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Studies on Viruses in Water

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STUDIES ON VIRUSES IN WATER

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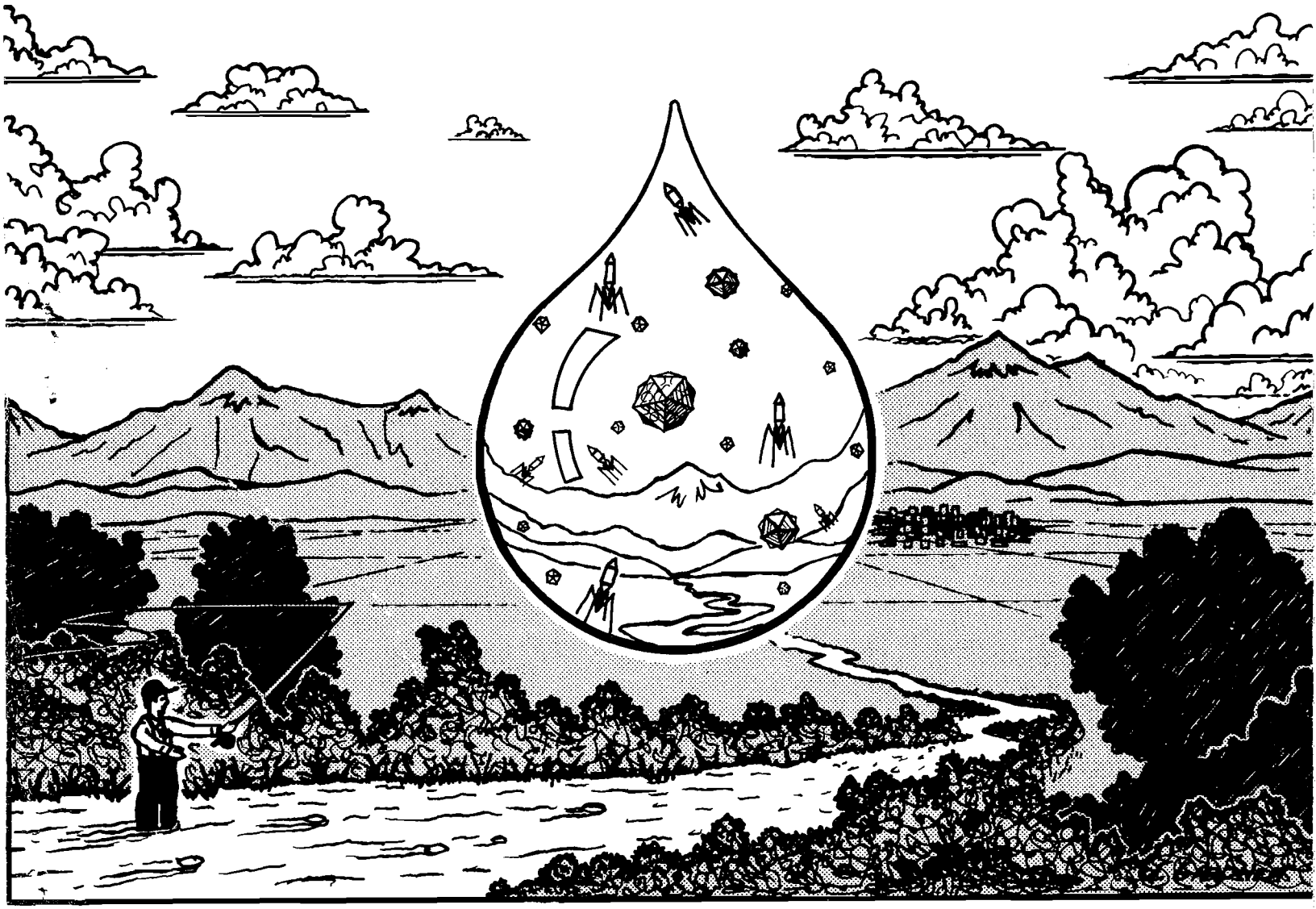

