THE REMOVAL OF LOW LEVELS OF POLIOVIRUS FROM WATER BY COAGULATION WITH ALUM

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Environmental Sciences and Engineering

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December, 1977

Blacksburg, Virginia

ACKNOWLEDGEMENTS

I would like to express my deep appreciation for the help and guidance of Dr. Robert C. Bates and I would also like to thank Dr. Robert C. Hoehn and Dr. Gregory D. Boardman for their help and encouragement.

I am also deeply indebted to Dr. Pete Shaffer and The Carborundum Company for their free donation of information, time and material, all of which helped make this work a reality. This help is most gratefully acknowledged.

I would also like to thank Ms. Susan Sutherland for her help with the cell cultures and experimental procedures; Mrs. Warren Fenton for the final typing of this thesis; and my parents for all their support.

Finally, I would like to express special thanks to all my friends at Hillside who for the past year and a half have helped me grow in so many ways.

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I. INTRODUCTION

Human enteric viruses are known to be contaminants of potable water supplies. These viruses are present at very low concentrations in water supplies, but can be very numerous in domestic sewage and sewage treatment plant effluents. These viruses have a very low minimum infective dose. It follows then that the low virus levels found in drinking water sources can be of public health significance.

With an ever increasing demand for biologically safe, potable water, the recycling of domestic wastewater effluents is becoming a greater reality. The need for effective virus removal procedures is therefore increasing.

Viruses, by their very nature, are obligate intracellular parasites. This means that human viruses present in the aquatic environment are incapable of reproduction. Therefore, their numbers would be expected to decrease with time once the viruses have entered the aquatic environment.

The very low numbers of human enteric viruses present in aquatic systems makes their study very difficult. There currently are no techniques for successfully enumerating viruses at these low concentrations (or titers). In order to obtain an estimate of the virus titer, one must concentrate the viruses present by some technique.

Water treatment plants have the responsibility of making a water source safe for human consumption. In so doing, a variety of contaminants must be eliminated by various different treatment processes. The potential for virus removal by any of these processes

is poorly understood at this time.

One commonly used treatment process is coagulation-flocculation with alum. This process removes primarily colloidal matter from water. The purpose of this research was to determine the virus removal efficiency of alum coagulation-flocculation in water containing low numbers of virus particles. Monodispersed poliovirus LSc1 was chosen as the model human enteric virus. Virus titers of approximately 10^3 plaque forming units (PFU) per liter were used during a portion of the study. The results at 10^3 PFU per liter were compared to the results obtained from studies with water containing approximately 10^6 PFU per liter. Coagulation-flocculation was achieved in jar tests after the addition of alum to a concentration of 30 mg per liter.

Concentration of the virus containing water samples was necessary in order to assay such low virus titers as were present before and after coagulation-flocculation. A microporous filter technique was used for this concentration. Preliminary work was done to determine the efficiency of this concentration technique at virus titers of 10^6, 10^5, 10^4 and 10^3 PFU per liter.

The objectives of this work were:

1. To determine the effectiveness of the virus concentration technique at various virus titers.

2. To determine the virus removal efficiency of alum coagulation of water containing approximately 10^3 PFU per liter.

3. To compare the virus removal efficiency at 10^3 PFU per liter using two different experimental coagulation techniques.

4. To compare the virus removal efficiencies found at 10^3 PFU per liter with the efficiency found at 10^6 PFU per liter.

II. LITERATURE REVIEW

Virus Distribution in Water

More than one hundred different enteric viruses which have the potential to be transmitted by water are recognized as being able to infect man (1). Coxsackie viruses, reoviruses, echoviruses, adenoviruses, and polioviruses (1,2) all have the potential for waterborne transmission but the infectious hepatitis virus is the only human enteric virus which has been shown conclusively to be spread by contaminated water (1,2). Though studying the epidemiology of waterborne virual diseases is very difficult, many cases of the spread of viral hepatitis by water have been well documented. These outbreaks have been recorded from 1895 up to the present time (3,4). Non-bacterial, acute, infectious gastroenteritis also appears to be viral in nature (5) and is suspected to be transmitted by the water route. The disease is generally not life threatening and isolating the etiological agent is very difficult, so little information is reported on this disease.

Because the viruses under consideration in this thesis can act on the gastrointestinal tract, the mode of transmission of concern is the fecal-oral route. Thus, the primary route of infection would be a public drinking supply that had been contaminated with human feces. The possibility of infection by contact with water in recreational or bathing settings also exists. This mode of transmission

would be of minor importance compared with the ingestion of water.

The levels of human enteric viruses found in natural water systems are very low. There are two main reasons for this. First of all, viruses are by their very nature and definition obligate, intracellular parasites. This means that they are incapable of growth and reproduction unless they are within a host cell. For human enteric viruses this would mean that a human cell or some similar cell would be needed and no such cells are available in the aquatic ecosystem. All of this means that human enteric viruses in natural water systems may either degrade to a noninfectious state or remain infectious. Since this environment is far from ideal for these viruses many of them will do the former. The second main reason is that the ultimate source of human enteric viruses in water systems is human feces, and most human feces passes through sewage treatment plants before it can reach a natural water system. The treatment of human feces receives at the sewage treatment plant can both remove and degrade viruses present. Sproul (6) estimated that surface waters in the midwestern United States contained three PFU per gallon of water. Sproul further estimated that a properly operated water treatment plant with commonly used facilities might reduce this level of contamination to one PFU in every 30,000,000 gallons of water that is distributed to the public. Sproul, however, stated that he believes one PFU per every 3,000 gallons of water distributed to the public to be a more realistic estimate of the actual virus removal that occurs during water treatment. This seems to be a very low level of contamination

but one must realize that as the population increases, the initial virus concentration in water after sewage treatment increases. Thus the possibility that greater numbers of viruses will survive after water treatment.

The minimum infectious dose for these viruses must also be considered when determining the public health significance of a particular virus concentration found in a water supply. Assigning a number to a virus level that is significant to human infection is difficult. Human volunteer studies directed toward this goal of necessity have been conducted with attenuated virus strains that may be less infectious than the respective wild types (7). The difficulty of studying the epidemiology of human viral diseases is a further problem. Thus the absolute proof of a minimum infectious dose is not yet at hand. However, a number of researchers (7,8,9) believe that the human system is just as susceptible to infection by these types of viruses as are cell culture systems. This means that a level detectable by cell culture techniques should be considered potentially dangerous. A concentration of one PFU per 100 gallons of water has been proposed as a drinking water standard. Melnick (10) states, however, that if better detection technology develops, a level of one PFU per 1000 gallons could and should be set.

Current work which points to a link between cancer and viruses might be of importance for water treatment in the future. It would be unlikely that most tumor viruses would be able to survive very long in the aquatic environment (11). However, adenoviruses which have been implicated in cancer may currently be an undetected threat to

health in our water supplies (12). The slow-acting nature of these viruses in producing these diseases means they could already be causing damage which might not be visible for many years. Tracing the origin and route of the viral transmission could be impossible when the damage is noticed.

Water Treatment and Viral Removal

The water treatment process. Hammer (13) states that the purpose of a water treatment plant is to "provide a potable supply" of water. Potable water is that which is safe for human consumption. Water for drinking purposes must be both biologically and chemically safe in order to meet specific water quality criteria in this country. In addition, the water must be aesthetically pleasing to the water service customers, and most importantly, all these goals must be met with as little cost to the consumer as possible.

In a common water treatment plant that uses surface water sources, there are many possible treatment processes. After an initial screening to remove very large material from the water, the water may first be aerated. This aeration step may be used in order to improve the taste, remove undesirable gases and volatile acids or to oxidize iron and manganese to their oxides which are insoluble. The treatment plant may then use chlorination to begin disinfecting the water as it proceeds through the plant. Next, chemicals may be added in a rapid mixing chamber. A flocculation chamber would follow this chemical addition. This chamber allows for the interaction between the chemicals which have just been added and colloidal material in the

water. Chemical coagulation may occur and the resulting coagulated "floc" particles may precipitate out of solution, thus clearing the water. A filtering system may follow to remove unsettled flocculent material. Filtration is followed by final disinfection, usually with chlorine, to meet public health standards. In some communities, fluoridation is practiced to reduce the occurrence of dental caries.

Virus removal potential in the water treatment plant. Virus removal in this discussion means either the actual removal, destruction, or rendering non-infectious of the viruses present in the water. One of the simplest treatments for effective virus removal is storage for long periods. It has been shown that the time required to reduce the virus content of water by 99.9 per cent may vary from a few days to several weeks or months depending on temperature and the type of virus involved (14). The pH of the water also may contribute to the survival time of the viruses.

