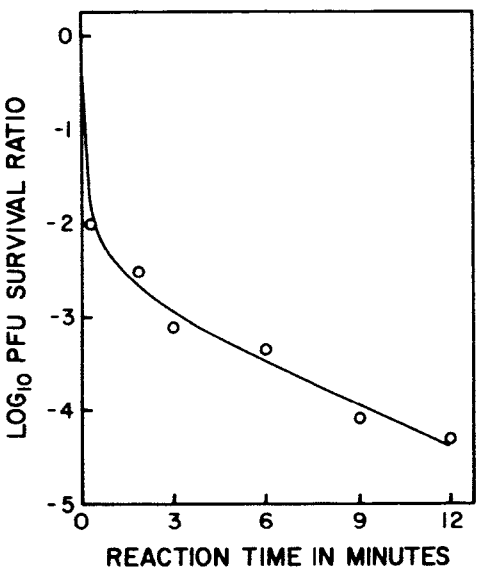


Fig. 26 Inactivation of poliovirus, dialyzed crude preparation, by 10 μM bromine at 2C.

The resistance of plaque titer to the action of bromine on suspensions of aggregated poliovirus has been compared with that of single particles.

In Fig. 26 are the results with 10 μM HOBr at pH 7 acting at 10C upon crude virus rendered essentially demand-free by dialysis across Amicon XM 300 membranes. The semi-log plot of survival in HOBr was very similar to that for aggregated reovirus. After the curvature begins, at the 2 log level, the survival of aggregated PFUs quickly becomes several factors of 10 greater than the projected survival of singles (Fig. 12). This discrepancy gives every indication of increasing with continued treatment.

Most of our virus preparations above started with the release of the virus from infected cells by freeze-thaw cycles in buffered saline solutions. Virus in field water will probably not come about that way. So we have collected some virus by freeze-thaw release directly into distilled water. Such preparations have some unexpected characteristics, as revealed by the zonal centrifuge. After velocity spectrum runs like that of Fig. 6, titrations were made on the water virus and there was no peak where the single particles ought to be (Fig. 27). A repeat of this same experiment with another virus preparation months later gave the same results. A broad peak of PFU appeared where groups of 4, 5, or 6 would be expected, but no peak appeared where it did when dispersed virus was centrifuged the same way. We cannot count poliovirus at these concentrations in the EM, but these titration experiments show that it is aggregates with which we must deal when this work moves into the field where the water is not nicely buffered with appropriate salt solutions at physiological concentrations.

These experiments and some suggestions by Dr. Stephen Schaub led us to examine the state of poliovirus put into water at about the concentration level of 10^4 PFUs/ml. This involved preparing virus that was monodisperse and virus that consisted mostly of natural aggregates. Each of these was into water at about 10^4 PFUs/ml, and an appropriate test was made immediately and again after 1 h to tell whether either preparation had become more or less aggregated.

The "appropriate test" we chose was the one shown Fig. 28 involving a small plastic horizontal centrifuge tube with 1 ml of 50% sucrose in the bottom, 2½ ml of 20% sucrose above it, and 2 ml of the dilute virus sample to be tested at the top. A control tube is prepared the same way except that

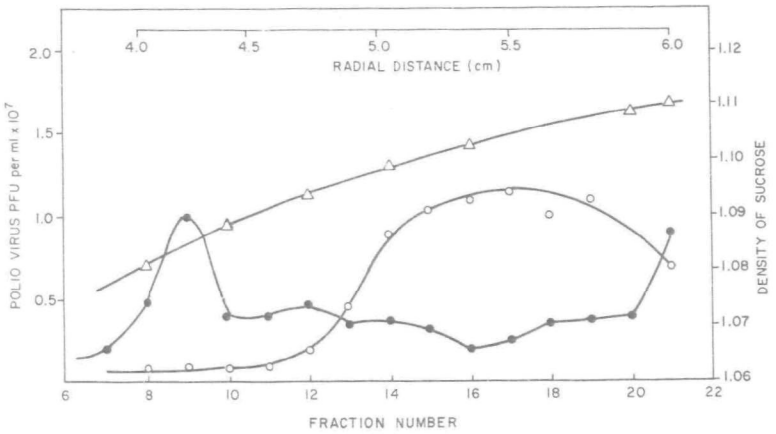


Fig. 27 Zonal Sedimentation Velocity spectrum of the poliovirus PFU in a 15-30% w/w sucrose gradient. Peak of single particles shows clearly in the freon-extract preparation (open circles), but when virus was released from infected cells by 3 freeze-thaw cycles, there was no single peak, only a broad band in which there were small aggregates (open squares).