Utah Water Research Laboratory
Utah State University
Logan, Utah 84322

July 1979

WATER QUALITY SERIES

UWRL/Q-79/02

STUDIES ON VIRUSES IN WATER

I. Analytical Procedures for Detecting Viruses and Viral Antigens in Water

   A. A Solid Phase Fluorescence Immunoassa

   B. Concentration of Infectious and Potentially Infectious Reovirus Particles from Polluted Waters Using Protamine Sulfate

II. Inactivation of Infectious and Potentially Infectious Reovirus Particles by Chlorination

III. Removal of Viruses from Water by Filtration Systems

by

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ABSTRACT

A new procedure for the detection of viral antigens in fecal material was developed. The test is performed by first diluting a fecal sample with phosphate buffered saline to give a liquid consistency. The pH is then adjusted to 8.5-9.0 and the solids are allowed to settle for five minutes. Supernatant fluid from above the fecal sediment is placed on the upper surface of a well of an inverted Immulon microtiter plate and incubated for one hour at 37°C to allow virus to adsorb to the plastic. The Immulon plate is then washed three times with a Tween 20 solution and dried. Adsorbed virus is stained with fluorescein labeled antiviral antibody containing Evan's Blue dye. The stained preparations are examined by epi-fluorescence microscopy for the presence of viral aggregates and virus-containing cellular membranes.

The test is applied in a continuous water monitoring procedure that can be used to supplement methods in which infectious viruses are isolated from water.

In another study a protamine sulfate procedure for concentrating and an immunofluorescent cell procedure for assaying infectious virus (IV, reovirus that is infectious without proteolytic enzyme treatment), and potentially infectious virus (PIV, enzyme enhanceable reovirus) from polluted waters have been developed. The presence of PIV in the environment had not previously been investigated.

In following these procedures, protamine sulfate concentrations of 0.005 percent for the first precipitation of the sample, and 0.0025 percent for the second are used. With these protamine concentrations and 0.25 percent fetal bovine serum, IV and PIV are concentrated over 500-fold from river water inoculated with virus. Virus recoveries are between 80 and 100 percent. The IV and PIV fractions are assayed respectively before and after treatment with 200 µg of chymotrypsin per milliliter. When PIV is precipitated from river water, and the precipitate is dissolved and stored at 20°C as a protamine-virus concentrate, only 5 percent of the viral infectivity is lost after 14 days. Therefore, reovirus can be precipitated from water at the sampling site, and only the protamine concentrate needs to be taken to the laboratory to be examined for virus content.

When reoviruses are treated with chlorine, PIV is more resistant to inactivation than IV, and PIV appears to be at least as resistant to chlorination as poliovirus and coxsackievirus A-2.

Granular media filtration systems (i.e., sand, anthracite coal and sand; anthracite coal; sand and garnet) are ineffectual in the removal of the bacteriophage MS 2 from water when used as in-line direct filters. Batch assays have indicated a 93 percent reduction of MS 2 can occur when polyelectrolytes are added to the water. In addition, alum concentrations of 20, 30, 40, and 50 mg/l remove 80 to 98 percent of the virus by precipitation. No reduction of MS 2 was observed at alum concentrations from 1 to 10 mg/l.

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INTRODUCTION

Increasing populations and the waste disposal problems they create continue to threaten our vital fresh water resource. The protection of water supplies from pathogenic agents has required a major effort in the past and will probably require an even more intense technological effort in the future. This effort is especially needed in the case of public health protection from water-borne viral pathogens. In comparison to other microbiological disease agents, viruses have very simple morphology (nucleic acid inside a protein coat) and because of this characteristic they are not always subject to natural decay processes which kill other pathogens in the aquatic environment. The reuse of treated wastewaters for recreational and culinary use will demand strict standards for pathogenic virus removal or inactivation. Although there is presently a high level of research interest, the problems of effectively detecting, identifying, and removing viruses from water are far from being solved.