Disinfection procedures have traditionally been the key to the removal of inactivation of biological hazards such as the bacteria present in water supplies. These procedures are also of key importance in the removal of any viral hazard (14). The present use of chlorination for the disinfection of water sources seems to be effective for high level virus removal (15).

The chlorination procedures routinely used at the discharge point in waste water treatment plants do not seem to do an effective job of virus removal however. The use of other halogens (16,17,18) and ozone (19) also show potential for virus removal in drinking water.

Filtration has been investigated and has also been found to remove viruses (19). However, the efficiency of the sand filters commonly used in water treatment seem to be highly variable in their virus removal capabilities. Floc particles breaking through the filter, even at very low turbidity levels, generally means that viruses are breaking through as well. This means that turbidity levels after the filters would have to be monitored very carefully and very often in a real water treatment situation.

Absorption by activated carbon, often an adjunct to the filtration step, seems to be variable and undependable virus removal as the viruses absorbed are always in competition with organic matter in the water for absorption sites. For this reason the viruses are in an equilibrium state rather than being ultimately removed (19).

Chemical coagulation also seems to be effective for removing viruses at water treatment facilities. This is the process evaluated for its virus-removal capacity in this thesis. Under properly controlled conditions, coagulation-flocculation is capable of removing high percentages of viruses from water (18). The results for this process are also variable. The virus being studied, the coagulant or synthetic polyelectrolyte used and the pH of the system all affect the results one obtains. This process, though capable of removing high percentages of the viruses present, is not capable of removing all viruses present when it is used as the sole treatment (21).

Alum Coagulation

Alum ($Al_2(SO_4)_3 \cdot 14.3H_2O$) was chosen as the coagulant for this study of virus removal by the coagulation-flocculation water treatment process. The entire hydrolysis reaction that occurs when alum is added to water is not completely understood. The presence of natural alkalinity in water is known to aid in the formation of the aluminum hydroxide alum floc by raising the pH of the system. The presence of hydrogen ions tend to suppress the formation of aluminum hydroxide (22).

The reaction involved, utilizing calcium bicarbonate alkalinity is as follows:

$$Al_2(SO_4)_3\ 14.3H_2O + 3Ca(HCO_3)_2 \rightarrow$$
$$2Al(OH)_3\downarrow + 3CaSO_4 + 14.3H_2O + 6\ CO_2 \qquad (1)$$

In alkaline solutions, species such as $Al(OH)_4^-$ and $Al(OH)_5^{2-}$ have also been shown to exist (13).

Virus Enumeration Methods

Qualitative considerations. Since viruses are obligate intracellular parasites, a host cell system must be utilized in order to detect their presence in a water sample. This host cell system may be provided in the form of live animals susceptible to the virus in question, the use of chick embryos, or the use of cell culture techniques (23,24,25,26). Cell culture methods are the most extensively used at this time. These techniques are relatively easy to use and take less space. However, these techniques use transformed cells from animals and for this reason they are not true in vivo tests. This means that the true effect the virus has on its true host may

not be exhibited. Therefore, in certain circumstances animal or chick embryo inoculation may be preferred.

When animal or chick embryo inoculation is used, the death of the host as well as other visible signs of infection may be the means of determining viral activity. With cell culture techniques, viral activity is determined by either microscopically visible changes in the cells themselves or by macroscopically visible areas in a sheet of cells called plaques. Plaques are loci of infection which are made up of round areas of dead cells in a cell monolayer. These areas are visible with the naked eye because after a single cell is infected by the virus it produces more virus particles and they may spread out in all directions infecting cells nearby and repeating the process until an area large enough to be seen is formed. The plaque assays must be done with a solid of semi-solid medium over the actual monolayer of host cells. This keeps the newly formed viruses from the originally infected cell from spreading all around the cell layer unchecked and starting new foci. Theoretically the plaque is the visual representation of a single cell being originally infected by a single infectious virus particle. Viral activity may also be exhibited by the ability of the cells to absorb certain types of red blood cells. This technique is known as hemadsorption and the technique is made possible by changes in the surface structure of cells after infection by a virus such that certain red blood cells can attach at newly open surface sites (23,24).

Quantitative methods. Whenever one is determining the number of viruses or infectious units present in a sample, a series of dilutions of the sample must first be made. The number and type of dilutions

are determined either by experimentation or previous experience. Samples from the appropriate dilutions are then used to infect the assay system being used. When using animal or chick embryo injection as the assay system the relative number or percent of the individuals at each dilution which display a predetermined indication of their infected state are determined. From this a value for the dilution at which a certain amount of infection occurs is determined. This is usually expressed as a percentage. Many names for this infectious dose determination exist and it is used at times with tissue culture systems as well as chick embryos or live animals. When cell cultures are used the tissue culture infectious dose (TCID) at which fifty percent of the individual cultures exhibit some sort of cytopathic effect (CPE) is determined. This yields a $TCID_{50}$ which gives us an idea of the infectious strength of the original sample. When utilizing tissue cultures in this way many small cultures are used and observed under a microscope for cell death or changes in the cells. With animals or chick embryos a macroscopic indication of infectivity such as the death of the individual is used. Macroscopic indications of infection are also possible when using cell cultures. A plaque assay is used to accomplish this. In this type of assay a solid or semisolid medium is put on top of a monolayer of cultured cells and a vital stain is added. This allows one to see individual loci of infection as clear areas in the cell monolayer which are formed by dead cells which do not take up the vital stain. Each of the cleared areas are called plaques and are assumed to represent a single infectious viral unit which infected a single cell and propagated within that cell releasing viruses

which spread to surrounding cells. The solid overlay medium keeps the viruses released from the infection of a cell from being spread all over the cell culture at random. With a knowledge of the inoculum size, the dilution of the sample used, and the number of plaques observed, a determination of the sample titer may be made in terms of plaque forming units (PFU) per unit volume.

The ability of viruses in a sample to adsorb to red blood cells may also be used to determine a virus titer in terms of hemagglutination units per unit volume. This is known as a hemagglutination test.

Virus Concentration

The need for concentration. The enumeration techniques previously described all assume that the sample actually being assayed contains enough virus particles to permit their presence to be detected without any concentration. This may not always be the case when a natural water is to be tested. Such samples may be so dilute in terms of viruses present that a sample of five, ten, one hundred or even several hundred liters may not contain any viruses. This means that concentration of the original sample to a smaller volume becomes necessary in order to allow for reasonable sample inoculum size and for the use of a reasonable number of animal hosts, chick embryos or cell cultures.

Potential concentration methods. Many methods for concentrating viruses exist. Some are much more efficient than others. The cost and adaptability to use in the field also vary immensely. The "gauze-pad technique" (27,28) was one of the earliest methods used by environmentalists for studying viruses in water. This technique is

very simple but is not quantitative. This procedure entails placing a gauze pad in flowing water for as long as is desired. A huge quantity of water can pass through the gauze pad, and viruses that may be present in the water tend to adsorb to the pad. These viruses may be eluted from the pad at a later time in the laboratory with a relatively small volume of a buffered solution having a pH somewhat greater than neutrality. This sample may then be assayed by previously mentioned techniques, and by immunological methods to determine the types of viruses present. The actual volume of water which passed through the pad remains uncertain so quantification is impossible. The effectiveness of the pads adsorption capability also is unclear.

There are many other concentration methods that can be used in attempting to quantitate the viruses present. Adsorption of viruses to susceptible cells and hydroextraction (essentially a dialysis technique), along with some other more complex procedures, can be used only when handling very small volumes (29). High speed centrifugation is another method that possibly could be used and could even be adapted to handle large volumes. This technique could separate the viruses from other material on the basis of density and concentrate them as well. The equipment needed however, is not only complex and delicate, but very expensive as well. These factors virtually eliminate this method as economically feasible for widespread generalized use. This technique would also be difficult to use in the field. Protamine sulfate precipitation has been found to be an effective method of concentration, especially for adenoviruses and reoviruses (30). This is a simple technique and could be used in the field, but its use

requires samples that are clearer than most natural waters. The technique is also limited in that the volume that can be easily handled is small. The protamine sulfate technique is to some extent selective for adenoviruses and reoviruses and can therefore eliminate the problem of overgrowth by some other enteric viruses in cell cultures. For this reason this might be the method of choice for concentrating these particular viruses.