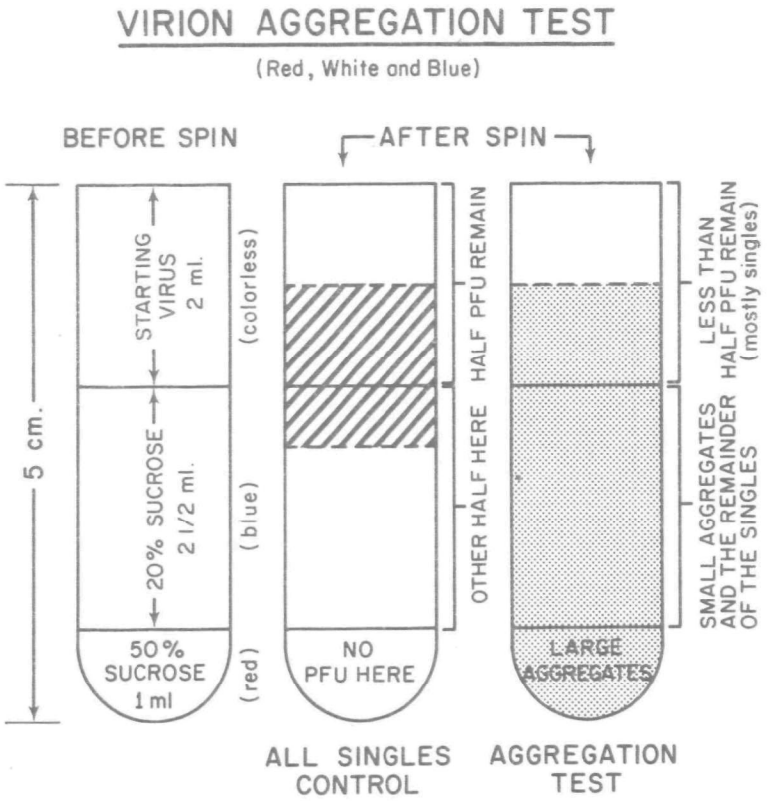


Fig. 28 Virion aggregation test (red, white, and blue) using centrifugation in small swinging bucket tubes with step gradient of sucrose. PFU titer of the 3 colored regions after centrifugation gives a measure of virion aggregation.

TABLE 2. THE PHYSICAL STATE OF POLIOVIRUS IN WATER.
DATA FROM THE RED, WHITE AND BLUE EXPERIMENT OF FIG. 28.

Water Type		Distilled (%)	University Lake Turbid (%)	University Lake Clarified (%)	Alum-Treated (Finished) Dechlorinated (%)	Phosphate Buffered Saline (%)
Input-Single Virions						
1 min	W	36	57	57	6*	60
	B	62	31	39	51*	37
	R	2	12	7	43*	3
60 min	W	32	58	51	6*	55
	B	62	33	45	50*	44
	R	6	9	4	44*	1
Input Aggregated Virus						
1 min	W	7	8+	15	10	54+
	B	21	22+	34	19	42+
	R	72	70+	51	71	4+
60 min	W	5	16+	14	7	60+
	B	26	30+	43	19	37+
	R	69	54+	43	74	3+

* Average of 4 experiments.

+ Average of 2 experiments.