Most viral diseases transmitted by water are not easily recognized and probably result in subclinical infections which manifest themselves as a mild diarrhea or low grade fever, and have a low level of transmission. It is interesting to note that hepatitis A virus has only recently been described. The rotaviruses (duoviruses) have been only recently recognized as the most important cause of diarrhea in hospitalized infants, and enteritis in animals. These viruses and others have not been susceptible to culture by normal tissue culture methods, and are recognized only by immuno electron microscopic techniques. The recognition and quantification of these and other important etiological agents in aquatic systems are difficult and expensive.

If the public is to be adequately protected, treatment procedures currently in practice in removing or inactivating viral pathogens must be understood. The principles of virus removal and/or inactivation under all conceivable operating conditions of treatment facilities must be described so effective operation of facilities for virus removal can be accomplished.

The major goals of this study were: 1) to convert a fluorescent virus precipitin test to a solid phase fluorescent antibody procedure that has potential use in continuous monitoring of viruses in water, 2) to adapt the protamine sulfate precipitation procedure to recover infectious reoviruses and potentially infectious (chymotrypsin enhanceable) reoviruses from water, 3) to study the inactivation kinetics of infectious reoviruses and potentially infectious reoviruses by chlorination, and 4) to determine the efficiency of sand filters in removing viruses from water. The report of research on goals 1 and 2 make up the first section (section I) of this report; the report on goals 3 and 4 is found in sections II and III respectively.

SECTION I. ANALYTICAL PROCEDURES FOR DETECTING VIRUSES AND VIRAL ANTIGENS IN WATER

A goal of this study was to evaluate the fluorescent virus precipitin test as a means of detecting viruses in water. It soon became evident that the procedure would have greater utility in water quality control testing if it were used as a solid phase antibody test, i.e., it could be used in continuous monitoring of water without first using filtration or precipitation procedures to remove the viruses from water. Therefore, the fluorescent virus precipitin test was modified and used as a solid phase antibody procedure. The ultimate goal was to apply the test in a continuous water monitoring procedure that could be used to supplement methods in which infectious viruses are isolated from water. A major problem in detecting viral antigens in polluted water is the presence of debris that interferes with testing. Debris can bind nonspecifically with antibody or with virus so it cannot react with antibody. To meet this challenge directly, the test was developed using human and calf diarrheic stool specimens which contain an extremely high concentration of nonviral debris.

In another part of our work, the protamine sulfate method of England (1972) for precipitating viruses was modified and used as a concentration procedure for recovering infectious viruses from water. This work was done to provide a sensitive infectious virus assay against which the solid phase fluorescent virus test could be compared.

In the present section, the development of the solid phase fluorescent virus test is described, then a report on studies using protamine sulfate precipitation of reoviruses from water is presented.

A. A Solid Phase Fluorescence Immunoassay

Introduction

Many serologic tests that can be used for detecting viral antigens in water have been developed. Some that have potential in water quality testing are radioimmunoassay, the fluorescent virus precipitin test, and enzyme-linked immunosorbent assays.

Radioimmunoassays are immunological tests in which antigen, bound either to a solid support (Watanabe and Holmes 1977), to an antibody bound to a solid support (Catt, Niall, and Tregear 1967), or in solution (Hunter and Greenwood 1962), is reacted with radioactive antibody. The resulting antigen-antibody complex is quantified in a scintillation counter, i.e., the bound radioactivity is proportional to the antigen concentration. Antibody can also be quantified by competitive binding of labeled and unlabeled antibody to a known concentration of antigen.

In the fluorescent virus precipitin test (Foster, Peterson, and Spendlove 1975) viral samples are reacted with fluorescein labeled antibody in concentrations that allow for the formation of labeled antibody-virus aggregates, which can be detected microscopically.

The enzyme-linked immunosorbent assays are conducted by first adsorbing the antigen in the specimen being tested to a solid support or to an antibody adsorbed to a solid support (Voller, Bidwell, and Bartlett 1976). This bound antigen is reacted with enzyme labeled antibody and the supporting structure is rinsed. Substrate is then added to the reaction vessel and the hydrolysis of the substrate, with time, is related to the antigen or antibody concentration.