The microporous filter technique. The currently preferred method for the overall concentration of human enteric viruses is the microporous filter technique. It has been selected as a tentative standard method to be used in the monitoring of water for viruses by the American Public Health Association, et al. (32). Two other methods of membrane filter concentration are reverse osmosis and ultrafiltration. These two techniques reject the virus particles at the filter surface. When the microporous filter technique is used, the viruses are actually adsorbed within the lattice of the filter (33). All of these filtration procedures suffer from the problem of clogging if the original sample is not clear. The problem of clogging is especially great when one considers the suggested sample size for the detection of viral presence in a water sample is at least 380 liters (100 gallons) (32,34,35) with 1900 liters being needed in order to detect with confidence the presence of one or two PFU per 380 liters (32,34). In order to clarify the original samples, prefilters may be used. They may be prepared by pretreatment with substances that compete with the viruses for binding sites on the filter. Such substances include serum, whey proteins, or polyvinyl-pyrolidone (33). The viruses have a high

molecular weight and are therefore not apt to displace the above mentioned substances as they pass through the prefilter, leaving much of the clogging material behind. Another method of clarification is the addition of a filtering aid rather than a separate prefilter. An example is Celite (a product of Johns-Manville) that forms a prefiltration complex on top of the filter pad and serves the same purpose as a separate roughing filter (36).

The microporous filter technique, as previously stated, depends on the adsorption of the viruses to the filter surface. This adsorption depends on many types of interactions. The surface of the viruses being studied as potential problems for man in the aquatic milieu are essentially made up solely of protein. Because they are protein, they may have both polar and nonpolar regions. The nonpolar regions can react hydrophobically with nonpolar regions on the filter surface. Hydrogen bonding between polar regions of the protein surface of the virus and the filters can also displace water molecules if the bonds are sufficiently strong and thus aid in adsorption (33). When these virus particles are in solution at a neutral pH they carry a net negative charge and can cause a strong ionic repulsion from negative areas on the filter surface. For this reason, the pH of the sample solution is generally changed so that the viruses being sought are at their isoelectric pH. This creates a net neutral charge although there are still microzones on the viral surface that allow for the various small charge-related interactions already described as having a role in virus adsorption to the filter. At the isoelectric point the net overall charge is eliminated and the viruses tend to aggregate and

precipitate out of solution. The addition of polyvalent metal cations can also aid in the adsorption process. These cations adsorb to hydroxyl groups on the membrane surface, and, if there are extra positive charges on the ion, can link to the virus particles (33). The polyvalent cation addition can make up for an isoelectric point pH adjustment that is less than perfect. The human enteric viruses being considered here fall within the size range of colloidal particles (13,22,37). Due to their size and the fact that the viruses have a net negative charge in their natural state, when metal cations are added to help precipitate the viruses out of solution, the Schulze-Hardy rule applies. This rule states that as the number of charges on the cation increases, the ability of the cation to cause the colloidal particle to precipitate out of solution increases markedly. For this reason trivalent aluminum or iron can be used at much lower concentrations than divalent or monovalent cations with the same results.

All of the previously discussed factors can be manipulated to achieve the desired concentrations of viruses in large volumes of water. The microporous filter technique is capable of handling the large volumes (ranging from 400 to 2000 liters) now considered as ideal for virus monitoring in water. The actual efficiency of recovery for the original number of virus particles present in controlled situations is variable and generally is not too high. When the sample size and the actual amount of concentration occurring are taken into consideration, however, the microporous filter technique demonstrates good potential in virus detection in large samples of water, even

though the technique is still not capable of reliably detecting the presence of one or two virus particles in 400 liters every time.

Once the viruses are adsorbed onto the filters, they must be eluted into a smaller volume for qualitative and quantitative assay. When concentrating very large volumes, the initial elutions may even be reconcentrated in order to further reduce the size of the sample to be assayed. The elution is accomplished by passing a high pH solution through the filter so that the viruses no longer have a high affinity for the filter, and they become free of the filter and float off. The most commonly used isoelectric pH for the concentration of enteric viruses is 3.5 to 3.6 (32) and the elution pH can vary from around 9 (36) up to around 11.5 (32,31). A high pH glycine solution is often used in the elution step.

Once the concentrated sample is eluted from the filter by the higher pH solution, the pH is adjusted to neutrality because the high pH can be virucidal and the virucidal effect becomes greater with increasing time. The isotonicity of the final eluant is adjusted with sodium chloride (31) once the pH has been adjusted. After the final sample is obtained it can be assayed for the presence of viruses by whatever method is appropriate. Standard Methods for the Examination of Water and Wastewater (32) suggests the use of cell cultures or suckling mice as the host system.

It should be remembered that the microporous filter technique, though very useful, may not be the best method of concentration in all situations, as was previously noted in the description of the protamine sulfate precipitation method for the concentration of

reoviruses and adenoviruses. None-the-less, the microporous filter method does seem to be the most widely applicable for concentrating the human enteric viruses that are of interest as potential hazards in water supplies.

III. MATERIALS AND METHODS

Cell Culture Preparation

Buffalo Green Monkey Kidney (BGM) cells were used for all the cell culture work done in this study. The original cell stocks were supplied by The Carborundum Corporation. These cells were grown using Earle's based minimal essential media (MEM) (Appendix A), which was supplemented with fetal calf serum (Flow Laboratories) at a concentration of 10 per cent by volume. Tissue culture grade l-glutamine, in a 100 millimolar solution, was also added to the growth medium to a final concentration of one percent by volume. Penicillin and streptomycin also were added. Stock cultures were grown in 32-ounce (oz.)(1000 ml) glass bottles (Brockway). The BGM cells were subcultured from these bottles by treating the cell monolayers with 0.25 percent trypsine-versene (Appendix A) for 10 to 15 minutes at 37°C in order to release the cells from the glass surface. The cells were then pelleted by centrifugation at 800 x g. The supernatant was discarded and the cells resuspended in MEM. The cells were then counted using a hemocytometer and seeded at a density of from four to five million cells per 40 ml growth media in each bottle. Alternately, the cells were planted in 60 mm diameter tissue culture grade Petri dishes (Falcon, Corning) at a density of $0.8\text{-}1.2 \times 10^6$ cells per plate with a total of 5 ml of growth medium per plate. Bottles seeded for continuation of the stock cell cultures were grown at 37°C with air tight seals. The cultures were observed daily and any change in the cultures pH was indicated by the indicator phenol red which was present

in the MEM. When necessary the pH was adjusted using a sterile 7.5 per cent $NaHCO_3$ solution. The cells grown in Petri dishes were incubated in a water-jacketed, humidified incubator with an atmosphere containing five per cent CO_2 for pH control. These Petri dish cultures were incubated for 48 to 96 hours prior to use in a virus assay procedure. All of the above procedures were undertaken using sterile techniques with the aid of a laminar flow hood (Troemner) for the production of bacterially filtered air within the work area.

Virus Purification Procedure

Poliovirus LScl was obtained from the Baylor School of Medicine for use in the experiments described in this thesis. The poliovirus was purified according to the following procedure. (See Figure 1.) Initially the poliovirus stock was used to infect BGM cell cultures and allowed to propagate. The cytopathic effect (CPE) of the cells rounding up and then coming off of the surface of the tissue culture bottles was used to detect infection of the BGM cells and the subsequent reproduction of the virus within them. The virus stock was added to the cell cultures in five milliliters of growth medium and 30 minutes was allowed for the virions to adsorb to cell receptor sites. Sufficient growth medium was then added to bring the final volume to 30 to 40 milliliters. The cultured were then incubated and observed for the next 24-48 hours for CPE. When 95 per cent CPE was observed, the cell cultures were frozen and thawed three times in order to rupture the cells and release as many virions as possible. The cell lysate was then centrifuged at 10,000 x g for 10 minutes to remove

Infect BGM[a] cells with poliovirus LSc1.

↓

Observe cells for 95% cytopathic effect.

↓

Freeze and thaw the cell lysate three times.

↓

Centrifuge the cell lysate for 10 min. at 10,000 x g[b] to remove cell debris.

↓

Centrifuge the above supernatant for 4 hours at 135,000 x g to pellet the virus particles.

↓

Discard the supernatant and resuspend the virus pellet from the last step in PBS[c] and sonically disrupt it.

↓

Layer the virus suspension from the last step on a 10-30% discontinuous sucrose gradient and centrifuge for 2 hours at 90,000 x g.

↓

Split the above gradient into two halves of equal density and centrifuge for 4 hours at 135,000 x g to repellet the virus particles.

↓

Discard the supernatant and resuspend the virus pellet from the last step in PBS and sonically disrupt it.

↓

Band the virus suspension at a density of 1.34 g/cm^3 by centrifugation of the virus suspension in a cesium chloride gradient for 24 hours at 135,000 x g.