the top section contains the best preparation of single virions we can produce. A little neutral red in the bottom section and some trypan blue in the middle section helps locate all three (red, white, blue) for plaque titration after centrifugation. The pairs of tubes are spun long enough to sediment single particles about half way through the top (white) section. This has been determined by experiment, and the value (30,000 RPM at 20C for 20 min) agrees quite well with that calculated for polio whose sedimentation constant is 160×10^{-13} S. When this is done, the other half of the PFUs in the control tube appears in the middle (blue zone) and only traces have been found in the red. Samples containing aggregated virus together with singles will leave less than 50% of the PFUs in the white zone with the rest distributed between blue and red, according to the frequency of small and large aggregates. The red, white, and blue (RWB) diagnostic technique has been used also to separate a single crude (freeze-thaw) virus preparation into two parts. The white part will contain practically all singles, while the red zone contains only aggregates with very few singles. These two fractions have been diluted about 5000 x with distillled water, one drop in 200 ml in 250 ml Erlenmeyer flasks with magnetic stirring bar. These were stirred gently and held at 25C. One sample of each was removed after 1 min and another after 1 h. Each was put in an RWB tube, and spun at 30,000 RPM for 20 min at 20C. All three sections of each tube were then titrated on monolayer cultures of HEp-2 cells, and the results were compared with previous runs with specially prepared singles. The results showing the aggregation spectrum of virus in distilled water are in Table 2, where it can be seen that single particles put in distilled water remain singles and that natural aggregates remain aggregated at least for the 1 h of the experiment. Similar data were obtained with natural resource water (University Lake - water supply for the city of Chapel Hill) with and without particles. But the results were very different when the finished water from the filter plant was used. The table shows that this water aggregated the virus. This experiment has been repeated 4 times with the same result. It is possible that trivalent aluminum and divalent calcium ions left over from the lime and alum treatment of the water are causing the virus to aggregate when it is put into this treated water. We expect to investigate this soon.

DISCUSSION

INACTIVATION OF REOVIRUS

Reovirus was chosen for a part of this work from among the many water viruses because of its size which makes it readily amenable to physical assay by two essentially independent methods. Earlier work [1] has shown that the reo particle counts and aggregation analyses made by the KA method of preparation for EM yield the same results with suspensions of 10^{10} virions/ml as those obtained by the sedimentation or agar pseudoreplica method on the same suspension after approximately 100-fold dilution. Demonstration of this fact is essential, of course, for if it were not so, the dilution of suspensions of aggregated reovirus for plaque assay would yield equivocal information about the bromine resistance or plaquing efficency of the aggregates seen in the EM. For this reason, reovirus may provide a useful model of refer-

ence when experiments of this kind are made with the many smaller picorna viruses that are of greater significance as water pollutants but which are more difficult to assay by physical means.

The reaction of bromine (3 μM) with preparations of reovirus in water at pH 7 is shown here to destroy 99.9% of the PFUs in 1 sec at 2C if the preparation contains predominately single virions. After the most strenuous efforts to remove all aggregated virus, the logarithm of the survival ratio is an almost linear function of the time of exposure to the bromine at least to the level of 1:10,000 surviving PFUs, the titer falling to the rate of 3.0 $\log_{10}$/sec. This is 25 x faster than the rate observed with poliovirus under similar conditions of bromine exposure.[6] It is more than 100 times faster than rates indicated by Scarpino et al[15] and Weidenkopf[16], respectively, for chlorine at pH 6 and the same molar concentration and approximately the same temperature. Comparison of bromine rates at pH 7 with those of chlorine at pH 6 seems most appropriate for comparison of the effects of HOCl and HOBr.

While reovirus, velocity banded in a sucrose gradient, is usually 90 to 95% single particles, it gradually aggregates in such a way that the log of the frequency of any group size becomes and remains a linear function of the log of the number of particles in the group (Figs. 3 and 18). Inactivation by bromine of such aggregated virus is characterized by a continuously decreasing decay constant. This indicates that plaque-forming aggregates are more resistant to bromine than single virions.

SURVIVAL OF REOVIRUS PFU THROUGH VIRION CLUMPING - A CLOSER LOOK

If about one PFU in 10,000 of the starting titer were an aggregate large enough to protect one potential plaque-forming virion from destruction by the bromine,[5] this would be enough to account for the persistent infectivity seen in the experiments of Fig. 2. If such an aggregate consisted only of virions (no extraneous material) there would seem to be no way that such small groups as 2, 3, or 4 could conspire to protect even one of their number from attack by the large number of small bromine molecules or ions. Effective protection would not seem possible with much less than a complete monolayer of particles surrounding the protected one, and this can happen with spheres of equal size only if there are at least 17 in the clump. Clumps of this size would be easy to see with the EM, but at a frequency of 10^{-4} they might go unnoticed. The pictures did show that no groups larger than 7 virions were present at a frequency greater than 1:1000 and such a cluster could not quite cover one half of the surface of one of them. It could not afford that one much protection. How then, can such a survival curve as that shown for the slightly aggregated reovirus of Fig. 4 come about? There have been suggestions[3,4].