A supplemental water quality control test has been developed. A brief description of the test as it was originally conceived follows. A plastic that adsorbs protein (antibody) is secured to a support. Antiviral antibody is adsorbed onto the plastic, after which albumin or Tween 20 is added to the plastic surface to saturate any protein binding sites not coated with immunoglobulin. A fecal extract or polluted water to be tested for the presence of virus is added to the antibody coated plastic. If infected intestinal cells, infected cell debris, and/or fecal virus are present, they are bound to the antibody. The presence of bound viral antigen is detected by staining with antiviral fluorescent antibody (Figure 1).

Literature Review

Adsorption of proteins by plastics

The adsorption of proteins by plastics was first studied by Leininger (1965) and Leininger et al. (1966) in connection with the use of plastics in artificial organs in contact with blood. The study of Leininger's group was concerned with the thrombogenic nature of the plastics. Ponder (1965) attributed thrombus formation to either the adsorption of blood proteins and perhaps other blood components to the plastic surfaces or the "sticking" of formed elements such as leukocytes and platelets to the surface. In the studies by Leininger (1965) and Leininger et al. (1966), the zeta potential of the plastic surface was used to monitor adsorption of the antibody to the plastic. It was noted that changes in the zeta potential occurred during contact of polystyrene surfaces with fibrinogen, gammaglobulin or albumin, at one-thousandth the usual physiological concentrations.

Herrmann and Collins (1976) did a quantitative study on the adsorption of immunoglobulins to plastic. They labeled rabbit IgG with iodine-125, and studied its binding to cellulose nitrate, polyallomer, polystyrene, and polyvinyl. Concentrations of 100, 10, and 1 µg protein/ml at pH 9.5 and incubation times of 1, 5, and 18 hours were used. It was reported that the different types of plastic adsorbed IgG to approximately the same degree, with the exception of cellulose nitrate, which adsorbed considerably less at all protein concentrations and adsorption times

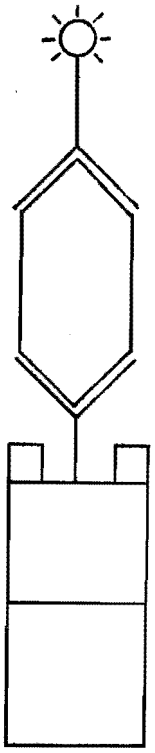


Figure 1. Diagram of solid phase fluorescent virus test.

tested. Concentration was reported to be the most important factor in the adsorption of protein in absolute amounts (µg), however, the percent adsorbed was reported to decrease rapidly with increasing concentration.

Clark and Adams (1977) reported that concentrations of 1-10 µg of gamma globulin/ml optimally coated the wells of microtiter plates, despite differences in antisera titers. Concentrations above 10 µg/ml were reported to reduce the strength of the virus specific reactions and increase nonspecific reactions. Voller, Bidwell, and Bartlett (1976) reported that a pH between 9.0 and 10.0 gave the best results when attaching antibody to microtiter plates.

Solid phase radioimmunoassay

Before the advent of "solid phase" techniques, radioimmunoassays required that one include a procedure to separate free and bound tracer antigen after antigen-antibody equilibrium had been reached. Separation techniques include: precipitation by a second antibody (Morgan and Lazarow 1963; Hales and Randle 1963); electrophoresis (Hunter and Greenwood 1962; Fitschen 1964); chromatoelectrophoresis (Yalow and Berson 1959); ion exchange (Meade and Kliterguard 1962); solvent fractionation (Odell, Wilber, and Paul 1965); gel filtration (Genuth, Frohman, and Lebovitz 1965; Haber, Page, and Jacoby 1965); salt precipitation (Grodsky and Forsham 1960); and adsorption to charcoal (Herbert et al. 1965). The use of these isolation procedures not only increased the length and complexity of this technique but invariably increased the possible sources of error.