↓

Collect the fractions at the buoyant density of poliovirus LSc1 (1.34 g/cm^3) and observe with an electron microscope using a negative staining technique.

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[a] BGM means Buffalo Green Monkey kidney cells.

[b] g indicates the force of gravity.

[c] PBS means phosphate buffered saline (See Appendix A).

Figure 1: The Procedure Used to Obtain Purified Monodispersed Poliovirus LSc1.

cell debris. The supernatant from the previous centrifugation was then centrifuged for four hours at 135,000 x g to pellet the virus particles present. The resulting pellets were resuspended and sonically disrupted in pH 7 phosphate buffered saline, 0.15 M NaCl, 0.01 M KPO_4 (PBS). This suspension was then placed on the top of a 10-30 per cent discontinuous sucrose gradient. This gradient was made by pipetting aliquots of sucrose solutions on top of each other. At the bottom of the gradient 30 per cent sucrose was placed in the centrifuge tube. Then aliquots of 25, 20, 15 and finally 10 per cent sucrose were layered on to make the discontinuous gradient. This gradient was then centrifuged at 90,000 x g for two hours. The sucrose gradient was then divided into equal density halves and centrifuged at 135,000 x g for four hours in order to repellet the virus particles. The pellets were once again resuspended and sonically disrupted in PBS. The virus suspension was then used along with PBS to make a cesium chloride (CsCl) solution with a density of 1.34 g/cm^3. This solution was then centrifuged for 24 hours at 135,000 x g. During the centrifugation a continuous CsCl gradient was formed with the virus particles migrating to a point along that gradient which was equal to their buoyant density and forming a band there. After centrifugation, the gradient was fractionated and 0.5 ml fractions were collected. The refractive index of each was determined using an Abbe refractometer (Bausch & Lomb, model 3-L). The densities corresponding to the measured refractive indices were determined from tables. The fractions that were closest to the buoyant densities of the poliovirus were utilized for the purified virus in these experiments.

Electron Microscope Observation of the Purified Virus

The concentrated, purified virus samples were negatively stained with uranyl acetate on carbon backed sample grids. When fields from these grids were observed with an electron microscope, almost all of the virions present were in a monodispersed form. A very few were observed in pairs and even less in triplets. Less than five per cent of the virions were not monodispersed. Following observation with the electron microscope the purified virus samples were diluted with PBS, their virus concentrations (or titers) were determined by plaque assay and the purified stock virus was stored at $4^{o}C$.

Concentration Techniques

The procedures used for the virus concentrations were described by the Carborundum Corporation (32) and are very similar to those described in Standard Methods (33). (See Figure 2.) Initially, the sample to be concentrated was adjusted to a pH of between 3.5 and 3.6 to bring the sample to the isoelectric pH of the test poliovirus. Samples were taken just before and just after this pH adjustment in order to check the virus titer detectable in both the original sample and the one adjusted to its isoelectric point. This was only done when using original virus titers where virus detection was feasible. After pH adjustment, sufficient $AlCl_3$ was added to produce a 0.0005 molar (M) solution of $AlCl_3$ (1 ml of a 0.5 molar solution in one liter of sample). Then the sample volume was determined and the sample was added to a one liter, glass, separatory funnel that had previously been cleaned and rinsed with HCl diluted 1:10. The samples to be

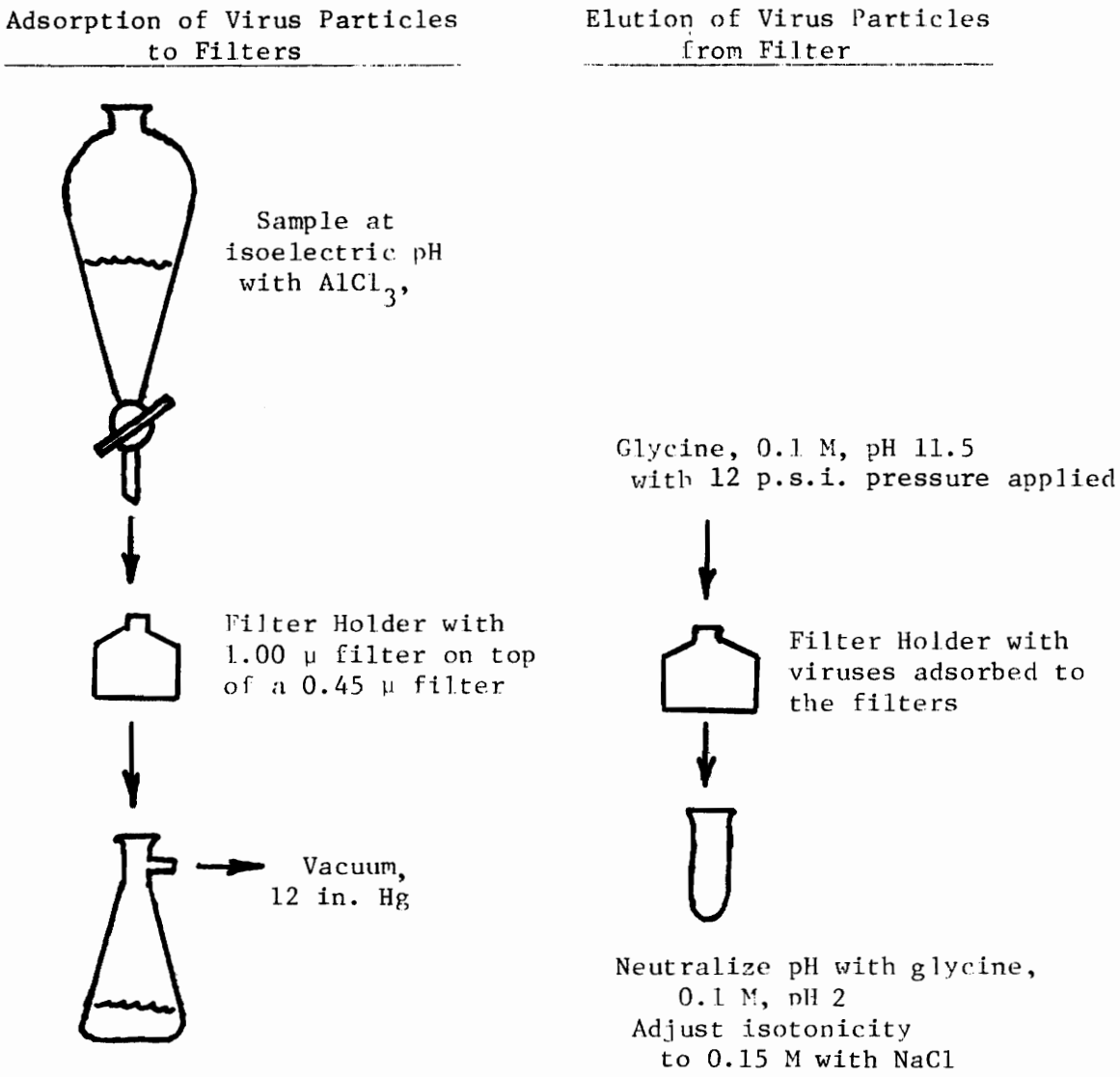

Figure 2: Microporous Filter Concentration Procedure.

concentrated varied in volume from slightly less than 500 ml to as much as 1000 ml.

Next, the samples were dispensed from the separatory funnels into a filter holder (Millipore Corp., Swinnex 47 mm) containing a 1.00 μ filter laying above a 0.45 μ filter (Cox Filter Corp.). The samples were drawn through the filters by applying a vacuum of approximately 12 in. Hg. After completion of the filtration, the vacuum line and separatory funnels were disconnected from the filter holder.

Next, viruses were eluted from the filter by forcing 0.1 M glycine, pH 11.5, through it. A pressure of approximately 12 pounds per square inch (p.s.i.) was applied to the top of the filter and the filtrate was collected in a 15 ml tube (Falcon Plastics) that already contained 2 ml of 0.1 M glycine, pH 2.0. The eluting glycine solution was added and forced through the filter in increments of 2 ml until a total of 8 ml had been used. The pH of the final concentrate was adjusted approximately to neutrality by the addition of the low pH glycine solution. The pH was determined by color change in the indicator dye phenol red (Fisher Scientific Company) that was added to both glycine solutions to give a final concentration of 0.0008 per cent. After the pH adjustment, the isotonicity of the sample was adjusted by adding sufficient 3 M NaCl to produce a final concentration of 0.15 M in the final concentrates (0.5 ml for every 10 ml of concentrate). Then 0.5 ml of fetal calf serum was added and the final volume determined. This final concentrate then was either analyzed by plaque assay immediately or was stored at 4^{o}C until used.