A part of the graph (Fig. 4) has been repeated twice for demonstration of two different qualitative analyses. If all the virions are alike in this mixture of singles and aggregates and the probability that any single particle will make a plaque is E, then the starting titer when t = 0 (before bromine treatment begins) must be given by:

$$T_0 = E(N_1 + C_2N_2 + C_3N_3 + \cdots C_mN_m)$$

where N_1, N_2, N_3, etc., are the numbers of singles, pairs, triplets, etc., M is the maximum clump size present in significant numbers, and C_2, C_3, C_4, etc. are coefficients, the values of which we will now seek.

If we were dealing with perfect virus (E=1.00), then each single, each pair, each triplet, etc., would make just one plaque and no more; $C_2=C_3=C_4$ etc. =1. But the reovirus used here had about 40 X more particles like those of Fig. 17 than the plaques it produced. This is saying that on the average only one virion in about 40 does make a plaque. If random pairs, triplets, etc., are made from such singles there is only 1:40 chance of finding 2 of the plaque-forming singles in one such pair so there will be little loss through redundancy, and the probability of plaque formation by a pair, triplet, etc., would seem to be very nearly 2 X, 3 X etc., greater than that for single particles. Briefly, if the plaquing efficiency of the virus is low, the probability of plaque formation by a small clump of i particles would be approximately i times that for any one particle and equation 1 above can be approximated by:

$$T_0 = E(N_1 + 2N_2 + 3N_3 \cdots MN_M)$$

If this is the starting titer, its logarithm will be subtracted from that of each survival titer so the E, whatever its value, does not appear in the difference that is the logarithm of the survival ratio which is plotted on the disinfection graph. The numbers N_1, N_2, N_3, etc. are supplied by the EM (like Fig. 17), and the logarithms of the corresponding set N_1, $2N_2$, $3N_3$, etc., up to MN_m are plotted as shown (Fig. 29) along the vertical zero time axis. (The set used to plot the dotted line on Fig. 4 as it appeared in the original publication[7] was simply N_1, N_2, N_3, etc.). The EM data do not go much beyond 1 group per 1000 particles so this analysis probably should not include groups of greater than 7 particles (M=7) and the approximation to the experimental curve should be accurate until single particles have been reduced 99.9% by the bromine.

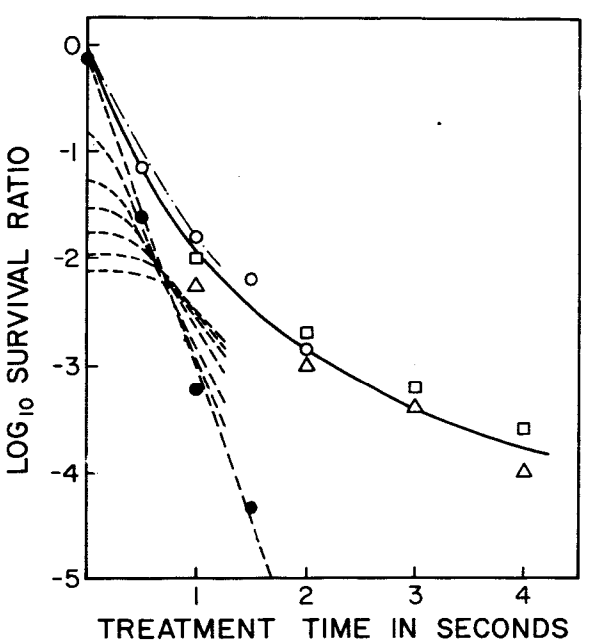


Fig. 29 Poisson approximation to the reovirus disinfection curve (Fig. 4) using aggregate frequencies observed by EM.