Catt, Niall, and Tregear (1967) eliminated antigen isolation procedures with the innovation of the solid phase radioimmunoassay. In the initial study, rabbit antibody against human growth hormone was coupled to an insoluble polymer (diazo-polystyrene) to which iodine-131 labeled tracer antigen and unlabeled antigen were added. It was found that the amount of tracer antigen bound, varied inversely with the amount of unlabeled antigen present, thus allowing for quantification of the unlabeled antigen in a sample over a range of 1 to 10 µg. They then used a different solid phase support consisting of the isothiocyanate derivative of a graph copolymer of poly tetrafluorethylene ('fluon G-4) and styrene. It was noted that the addition of vast quantities of unlabeled human growth hormone to solid phase antibody which had been allowed to bind labeled growth hormone does not result in displacement of the tracer, therefore, binding of the antigen to solid phase antibody is not readily reversible.

From these initial studies, the use of solid phase radioimmunoassays spread to many fields of study and led to an array of solid phase tests varying markedly in solid supports used, incubation times, volumes and concentrations of reagents, and sensitivity and specificity. The basic principles, however, remain the same: attachment of the antibody to the support medium, binding of the antigen, addition of the radioactive antibody, measurement of the bound radioactivity, and comparison to a standard curve.

One of the most important innovations in solid phase tests is the utilization of microtiter plates as the support medium. Purcell et al. (1973) first

developed the micro solid-phase radioimmunoassay (micro-SPRIA) for hepatitis B antigen (HB-Ag). This procedure involved the coating of polyvinyl V-bottom microtiter plates with hyperimmune guinea pig serum, followed by a coating with albumin. These sensitized plates were reacted with samples to be tested for HB-Ag, rinsed, and reacted with purified antibody labeled with iodine-125. The wells were then cut apart with a hot spatula or dissecting needle and counted in a gamma spectrometer. The principal advantages to this test are: 1) ability to test sera for HB-Ag with sensitivity comparable to other tests, but with a reduced volume; 2) ability to test large numbers of samples simultaneously with greater ease than other comparable tests; and 3) economy.

Enzyme linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) are very similar in principle to radioimmunoassays, with the main difference being that an enzyme rather than a radioisotope is conjugated to the final antibody. The first ELISA test was developed by Engvall and Perlmann (1971a) for the detection of specific antibodies. In this study, the immunoglobulin fraction of sheep anti-rabbit IgG was conjugated to microcrystalline cellulose. Alkaline phosphatase from calf intestinal mucosa was conjugated to rabbit IgG.

In a second paper by Engvall and Perlmann (1971b), polystyrene tubes coated with antigen were incubated with antiserum followed by an alkaline phosphatase linked preparation of anti-immunoglobulin. The enzyme-linked immunoglobulin that remained in the tubes after washing thus provided a measure of the specific antibodies in the serum.

From these initial studies, many test variations were developed and used in a wide array of combinations giving rise to a multitude of tests suitable for many fields of study and having a wide range of specificity and sensitivity. Voller, Bidwell, and Bartlett (1976) have reviewed the theory and practice of ELISA tests. They explained the principles of the three basic ELISA techniques. In the first (the indirect method for the detection of antibody), antigen is coupled to a solid phase support. Serum to be tested for antibody is incubated in the sensitized carrier. After appropriate washing steps, enzyme labeled antibody against the antigen (antibody in the serum) is added. The amount of enzyme present is then measured by the amount of substrate it hydrolyzes.

In the second method, a double antibody is used to detect the antigen. Antibody is used to sensitize a carrier surface; a solution containing antigen is added, the mixture is then incubated and washed to remove the unbound portion. The enzyme labeled specific antibody is then allowed to react with the bound antigen and becomes "captured" by the sensitized surface. After rinsing, the bound enzyme can be measured by the amount of hydrolysis of substrate which is related to the antigen concentration.