If stored the quantitative assay would be performed within the next 24 to 48 hours.

Virus Assay Techniques

The plaque assay was utilized to determine virus titer in all these experiments. Monolayers of BGM cells in 60 mm Petri dishes were used for these plaque assays. To begin the assay virus dilutions were made in MEM. Depending on the virus titers in question, dilutions were made either on a 1:1 basis with double strength MEM or on a serial 10X dilution basis with single strength MEM.

After the sample dilutions were made the growth media was removed from the Petri dishes with the BGM cell monolayers by suction. Next, 0.25 ml to 0.5 ml of the 10X dilutions and 0.5 or 1.0 ml of the 1:1 dilutions was added to the cell monolayers. The smaller inoculum sizes were given 30 minutes for the virus particles present to adsorb to the cells and the larger inoculum sizes were given one hour for adsorption. The adsorption period took place in a CO_2 incubator at $37^{o}C$.

At the end of the adsorption period a solid overlay medium was added. This medium consisted of MEM containing the previously mentioned antibiotics, 5 per cent fetal calf serum, one per cent of a one per cent solution of $MgCl_2$, one per cent of a 100 millimolar solution of l-glutamine, 18 mg of neutral red per liter of overlay, and one per cent Noble Agar (Difco). The overlay medium was prepared in two double strength portions which were added together immediately before use to give the final medium with all its components in the

proper concentrations. One portion was double strength agar which was sterilized by autoclaving and the other half contained all the growth media and supplemental components and was filter sterilized and made up under sterile conditions. Just before use the pH of the later half would be adjusted up and down by the addition of $NaCHO_3$ and HCl in order to increase the pH buffering capacity of the medium. When the overlay medium was mixed together it was added to the Petri dishes with a pipet by pouring in down the side of each dish while the media was still between 42 and 45°C. Four to five ml were added to each plate. Once the overlay was added the plates were gently tilted back and forth to mix the media completely with the virus inoculum, thus eliminating the possibility of any soft spots in the agar. When 1.0 ml inocula were used, the final agar concentration was increased to 1.1 per cent to ensure that the agar would harden after mixing with the virus inoculum.

Once the overlay had hardened the plates were inverted and placed in a CO_2 incubator. On the second day of incubation the plaques were counted. The neutral red in the overlay medium is a vital stain for the BGM cells so that plaques appear as clear areas on a pink to purple background. The plaques were counted on each succeeding day for up to six days to see if an increase in plaques was found. Virus titers were then calculated (Appendix B). On some occasions, the overlay medium did not contain the dye but after two days of incubation 1.0 ml of a solution of 1:1 neutral red and MEM was added three hours prior to the time when plaques were counted. This procedure provided sufficient contrast so that plaques were easily observed. All the

titer calculations were done using plaques counted on the second day as the remaining days did not show an appreciable increase in the number of plaques present, and this allowed for comparison of the experiments in which the neutral red was not present in the overlay medium.

Experimental Procedures

As a prerequisite to the other experiments, initial experimentation was performed to determine the efficiency of the virus concentration technique. Then the coagulation-flocculation tests were performed. These tests were done under two separate sets of conditions. These will be defined as Method 1 and Method 2.

Determination of concentration efficiency. In order to determine the efficiency of the concentration technique, concentration runs were made at initial virus titers of 10^6, 10^5, 10^4 and 10^3 PFU/liter, as previously described (pages 24-27).

The desired virus titer in the test samples was made by adding to them a portion of stock virus. Once the dilution was made, the titer was verified by assaying directly the original sample and assaying the sample after it had been adjusted to the isoelectric pH. These two samples along with the final concentrates were assayed to determine their titers. The results were compared to determine the efficiency of the concentration procedure (Appendix B). The original samples and the final concentrates were compared as well as the samples taken at the isoelectric point and the final concentrates. There were four replicate concentration experiments performed at an original

titer of 10^6 PFU/liter, six at 10^5 PFU/1, nine at 10^4 PFU/1, and six at 10^3 PFU/1.

Coagulation tests: Method 1. Water that had been distilled, deionized and then glass distilled was used as the stock water. One liter was added to each of six battery jars that had been washed and rinsed with 1:10 HCL. Then, 30 mg of aluminum sulfate (alum) was added to each jar except the first one, and the jars were positioned on a six-place jar-test apparatus (Phipps & Bird). High-speed mixing was initiated, and the pH of each jar, as determined with a pH meter, was adjusted to neutrality with 0.25 M $NaHCO_3$. Approximately 4.5-5.0 ml of the $NaHCO_3$ solution was required to reach neutrality. After the pH adjustment was made, the virus inoculum was added. All adjustments were completed within two minutes after alum was added, and an additional 30 seconds flash mixing time was provided during which the virus was added. Then the stirrer was reduced to the point where floc could be seen to be circling upward from the bottom of the jars to the top along the outside and back down in the center.

Flocculation was allowed to continue for 20 minutes after which the stirring was stopped, and settling of the floc was permitted for 30 minutes. Next, 500 ml samples were taken from the jars with a 50 ml volumetric pipet that had been altered (Figure 3) to permit removal of the sample with a minimum of disturbance of the flocculant material on the bottoms of the jars. These samples were centrifuged for 10 minutes at 7150 x g in order to remove any unsettled floc or any which might have been resuspended by the sampling procedure. After centrifugation was complete, the volumes of the samples were determined

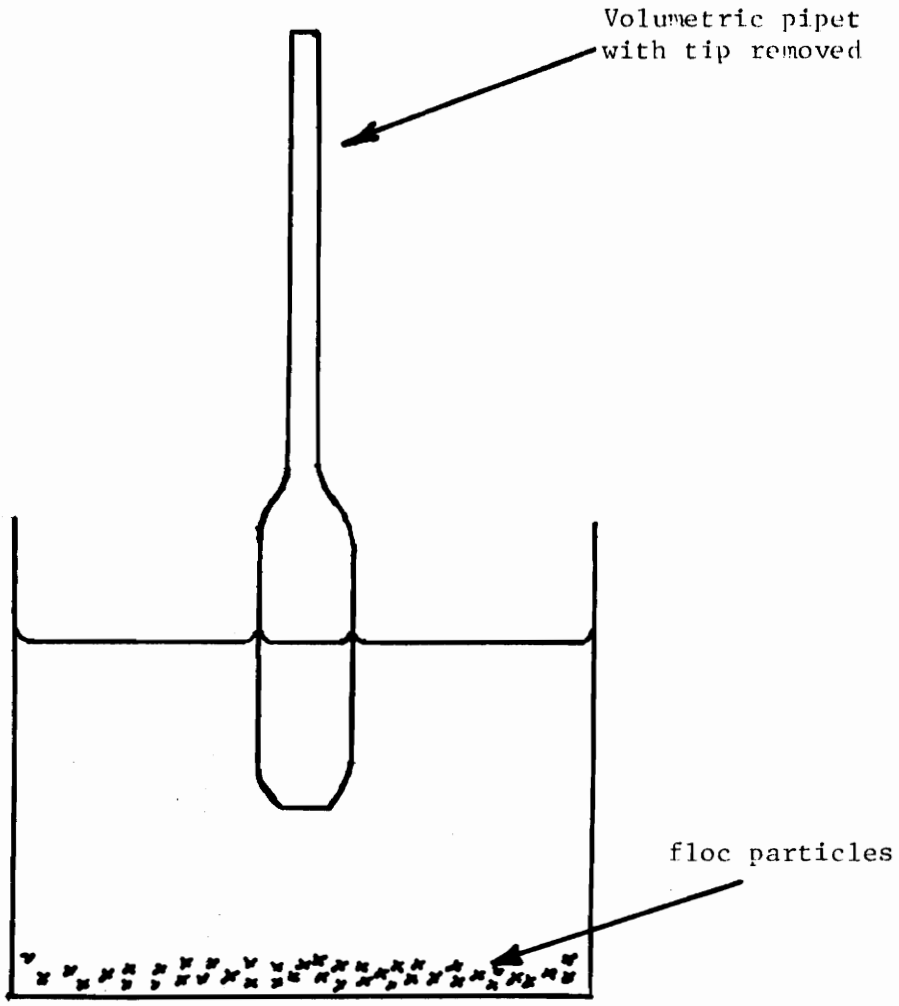

Figure 3: The Modified Volumetric Pipet as Used for the Removal of Samples From Test Jars at the End of the Settling Period.

to the nearest 1.0 ml and were concentrated as previously described, except that the filters were eluted twice so that two final concentrate samples were obtained. These two final concentrates were assayed separately and the total number of viruses found combined in the per cent recovery calculations. Samples assayed were taken from the jars just after addition of the virus (original sample), after centrifugation of the settled supernatant (post treatment sample), after the isoelectric pH adjustment had been made (isoelectric sample), and after the final concentration step had been completed (two samples). The procedure just described was followed for two experiments where the original virus titer was approximately 10^3 PFU/l.