Having gotten the starting points for each group size in the mixture, one can draw immediately the best straight line through the data points for inactivation of single particles. This would indicate a titer reduction from To to Tm after a mean dose m of bromine; $T_m = T_o e^{-m}$. If one assumes that the survival of a group of i particles is predicted by the multi-hit target concept then the Poisson limit gives

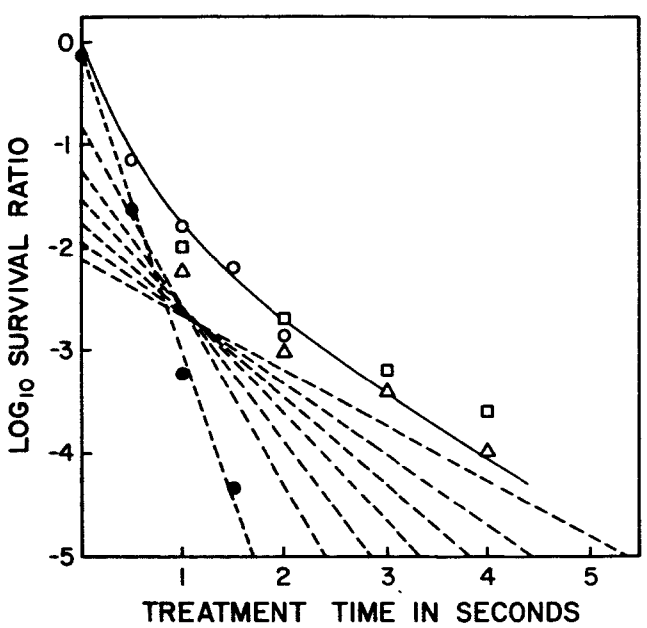
$$T_m = E\left[1 + m + \frac{m^2}{2!} + \frac{m^3}{3!} + \cdots \frac{m^{(i-1)}}{(i-1)!}\right] e^{-m}$$

and the survival titer of the whole mixture will be

$$T_m = E\left[N_1 + (1+m)N_2 + \cdots\right] e^{-m}$$

We can now use the straight single's line to determine m in terms of t, then calculate from equation 3 and draw on Fig. 29 the predicted survival curve beginning at the proper point indicated above for pairs $T_{2m} = E\,(1 + m)e^{-m}$ for triplets $T_{3m} = E(1 + m + \frac{m^2}{2!})e^{-m}$ etc. making 7 lines in all. Now, for any chosen time, the predicted survival titer for the mixture will be given by the sum of the antilogs of the seven intersections with this vertical time line. The locus of the logarithms of these sums forms the curve drawn (Fig. 29) for comparison with the experimental data from Fig. 4. The fit of the predicted curve to the data is quite good.

Another, perhaps equally good, fit to the experimental data can be obtained by drawing straight lines (Fig. 30) from the same set of starting points that were used in Fig. 29. The slopes of these lines (except the single's line that has been determined by experiment) can be found by trial until the sums of the contributions from all the significant group sizes make a total the $\log_{10}$ of which falls on or near the experimental curve for all times within the range tested. By means of such graphical experimenting it is not difficult to achieve a good fit, as may be seen in Fig. 30, and each of the lines can be expressed as $T_{it} = T_{io}\, e^{-K_i t}$, where K_i is the reaction constant for groups of i particles. Unlike the multi-hit Poisson concept of virus disinfection which gives the exact curve for each group size, this method shows only that if all the reactions are first order (straight lines) a set of slopes can be found, consistent with the known numbers of each aggregate size present, to account quite well for the observed results.



Both of these graphical analyses provide a tolerable fit to the experimental

Fig. 30 Straight-line approximation to the reovirus disinfection curve (Fig. 4) using aggregated frequencies observed by EM.

disinfection curve down to the point where the survival ratio of single particles is 10^{-3} and that of the aggregated preparation is 10^{-2}. Beyond this point the EM does not supply the needed accurate data on the frequency of clumps containing 8 or more virions. One is tempted to extrapolate the observed linear relationship between Log Ni and Log i to provide a rational basis for extending the predicted disinfection curve, but this must be done with care because of increasing closeness of the lines (Fig. 30) or curves (Fig. 29) for each group size. This can be seen on either graph, and it signals a limit to useful extrapolation. While this might discourage those who seek a simple method of predicting the course of disinfection beyond any given experimental limit, the above exercise has provided a means of answering a significant question about the mechanism of survival through aggregation.