Detection of antigen by the labeled antigen competition method is the final method reviewed by Voller, Bidwell, and Bartlett (1976). The carrier surface is sensitized as above, but in this procedure various dilutions of solution to be tested for antigen are incubated with various dilutions of labeled antigen. The amount of enzyme labeled antigen attached to the sensitized carrier is again measured by the rate of hydrolysis of its substrate. The amount of labeled antigen bound will vary inversely with the amount of unlabeled antigen present.

The most commonly used carrier surfaces were also reviewed by Voller, Bidwell, and Bartlett (1976). Among those discussed were Sepharose beads, which permit the covalent linkage of either antigen or antibody; polystyrene tubes; and polystyrene microtiter plates. The microtiter plates were noted to be much more suitable for large scale use because of their economy and small quantities of reagents required. Conditions for sensitizing the microtiter plates were also discussed.

The characteristics of the microtiter double antibody technique for plant virus detection have been reported by Clark and Adams (1977). They used polystyrene microtiter plates from Cooke Engineering (M29AR). The plates were sensitized with gamma globulin at a concentration between 1 and 10 µg/ml regardless of the serum titer. It was noted in the sera tested that globulin concentrations exceeding 10 µg/ml reduced the strength of the virus specific reaction and increased that of nonspecific reactions. Adsorption of the gamma globulin was reported as being very rapid with little difference in extinction values obtained from wells coated 2 or 4 hours at 37°C or 18 hours at 6°C. Sensitized plates were stored at 18°C for several weeks or were dried over $CaCl_2$ for several days without apparent loss of efficiency. It was reported that the plates must be washed thoroughly between each reaction step to remove any soluble reactants that could cause nonspecific reactions. Plates were routinely washed three times with phosphate buffered saline (PBS)-Tween 20 solution. The Tween 20 was included to prevent post-coating adsorption of protein to the well surface. Incubation times were also studied and it was reported that samples (diluted in PBS-Tween 20) incubated at 18°C for 6 hours gave better results than did those incubated at 37°C for 1, 2, or 4 hours.

Fluorescent Virus Precipitin Test

Foster, Peterson, and Spendlove (1975) developed an immunological assay for the detection of small particulate antigen, in particular, viruses in stool specimens. This procedure reacted filtered virus samples with filtered fluorescein labeled antibody under conditions that would produce fluorescent antibody-aggregated virus particles that were detectable by the use of a fluorescence microscope with epi-illumination. This test made it possible to detect viruses rapidly and reliably without the expense of an electron microscope or use of dangerous isotopes. It was reported to be as sensitive as any other serological test and did not require the cultivation of the virus.

Recently, Yolken et al. (1977) pointed out that the fluorescent virus precipitin test was preferable to several other tests used to detect rotaviruses in stool specimens. The disadvantages cited for the other tests were: 1) electron microscopy is not readily available to most laboratories and cannot be used with large numbers of specimens; 2) the material in many stools is anticomplementary in the complement fixation test; 3) counter immunoelectrophoresis often fails to detect small amounts of rotavirus antigen unless special concentration or staining procedures

are used; and 4) fluorescent antibody staining of infected cells in smears made from stool specimens gives a large number of false positive results.

Potential importance of rotaviruses in water pollution studies

A recent report of an extensive 18 month investigation (Kapikian et al. 1976) stated that approximately half of the children surveyed who had gastroenteritis were infected with rotavirus. The World Health Organization (1978) has estimated that there are 5 to 18 million deaths in children under 5 years of age in underdeveloped countries each year caused by gastroenteritis. If rotavirus is causing half of these deaths rotavirus gastroenteritis is a major public health problem of the world. Even though rotaviruses are the single most important cause of gastroenteritis and they are potentially the most important viral contaminant of polluted waters, practically nothing has been done to determine if rotavirus infections are transmitted by water. Rotavirus was selected for use in these studies because of its potential importance in water quality control testing.