Coagulation tests: Method 2. In this procedure the stock water was made from distilled, deionized and glass distilled water to which 102.5 mg/l alkalinity (as $CaCO_3$) had been added in the form of $NaHCO_3$. The virus inoculum was also added to a large quantity of the water so that less pipetting error would be introduced and more uniform samples would result. One liter of the virus containing water was added to each of six jars. To all but the first of these jars, 30 ml/l alum was added and flash mixed. Flocculation and settling were then allowed to occur as described for Method 1. All remaining procedures in this method are similar to those described for Method 1. This method was used for three experiments in which the initial virus titers were 10^3, 10^4, and 10^6 PFU/l.

IV. RESULTS

Concentration Efficiency Using the Microporous Filter Technique

Virus concentration procedures were evaluated using the microporous filter technique. Starting virus titers of four different orders of magnitude were analyzed. These were approximately 10^3, 10^4, 10^5 and 10^6 PFU/l, and the number of replicate experiments at each level was six, nine, six, and four, respectively. The number of PFU in the original samples, in samples at their isoelectric point and in final concentrates was determined by plaque assay. This value could then be related to the size of the various samples which would give a number of PFU per some volume which could then be related to the original volume at each size to determine a total number of PFU at each step in the concentration procedure (for calculations see Appendix B). When the total number of PFU at each step was known a per cent recovery could be determined. The per cent recoveries obtained as well as the total numbers of PFU for all of the concentration experiments are given in Tables 1, 2, 3 and 4. The per cent recovery varied widely among the replicates for any one initial virus titer tested as can be seen by the standard deviations. The average recovery percentages for all the initial titers also varied when compared (Table 5). The average recovery percentages varied from 18.02 for the 10^5 PFU/l initial titer samples to 53.87 for the 10^3 PFU/l initial titer samples. When recovery percentages from the isoelectric point samples were determined, the values ranged from 14.16 to 47.82 at the same respective initial virus concentrations. There appeared to

Table 1. Concentration of Poliovirus LSc1 by the Microporous Filter Technique.

Experimental Run	Orig. PFU[a]	Iso. PFU[b]	Final PFU	Recovery Efficiencies, %, from Orig. Sample	Recovery Efficiencies, %, from Iso. Sample
1	3.4×10^3	3.2×10^3	1.1×10^3	31.67	33.10
2	3.2×10^3	3.4×10^3	2.7×10^3	82.25	78.62
3	3.8×10^3	2.6×10^3	1.1×10^3	28.05	41.00
4	2.4×10^3	3.2×10^3	0.6×10^3	23.87	19.90
5	1.8×10^3	3.4×10^3	1.6×10^3	90.43	47.88
6	2.2×10^3	2.2×10^3	1.5×10^3	66.92	68.44
		Average =		53.87	48.16
		Standard deviation =		29.57	21.98

[a] PFU = plaque forming units.

[b] Isoelectric point sample PFU.

Table 2. Concentration of Poliovirus LSc1 by the Microporous Filter Technique.

Experimental Run	Orig. PFU[a]	Iso. PFU[b]	Final PFU	Recovery Efficiencies, %, from Orig. Sample	Recovery Efficiencies, %, from Iso. Sample
1	6.27×10^4	3.07×10^4	1.51×10^4	24.13	49.30
2	1.80×10^4	1.20×10^4	4.55×10^3	25.28	37.92
3	1.45×10^4	2.10×10^4	3.45×10^3	23.76	16.40
4	1.47×10^4	5.33×10^3	1.36×10^3	9.23	25.41
5	1.03×10^4	1.10×10^4	3.96×10^3	38.38	36.05
6	5.30×10^4	---[c]	1.06×10^4	20.00	---
7	7.15×10^4	8.20×10^4	1.12×10^4	15.62	13.62
8	5.80×10^4	---	9.09×10^3	15.67	---
9	5.70×10^4	---	5.09×10^3	8.93	---
		Average =		20.11	29.78
		Standard deviation =		9.17	13.75

[a] PFU = plaque forming units.

[b] Isoelectric point sample PFU.

[c] Not determined.

Table 3. Concentration of Poliovirus LSc1 by the Microporous Filter Technique.

Experimental Run	Orig. PFU[a]	Iso. PFU[b]	Final PFU	Recovery Efficiencies, %, from Orig. Samples	Recovery Efficiencies, %, from Iso. Samples
1	9.50×10^{4}[c]	1.35×10^{5}	4.65×10^{4}	47.91	33.72
2	1.30×10^{5}	1.95×10^{5}	7.36×10^{3}	5.66	3.69
3	1.20×10^{5}	1.40×10^{5}	1.52×10^{4}	12.69	10.87
4	4.82×10^{5}	4.56×10^{5}	9.13×10^{4}	18.94	19.03
5	3.76×10^{4}[c]	4.80×10^{5}	3.48×10^{3}	11.93	9.35
6	3.46×10^{4}[c]	4.60×10^{5}	3.80×10^{3}	10.99	8.27
		Average =		18.02	14.16
		Standard deviation =		15.24	10.81

[a] PFU = plaque forming unit.

[b] Isoelectric point PFU.

[c] These values included in this table of 10^{5} PFU/l runs as they were seeded with that intended value and the ISO PFU samples indicate that many virions are present.

Table 4. Concentration of Poliovirus LSc1 by the Microporous Filter Technique.

Experimental Run	Orig. PFU[a]	Iso. PFU[b]	Final PFU	Recovery Efficiencies, %, from Orig. Samples	Recovery Efficiencies, %, from Iso. Samples
1	5.33×10^6	6.67×10^6	1.81×10^6	33.86	27.09
2	3.89×10^6	3.33×10^6	1.22×10^6	31.48	37.93
3	6.00×10^6	6.80×10^6	9.15×10^5	15.25	13.46
4	6.53×10^6	4.74×10^6	2.99×10^6	45.77	63.05
		Average =		31.59	35.38
		Standard deviation =		12.56	20.99

[a] PFU = plaque forming units.

[b] Isoelectric point PFU.

Table 5. Comparison of the Recovery Efficiencies of the Microporous Filter Technique at Various Original Virus Titers.

Approximate Original Titers	Average Recovery from Original Titers, %	Standard Deviation	Average Recovery from Isoelectric Point Titers, %	Standard Deviation
10^6 PFU[a]/liter (4 replicates)	31.59	12.56	35.38	20.99
10^5 PFU/liter (6 replicates)	18.02	15.24	14.16	10.81
10^4 PFU/liter (9 replicates)	20.11	9.17	29.78	13.75
10^3 PFU/liter (6 replicates)	53.87	29.57	48.16	21.98

[a]PFU = plaque forming units.

be no pattern to the relationship between recovery efficiency and the initial titers involved.

Coagulation-Flocculation With Alum

Method 1. For Method 1, two separate experiments utilizing one control jar and five test jars were performed. The experimental data, including initial virus titers, post treatment titers and the final number of PFU detected after concentration, concentration efficiency determined for the control jar and the per cent removals observed in the five test jars are presented in Tables 6 and 7. (For calculations see Appendix B). The recovery efficiency for the two experiments was found to vary by 4.8 percentage points with one efficiency being 62.7 per cent and the other being 57.9 per cent. The average removal percentage for the first experiment was found to be 93.6 per cent with a standard deviation of 4.39. The second experiment had an average removal percent of 97.9 with a standard deviation of 1.34. This indicates very similar removal efficiencies when the control recovery efficiency is applied to all the experimental removals.

Method 2. For the second method, three separate experimental runs were made. One experiment was done using 10^3 PFU/l as the initial virus titer, another used an initial virus titer of 10^4 PFU/l and the third used 10^6 PFU/l. The first two experiments had one jar as a control and four experimental jars. The latter had one control jar but due to contamination some of the cell cultures used in the final assay, the results were available for only three of the experimental

Table 6. Coagulation-Flocculation of Water Containing Poliovirus LSc1 with 30 mg/1 Alum, and the Virus Particles Added After Coagulant Addition (Method 1).