The question is whether small clumps of virus, even pairs, are more resistant to bromine action than single particles or whether, after exposure to bromine, the virions of a pair or small clump can help each other in the act of plaque formation that might not occur if they were acting separately. If we consider the point at which the PFU titer of single particles has dropped by a factor of 10^{-3}, some reason must be found for the observed survival titer of the aggregated virus which is 10 times greater. There are not enough large aggregates present to account for this, and only large aggregates can surround and protect one or more of their number from contact with the bromine. Even small aggregates are not frequent enough (Fig.3)--unless they survive much better than single particles. By either of the graphs it is clear that when the mean treatment (m=7) has reduced singles to e^{-7}, which is just about 10^{-3} of their original titer, pairs, for example, must be 8 to 10 times more resistant than singles to contribute enough to the total observed titer. This author thinks it unlikely that two virions can contrive to protect each other to this extent, therefore their demonstrated survival must be a synergistic effect resulting from some form of complementation in plaque formation. This is probably the dominant mechanism of reovirus PFU survival in small clumps down to 10^{-3}, and perhaps farther, but earlier work [3] showed that the large aggregates were dominant in survival at about the 10^{-4} level. It seems, therefore, that both modes of survival are operating with aggregated reovirus exposed to bromine.

Preparations of reovirus containing 90 to 95% single particles can be obtained easily from either freon-extracted lysates or infected L cells or directly from crude lysates in which virus has been released from the cells by 3 cycles of freezing and thawing. The freon extracts yield about 2 x as much virus, but both appear equally well purified when harvested from the sucrose gradient in the singles band in either the swinging bucket or the zonal centrifuge rotor. Slow spontaneous aggregation occurs in preparations of reovirus stored at 4-6C, and in these mixtures of aggregates there is a continuum of sizes, the logarithm of whose frequency is a linear function of the logarithm of the number of virions in the group. As aggregation continues, the slope of this line changes but the line remains straight. These aggregates appear to contain nothing but virions (like those in Fig. 19). They are "pure" aggregates. But the major part of the virus released from infected cells by freezing and thawing alone, without homogenization with freon, must consist of aggregates of a different kind. One difference is immediately seen in the sedimentation velocity spectrum of such crude

virus which is produced in the zonal centrifuge rotor. While about 1/5 of the total virions and 1/50 of the PFUs appear in a prominent "singles" band, there are very few groups of 2 to 10, such as those seen in spontaneously aggregated preparations (Fig. 6). The supporting experiment in the swinging bucket rotor supplied the quantitative data on the partition of the virus in the crude preparations. It indicates clearly that the fast-reaction kinetics established in this report, and certainly required to understand the bromine-virus reaction, have been obtained with about 1/5 of the virions normally present in crude extracts of infected cells, while the remaining 4/5 is in quite a different physical state, one that appears to be much more resistant to bromine action. One can reasonably expect that the virus found in polluted water will be in a state more like the crude. The disinfection rates for HOCl and HOBr on suspensions of single polio virions (Fig. 15) are not very different. Due to the small amount of chlorine experimentation we have done and the aggregation problems encountered at pH 6, we prefer to defer discussion of this point until more chlorine data are available.

PHYSICAL STABILITY OF VIRUS IN WATER

The earlier work with the EM has lead us to expect some of the results that we have since detected by sedimentation velocity techniques in work with very dilute virus in water. As the work progresses it becomes more and more apparent that by "water" we do not mean a buffered physiological saline solution. Nor do we mean water that has been distilled and/or demineralized. Ultimately, we must deal with water that has an ionic strength much lower than that commonly used to suspend virus in the laboratory (or much higher for sea water), and it may contain a substantial concentration of divalent and trivalent ions and it may have a very low buffering power. The experiments we have done by diluting stock virus in distilled water and in weak buffers to change the pH have, therefore, opened some doors and prepared us somewhat for the real problem of virus in natural resource water. One of the first things they have done is emphasize the importance of a "stock virus" preparation for such laboratory work (see page 1).

One must have a stock virus suspension as starting material for experiments involving halogen disinfection and the influence of virion aggregation upon survival and titration efficiency. The stock must have a high infectivity titer so that its decline may be observed for several powers of 10. Its infectivity must be stable so that it can be kept and used as starting material for several, perhaps many, experiments. It must not contain extraneous material which can exert halogen demand, and the virions must remain monodispersed. This author is confident that no one has or ever has had such a stock of virus. But the degree to which an investigator can approximate such a virus preparation will doubtless be a measure of the experimental success of his work. With such a stock virus he can make comparisons of the resistance of different viruses to a given disinfecting agent, etc., and he may induce aggregation in various ways and experiment with disinfection of such aggregates. Eventually, however, he will want one more stock virus of a different kind--that is aggregated exactly like it will be when it breaks out of an infected cell and is released into a water supply. Our attempts

to produce these stocks of virus and the resulting physical states attained when they have been diluted in water constitute a major part of this report.