Rotavirus gastroenteritis has been found in humans, monkeys, mice, calves, foals, lambs, rabbits, deer, antelope, and other animals. The wide range of species involvement is especially significant in water pollution studies since inter-species transmission has been demonstrated (Mebus et al. 1976). In regard to viruses of nonhuman origin in water, Berg (1973) made the following comment that is relevant to our studies with rotaviruses, "viruses that infect animals, especially those viruses that multiply in or about the gastrointestinal tract, must enter our waterways in large numbers from processing plants and with rural runoff. There is a need to identify these viruses and to determine their effects on people who consume them."

It seems likely that some rotavirus infections are transmitted by water. Individuals with rotavirus gastroenteritis excrete virus at concentrations estimated to be as high as 10^{10} viral particles/gram of diarrheic stool (British Medical Association 1975). An additional source of rotaviruses in polluted water is the individual who may not have a clinically recognizable infection, but who excretes the virus (Bolivar et al. 1978; Chrystie, Totterdell, and Banatvala 1978; Murphy, Albrey, and Crewe 1977). The rotaviruses appear to be relatively stable; i.e., they retain their infectivity in stool specimens held seven months at room temperature (Flewett et al. 1975), so virus that finds its way into water should survive for long periods.

Rotaviruses have not been isolated from water because they are almost all potentially infectious, i.e., they are highly communicable, but can rarely be grown in cell culture. For example, the human rotavirus has never, to our knowledge, been grown to a usable concentration in cell cultures. Interestingly, like the reoviruses, the infectivity of some bovine (Babiuk et al. 1977; Matsuno, Inouye, and Kono 1977) and porcine (Theil, Bohl, and Agnes 1977) rotaviruses can be enhanced by trypsin treatment. Evidently in nature rotavirus infectivity is enhanced by enzymes as the viruses pass through the body. Eventually ways will probably be found to simulate conditions found in the gastrointestinal tract, so rotaviruses of all species can be activated and grown in cell culture. When this time comes, the procedures used in this study will enable investigators to directly determine the role of water in disseminating rotavirus infections.

Lycke et al. (1978) reported on an epidemic of rotavirus gastroenteritis in a small Swedish town in which at least 30 percent of the population (3,172 adults and children) were infected within two weeks. They observed that the fresh water supply was contaminated with sewage effluent.

Materials and Methods

In the early part of our study, it was found that fecal virus would bind as efficiently to Immulon plastic as it would to antibody bound to plastic. Therefore, the solid phase antibody part of the procedure was eliminated. The procedure as it was developed is described below.

Antiserum preparation

Antiviral antiserum was prepared by inoculating rabbits with purified rotavirus. The virus suspension was mixed with Freund's adjuvant to give a 50-50 adjuvant-virus mixture before inoculation. Initially 5 µg of virus were injected into each hind flank (10 µg/animal). After six weeks, the animals were boosted with 5 µg of virus/animal. Two weeks later the serum was collected.

Plastic surfaces for adsorbing proteins

Polyethylene, polyvinyl, polystyrene and Immulon, a polystyrene derivative, were tested for their capacity to adsorb antiviral antibody. The Immulon plastic was selected for use in this study because it adsorbed protein effectively and it was available as a microtiter plate (Micro-Elisa microtiter plate, Dynatech Laboratories). The plates are not used in the conventional way. They are inverted, so the wells usually used in serologic testing are facing down. The flat round outer surface of each well which now faces up is the antigen (virus) adsorbing surface used in the test. Inverted plates can be easily examined microscopically. The cover originally made to fit the top of the plate was attached with a tape hinge to the bottom of the plate to make a humid chamber to confine the virus. The plates are especially convenient since they contain 96 wells that can be manipulated as a unit.

The adsorption of protein to the plastic was studied at different concentrations of protein and at different pHs. Evaluations of protein adsorption were based on the effects of the conditions on specificity and sensitivity in the test.

To examine these two factors, i.e., pH and globulin concentration, a plate was prepared in which both factors were varied in a "checkerboard titration." Concentrations of 0, 0.01, 0.1, 1.0, 5.0, 10.0, and 100.0 µg/ml, and pH values of 8.5, 9.0, 9.5, 10.0, and 10.5 were examined.