TEST 1

Jar	Orig. PFU[a]	PT[b] PFU	Final PFU	Recovery, %	Removal, %
1[c]	3000	3000	1882	62.7	--
2	2750	125	182	--	86.7
3	3140	250	196	--	93.8
4	3500	0	252	--	92.8
5	3860	290	104	--	97.9
6	4630	0	147	--	96.8
			Average =		93.6
			Standard deviation =		4.39

[a]PFU = plaque forming units.

[b]PT = Post Treatment, before concentration.

[c]Jar 1 acted as a recovery efficiency control.

Table 7. Coagulation-Flocculation of Water Containing Poliovirus LSc1 with 30 mg/1 Alum, and the Virus Particles Added After Coagulant Addition (Method 1).

TEST 2

Jar	Orig. PFU[a]	PT[b] PFU	Final PFU	Recovery, %	Removal, %
1[c]	2000	2125	993	57.9	--
2	2964	124	51	--	96.5
3	2750	0	7	--	99.5
4	2864	0	15	--	99.0
5	3250	0	54	--	96.7
6	1375	250	15	--	97.9
			Average =		97.9
			Standard deviation =		1.34

[a]PFU = plaque forming units.

[b]PT = Post Treatment, before concentration.

[c]Jar 1 acted as a recovery efficiency control.

jars. The results are presented in Tables 8, 9 and 10. For calculation procedures see Appendix B.

When using the Method 2 procedure, the concentration efficiency was much lower than it was in the Method 1 procedure. The concentration efficiency was also lowest at the lowest initial virus titer and seemed to increase as the initial virus titer increased. However, when the concentration efficiency values were used to calculate the removal percentages (Appendix B) the values obtained were very similar to those obtained for Method 1. Even though different initial virus titers were used in Method 2 and 10^3 PFU/l was always the initial titer for Method 1, the final removal percentages were very similar, even at an initial virus titer of 10^6 PFU/l which is three orders of magnitude larger than initial levels in Method 1.

Comparison of Method 1 and Method 2. The results of all five coagulation-flocculation experiments are summarized and compared in Table 11. The sum of the average per cent removal values yields an overall average removal of 95.2 per cent.

Table 8. Coagulation-Flocculation of Water Preseeded with Poliovirus LScl Using 30 mg/l Alum (Method 2).

TEST 3

Jar	Orig. PFU[a]	PT[b] PFU	Final PFU	Recovery, %	Removal, %
1[c]	2375	2256	91	3.8	--
2	1094	0	4	--	90.3
3	908	0	0	--	100.0
4	1094	122	0	--	100.0
5	1479	0	4	--	93.0
			Average =		95.8
			Standard deviation =		4.95

[a]PFU = plaque forming units.

[b]PT = Post Treatment, before concentration.

[c]Jar 1 acted as a recovery efficiency control.

Table 9. Coagulation-Flocculation of Water Preseeded With Poliovirus LSc1 Using 30 mg/l Alum (Method 2).

TEST 4

Jar	Orig. PFU[a]	PT[b] PFU	Final PFU	Recovery, %	Removal, %
1[c]	18,000	19,877	1722	9.6	--
2	18,750	0	264	--	85.3
3	20,875	0	84	--	95.8
4	18,857	125	98	--	94.6
5	6,875	0	49	--	92.5
			Average =		92.1
			Standard deviation =		4.7

[a]PFU = plaque forming units.

[b]PT = Post Treatment, before concentration.

[c]Jar 1 acted as a recovery efficiency control.

Table 10. Coagulation-Flocculation of Water Preseeded With Poliovirus LSc1 Using 30 mg/1 Alum (Method 2).

TEST 5

Jar	Orig. PFU[a]	PT[b] PFU	Final PFU	Recovery, %	Removal, %
1[c]	2,700,000	230,000	342,000	12.7	--
2	2,200,000	1,500	3,710	--	98.7
3	2,400,000	60,000	18,800	--	93.8
4	2,600,000	30,000	8,860	--	97.3
			Average =		96.6
			Standard deviation =		2.52

[a]PFU = plaque forming units.

[b]PT = Post Treatment, before concentration.

[c]Jar 1 acted as a recovery efficiency control.

Table 11. Comparison of the Poliovirus LScl Removal Efficiencies of Alum Coagulation Using Experimental Method 1[a] and 2[b].

TEST[c]	Approx. Initial PFU[d]/l	pH	Recovery, % Jar 1	Removal, % Jar 2	Jar 3	Jar 4	Jar 5	Jar 6	Average Removal, %	Standard Deviation
1[a]	10^3	7.0	62.7	86.7	93.8	92.8	97.9	96.8	93.6	4.39
2[a]	10^3	7.0	57.9	96.5	99.5	99.0	96.7	97.9	97.9	1.34
3[b]	10^3	7.2	3.8	90.3	100.0	100.0	93.0	--	95.8	4.95
4[b]	10^4	7.7	9.6	85.3	95.8	94.6	92.5	--	92.1	4.70
5[b]	10^6	7.5	12.7	98.7	--[e]	93.8	97.3	--	96.6	2.52
						Average =			95.2	
						Standard Deviation =			2.34	

[a]Method 1 = Virus added after coagulant addition.

[b]Method 2 = Virus preseeded in water sample.

[c]TEST refers to the experiments in Tables 6-10.

[d]PFU = plaque forming units.

[e]Not determined.

V. DISCUSSION

Efficiency of the Concentration Technique

All the data accumulated for determination of the efficiency of the concentration technique used in this study indicate that, at best, the procedure is highly variable. The per cent recovery values can range widely for the same virus titer being concentrated as indicated in Tables 1-4. Considering the trial concentration runs using an initial virus titer of 10^3 PFU/1, the titer closest to the majority of the jar test titers, a range of 23.9 to 90.4 per cent recovery with a standard deviation of 29.6 is found for six trials. This appears to be a tremendous variation; however, it may well be deceiving. For this reason total numbers of viruses (as PFU) are also included in the data tables. When examining the per cent removal values, it is important to keep in mind the total numbers of viruses being concentrated and to note that the number of viruses recovered was always in the same order of magnitude.

The wide range of recovery percentages could be accounted for in many ways. Very important among these would be the limitations of the plaque assay system used for these experiments. When using samples with very low virus titers and therefore using a very large inoculum for the individual Petri dishes in the plaque assay, it is very conceivable that even an hour would not allow all the virus particles in the inoculum to adsorb to the cell surfaces. This would allow some of the viruses to become enmeshed in the agar overlay and never appear as plaques. The pH adjustment after concentration could also be a

source of the large fluctuation in recovery percentages. This pH adjustment was done with a visual indicator and done very quickly so that the virions present would not be adversely affected by the initial high pH needed to elute them from the filters. So a difference in time of exposure to the high pH could have had an influence on the number of virions inactivated. This would be especially true when the samples were not assayed until the next day.

When the average recovery percentages (the average of all the individual runs at a particular starting titer) for all the initial virus titers tested are compared, a range of from 18.02 to 53.87 per cent is found. Again the variations were large, and there was no discernable pattern as to an increasing or decreasing recovery percentage with an increasing or decreasing initial virus titer. This indicates that the initial titer of the sample had no affect on the efficiency of the concentration when following the procedure used in these experiments.

Coagulation-Flocculation Tests

The primary goal of the experiments to determine the virus removal by the coagulation-flocculation procedure was to measure how many virions were still present in the supernatant water after flocculation and settling of the floc particles. To insure good floc removal, supernatant samples were carefully taken from the jars with a 50 ml volumetric pipet (modified as in Figure 2) and centrifuged, a step designed to eliminate the possibility that viruses could desorb from floc particles that had not settled. Though the centrifugation

step was routinely carried out, no pellet of any kind was ever noted in the bottoms of the tubes. For this reason, the technique of withdrawing samples from the jars was considered very effective for obtaining a clear supernatant sample.

In the jar tests, one jar was used as a concentration control to determine the concentrator efficiency for that particular experimental run. When all the variation in concentration efficiency previously described is considered, this may seem to be an ineffective means of determining the amount of concentration efficiency for all the jars. However, it was the only practical method by which to determine a concentration efficiency value for each jar which was necessary to calculate the virus removal efficiency for all of the experimental jars. (See Appendix B for calculations.) Also, any variation or error in the concentration efficiency would affect the removal percentage results equally for all of the experimental jars. It is interesting that when this calculation was made, the final removal percentages at all the different initial virus titers were very similar. This would indicate that the concentration efficiency had been more nearly constant than when the concentration efficiency experiments had been previously performed (Table 5). This could be due to the fact that the concentration efficiency experiments were performed using distilled water as the water stock, and the water for the jar tests also contained $NaHCO_3$ which may have exerted a stabilizing effect on the viruses.