While a maximum yield of single particles of either reo or poliovirus comes by way of freon extraction followed by velocity banding on a sucrose gradient, the virus preparations banded without the freon extraction step seem to be just as free of aggregation and physically indistinguishible, except for concentration. Therefore, by using virus released by freezing and thawing we have been able to get both kinds of stock virus from a single crude cell lysate under conditions somewhat like those that might obtain in nature. The fact that neither reo nor poliovirus aggregates rapidly in concentrated sucrose solutions has been most helpful for the single particle band, and the large aggregate band can be collected separately and kept right in the sugar in which they are banded. Since no bacteria grow in these preparations at 5C, there is no need to freeze them and jeopardize the monodisperse condition by the temporary forming of phase boundaries.

Dilution of stock virus with water is probably not like anything that occurs in nature, but it is a laboratory necessity. Sucrose, virus, and dissolved salts are all reduced in the process, and present data make it appear that there is a critical dilution range in which virion aggregation occurs. Others have reported the clumping of plant viruses [17] when infected sap is purified and the clumping of bacteriophage when it is put in seawater [18]. The latter authors did not find their bacterial virus stock clumping in water of low or zero salt content; only when the salts approached the concentration of seawater did clumping occur. Our two enteric viruses both show physical instability (colloidal instability) with decreasing ionic strength, even at pH 7, and if the virus particle concentration is still great enough when the sucrose and salts have been diluted to the critical range, rapid clumping occurs. In some cases, dilution with water can be done without clumping the virus if the first step of the dilution is big enough that the transport time for their getting together is long. One conspicuous exception to this is the 5000-fold, one-step dilution of stock virus in the finished water from the Chapel Hill filter plant. In this case, aggregation seems to be instantaneous on addition of one drop to a gently stirred 200-ml volume of water.

The two viruses we have examined show one conspicuous difference when aggregated by dilution from stock into water. The reo aggregates do not break up when diluted further with the balanced salt solutions that are used for plaque titration in tissue cultues. Poliovirus aggregates, formed in the same way, do break up when diluted with balanced salt solutions for titration. This behavior could, and probably has, led to confusion in the past when a virus stock has been diluted, become aggregated, and exposed to halogen disinfection in this condition, and then dispersed again during dilution to detect survivors by the plaque method.

The aggregation of both reo and polio viruses in weak buffers at pH 7 and at pH 5 is another observation that is doubtless significant because it means that survival of virus under these slightly acidic, but by no means unusual, conditions will certainly be complicated by the clumping. It probably will be prolonged and titration of the results will be complicated by dispersal

or non-dispersal of the clumps in the titration process. Present titration techniques do not permit the introduction of virus samples of low ionic strength and pH 5 directly onto monolayer cultures of cells.

In summary, some of these experiments on aggregation of virus in water--not balanced and buffered salt solutions but natural resource water--have revealed a means for extended survival of virus and a need for study of more than just the two viruses that have been investigated here.

REFERENCES

1. Sharp, D. G. Sedimentation Counting of Particles by Electron Microscopy. Proc. 4th International Conference on Electron Microscopy. Springer-Verlag, Berlin, 1960, pp. 542-548.

2. Sharp, D. G. Physical Assay of Purified Virus, Particularly the Small Ones. 32nd Ann. Proc. Electron Microscopy Soc. Amer. pp. 264-265.

3. Clark, R. M. A Mathematical Model of the Kinetics of Viral Devitalization. Mathematical Biosciences 2:413-423, 1968.

4. Wei, J. H. and S. L. Chang. A Multi-Poisson Distribution Model for Treating Disinfection Data, Chapter 2 in Disinfection-Water and Wastewater, J. D. Johnson, ed. Ann. Arbor Publishers, 1975.

5. Sharp, D. G., R. Floyd and J. D. Johnson. Nature of the Surviving Plaque-Forming Unit of Reovirus in Water Containing Bromine. Applied Microbiology 29:94-101, 1975.

6. Floyd, Roger, J. D. Johnson and D. G. Sharp. The Inactivation by Bromine of Single Poliovirus Particles in Water. Applied Microbiology 31:298-303, 1976.

7. Sharp, D. G., R. Floyd and J. D. Johnson. The Initial Fast Reaction of Bromine (HOBr) on Reovirus in Turbulent Flowing Water. Applied Microbiology 31:173-181, 1976.

8. Taylor, D. G., and J. D. Johnson. In A. J. Rubin (ed) Chemistry of Water Supply Treatment and Distribution. Ann Arbor Science Publishers 1974, pg. 369.

9. Sharp, D. G. and P. M. McGuire. Spectrum of Physical Properties Among the Virions of a Whole Population of Vaccinia Virus Particles. J. Virology 5:275-281, 1970.

10. Anderson, N. G. The Development of Zonal Centrifuges. National Cancer Institute Monograph 21. U. S. Government Printing Office, Washington, 1966.

11. Kim, K. S. and D. G. Sharp. Electron Microscopic Observations on the Nature of Vaccinia Virus Particle Aggregation. J. Immunol. 97:197-202, 1966.

12. Geister, R. G. and D. H. A. Peters. Quantitative Use of the Electron Microscope in Virus Research. Methods of Analyzing and Predicting Biologic Titers of Aggregated Virus Suspensions with a New Law of Aggregation. Laboratory Investigation 14:864-874, 1975.

13. LaPointe, T. F., Guy Inman and J. D. Johnson In: Disinfection-Water and Wastewater, J. D. Johnson, ed., Ann Arbor Science Publishers, 1975, Ch. 15.

14. Sharp, D. G. and M. J. Buckingham. Electron Microscopic Measure of Virus Particle Dispersion in Suspension. Biochim. et Biophys. Acta 19:13-21, 1956.

15. Scarpino, P. V., G. Berg, S. L. Chang, D. Dahling and M. Lucas. A Comparative Study of the Inactivation of Viruses in Water by Chlorine. Water Research 6:959-965, 1972.

16. Weidenkopf, S. J. Inactivation of Type I Poliomyelitis Virus with Chlorine. Virology 5:56-67, 1958.

17. Brakke, M. K. Dispersion of Aggregated Barley Stripe Mosaic Virus by Detergents. Virology 9:506-521, 1959.

18. Gerba, C. P. and G. E. Schaiberger. Effect of Particulates on Virus Survival in Seawater. J. Water Pollution Control Federation 47:93-103, 1975.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/2-76-287	2.	3. RECIPIENT'S ACCESSION NO.
4. TITLE AND SUBTITLE Virus Particle Aggregation and Halogen Disinfection of Water Supplies		5. REPORT DATE December 1976 (Issuing Date)
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) D. Gordon Sharp		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS School of Medicine, Department of Bacteriology, University of North Carolina, Chapel Hill, North Carolina		10. PROGRAM ELEMENT NO. TCC 614
		11. CONTRACT/GRANT NO. R 803771
12. SPONSORING AGENCY NAME AND ADDRESS Municipal Environmental Research Laboratory - Cin., OH, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268		13. TYPE OF REPORT AND PERIOD COVERED Final 06/75 - 08/76
		14. SPONSORING AGENCY CODE EPA/600/14

15. SUPPLEMENTARY NOTES

For supplementary information see "Virion Aggregation and Disinfection of Water Viruses by Bromine," EPA-600/2-76-163, PB 253 087.

16. ABSTRACT

Using a dynamic system the inactivation of polio and reo virus preparations containing essentially all single and preparations containing aggregated viruses was examined. Differences in resistance to bromine and chlorine were shown to be caused both by inherent differences between different virus groups and by state of aggregation. Electron microscopic observations of polio and reo viruses as they emerge from infected cells and in crude extracts indicated that substantial numbers of viruses may be released from infected cells in an aggregated state. Differences in stability of aggregates of polio and reo-viruses and effects of dilution, ionic strength, and pH on aggregation are also shown.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Viruses Agglomeration Water treatment Disinfection Halogens	Virus inactivation	13 B 6 M

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 59
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA Form 2220-1 (9-73)

☆ U. S. GOVERNMENT PRINTING OFFICE 1977-757-056/5564 Region No. 5-11