Methods 1 and 2 differed primarily in that in Method 1 the virus particles were added after the floc had begun to form. This meant

that the viruses would not be able to be entrapped in the floc as it began to form and, possibly, would be less tightly enmeshed in the floc particles. The technique involved in starting a jar test run using Method 1 also took a longer time and thus some jars had a much longer flash mixing period than others. This time differential was much smaller using Method 2. The results showed no difference in the removals with the increased length of time for the flash mix period. Also, when compared, the removal percentages observed for the two treatment procedures were similar.

Method 2 also was performed with different initial virus titers, and the results indicated there was no difference in removal efficiency for poliovirus when using alum coagulation over a range of 10^3 to 10^6 PFU/1 for the initial virus titer. These levels are all very low for most laboratory work done with these types of tests, however, and the relative similarity might not be present if initial titers as high as, for example, 10^9 PFU/1 (10^6 PFU/ml) were used.

When using Method 2 the per cent efficiency for the concentration as determined by the control jar was much less than that found with Method 1. This could be due to an aggregation effect created by the Na^+ ions present in the Method 2 supernatant which were not present in the Method 1 supernatant. Increasing concentration efficiency was noted as the initial virus titer increased using Method 2. This could be due to the fact that the much larger number of virions present would be less affected by the excess Na^+ ions present.

Finally, when considering the concentration efficiency in these jar tests it must be remembered that post treatment the virus titer is on the order of 10^2 PFU/l or less and, therefore, is at a level which was not tested with separate experiments for concentration efficiency.

These experiments indicate that with a clear water source and a relatively common water treatment process (alum coagulation), most polioviruses present in a monodispersed form will be removed. This is very significant for the water treatment plant with such a water source. With final chlorination of the water for disinfection, most of the threat of viral contamination could be eliminated by simple and commonly used means. However, most water sources do not meet these criteria and more experimental work under more natural conditions would be needed to make a more widespread and realistic statement on the real effectiveness of alum coagulation for virus removal.

VI. CONCLUSIONS

The experimental results of the research contained herein provide evidence for the following conclusions:

1. The results of virus concentration using the microporous filter technique are highly variable with Poliovirus LSc1 at initial titers ranging from 10^3 to 10^6 PFU/1.
2. The efficiency of the microporous filter concentration technique does not appear to be affected by the initial virus titer of the sample being concentrated within the range of 10^3 to 10^6 PFU/1.
3. Coagulation with alum at a dose of 30 mg/1 removes greater than 90 per cent of the poliovirus LSc1 present in a clear water sample.
4. The efficiency of removal of poliovirus LSc1 by coagulation with 30 mg/1 of alum is not affected by the initial virus titer within the range of 10^3 and 10^6 PFU/1.

VII. RECOMMENDATIONS

Studies involving water treatment processes and virus removal at low levels can contribute a great deal to our knowledge of the mechanisms involved in virus removal and inactivation in the actual application of water use. Potable water is becoming more precious every day and the knowledge necessary to assure that it is always available should be gained now while there is time and a crisis has not yet developed. Future work on virus removal by coagulation-flocculation should emphasize the utilization of low initial virus titers in the experimental work. Attempting to make the test water systems more similar to natural water sources should also be a top priority, as well as more widespread monitoring of actual water sources for human enteric viruses. Different types of human enteric viruses should be used and compared in future experimentation. And very close attention should be paid to the virus assay system itself in order to obtain as much consistency and comparability in these studies as is possible.

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APPENDIX A

Minimum Essential Medium (MEM), Earle Base

Ingredients per liter:

l-Arginine HCl	126.98	mg
l-Cystine	24	mg
l-Glutamine	292	mg
l-Histidine HCl·H_2O	41.88	mg
l-Leucine	52	mg
l-Isoleucine	52	mg
l-Lysine HCl	58	mg
l-Methionine	15	mg
l-Phenylalanine	32	mg
l-Threonine	48	mg
l-Tryptophan	10	mg
l-Tyrosine	36	mg
l-Valine	46	mg
Choline Chloride	1	mg
Biotin	1	mg
Folic Acid	1	mg
Inositol	2	mg
Ca-D-Pantothenate	1	mg
Pyriodoxal HCl	1	mg
Thiamine HCl	1	mg
Nicotinamide	1	mg
Riboflavin	0.1	mg
Sodium Chloride	8	g
Potassium Chloride	0.4	g
Calcium Chloride	0.2	g
Magnesium Sulfate	0.0977	g
Monosodium Phosphate·H_2O	0.14	g
Dextrose	1	g
Phenol Red	0.02	g
Sodium Bicarbonate	2.2	g
Streptomycin	0.1	g
Penicillin	100,000	units

Dulbecco's Phosphate Buffer

Ingredients per liter:

Sodium Chloride	8	g
Disodium Phosphate·H_2O	1.12	g
Potassium Chloride	0.2	g
Magnesium Chloride·H_2O	0.1	g
Monopotassium Phosphate	0.2	g
Calcium Chloride	0.1	g
Phenol Red 1%	0.0005	g
Streptomycin	0.1	g
Penicillin	100,000	units

Trypsin-Versene

Ingredients per liter:

Sodium Chloride	8	g
Sodium Bicarbonate	0.35	g
Phenol Red	0.02	g
Streptomycin	0.1	g
Potassium Chloride	0.14	g
Glucose	1	g
Trypsin (1:250)	0.5	g
Penicillin	100,000	units

Phosphate Buffered Saline (PBS), pH 7.0

0.01 M Potassium Phosphate
0.15 M Sodium Chloride

APPENDIX B

Calculations:

Plaque forming units per milliliter (PFU/ml):

$$\text{PFU/ml} = \frac{\text{number of plaques counted}}{(\text{dilution})(\text{inoculum size in ml})}$$

with several plaque plates of a given dilution

$$\text{PFU/ml} = \frac{\dfrac{\text{The sum of plaques counted on all plates}}{\text{number of plates counted}}}{(\text{dilution})(\text{inoculum size in ml})}$$

Total plaque forming units (Total PFU):

$$\text{Total PFU} = (\text{PFU/ml})(\text{Volume in ml})$$

The concentration efficiency in per cent (CE):

$$\text{CE} = \frac{\text{Total PFU in final concentrate}}{\text{Total starting}^{a}\ \text{PFU}}(100)$$

[a]This would be either the original sample or the isoelectric point sample.

Virus removal efficiency in per cent (RE):

$$\text{RE} = \left[1 - \frac{\text{Total PFU in final concentrate}}{(\text{Total starting PFU})(\text{CE}/100)}\right] 100$$

VITA

Richard G. Graham, Jr. was born December 17, 1952 in Neenah, Wisconsin. He attended public school in the Michigan, Wisconsin, Utah and Virginia school systems graduating from Christiansburg High School in the spring of 1971. He entered Purdue University in West Lafayette, Indiana in August of 1971 and received his Bachelor of Science degree in Microbiology with a biology teaching option from that University in May of 1975. In September of 1975 he entered the Graduate School of the Virginia Polytechnic Institute and State University and received his Master of Science degree in Environmental Sciences and Engineering in December of 1977.

Richard Gilbert Graham, Jr.

REMOVAL OF LOW LEVELS OF POLIOVIRUS FROM WATER BY COAGULATION WITH ALUM

by

Richard Gilbert Graham, Jr.

(ABSTRACT)

Human enteric viruses are present at low levels in sources of potable water. Due to the low minimum infective dose required for such viruses, these low levels are of public health significance and will become more important as water reuse increases. The effectiveness of alum coagulation for virus removal was evaluated using a synthetic water seeded with monodispersed poliovirus LSc1. Experiments were done using 10^3, 10^4, and 10^6 Plaque Forming Units (PFU) per liter. A microporous filter concentration technique was used post treatment to increase the virus titer to make possible virus enumeration by plaque assay on BGM cell cultures. Preliminary work was done to determine the efficiency of the concentration technique at 10^3, 10^4, 10^5, and 10^6 PFU per liter. An average of 30.9 per cent of the original PFU were recovered. Two coagulation test methods were used at the lower virus titers. In one, virus was added to the system after the coagulant during the flash mix period and in the other, the coagulant was added to water already seeded with virus. Removals were similar for both conditions. Alum coagulation was found to remove 95 percent of the virus present at the low titers. This efficiency of virus removal is similar to that

observed at high initial virus titers. Therefore, it appears that alum coagulation is an effective virus removal method.