The serum was diluted in a coating buffer (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; NaN_3, 0.2 g; and H_2O, 1000 ml). Thirty microliters of the antibody solution were placed on the upper surface of each

inverted well and incubated for two hours at 37°C.

Compounds used to block protein adsorption sites

The three most commonly used compounds for blocking sites on plastic that are not coated with immune globulin are albumin, polyoxyethylene sorbitan monolaurate (Tween 20) and polyoxyethylene sorbitan monooleate (Tween 80). These compounds were compared in tests using positive and negative fecal specimens. Concentrations of blocking compounds ranging from 0.1 to 1.0 percent and incubation times of 5 to 30 minutes were used to determine the minimum concentration and time required to block the unreacted sites. The PBS-Tween 20 mixture had the following composition: NaCl, 8.0 g; KH_2PO_4, 0.2 g; $Na_2HPO_4 \cdot 12\ H_2O$, 2.9 g; KCl, 0.2 g; Tween 20 or Tween 80, NaN_3, 0.2 g; and 1000 ml of H_2O.

Preparation of fecal specimens

Human and bovine stool specimens were obtained from children and calves. The presence or absence of rotaviruses in the fecal samples was determined by immunoelectron microscopy.

Studies were conducted to determine the purity required of viral antigens extracted from feces to give optimal test results. Five different extraction procedures were tested. 1) Feces were suspended in PBS after which the pH of aliquots was adjusted with 0.5 N NaOH to pH values from 7.4 to 9.5. The preparations were allowed to settle for 5 to 15 minues. 2) Fecal material was diluted in PBS to a liquid consistency and allowed to settle for 5 to 15 minutes. 3) Fecal suspensions diluted in PBS were centrifuged at 3,020 x g for 10 minutes. 4) Freon and fecal suspensions were mixed at a ratio of 1 ml of freon to 1.38 ml of fecal suspension. This preparation was homogenized at 10,000 RPM for 2 minutes in a VirTis homogenizer (VirTis Co. Inc., Gardiner, N.Y.), and centrifuged at 3,020 x g for 10 minutes. 5) Stool suspensions treated as described in 4 above were filtered through a 0.45 µm pore size Millipore filter. In procedures 2, 3, 4, and 5, the pH of the suspended feces was adjusted to 8.5 before fecal fluids were transferred to the plastic for adsorption.

After the various treatments, fluids above suspensions that had been allowed to settle, supernatant fluids in centrifuged suspensions, and filtrates were tested for their viral antigen content using the solid phase, fluorescent antibody test.

Fluorescent antibody procedures

Sera were fractionated with 35 percent saturated ammonium sulfate as described by Herbert, Pittman, and Cherry (1972). The gamma globulin fraction was then conjugated with fluorescein isothiocyanate using procedures described by Spendlove (1966). The conjugated antibody solutions were passed through a 0.45 µm pore size Millipore filter that had been pretreated with Tween 20.

With the use of Evan's Blue as a counter stain, positive samples were easier to identify, and the determination of negative samples was easier due to the bright red staining of cellular membranes and the elimination of background fluorescence. The stain was mixed with the antibody conjugates at a final concentration of 0.005 percent.

Serial dilutions of the conjugated antibody were tested for staining intensity and serologic specificity. The time and temperature for optimal staining using a 1:60 dilution of the conjugate was also determined. Three microtiter plates were prepared identically with five sets of tests, each set contained positive and negative viral fecal specimens. Fluorescent antiviral antibody was added; one plate was incubated at 4°C, one at 22°C, and another at 37°C. After 15, 30, 60, 120, and 240 minutes respectively, one set of preparations on each plate was stained, washed three times, and dried. Washing the inverted well involved removal of the liquid from the test surface, covering with PBS-Tween 20 and agitating for 30 seconds. Negative samples were given a rating of (-). Positive samples were rated on a scale from 1+ to 4+. Viral aggregates and viral antigen in membranes from infected cells were both used in this rating. A 1+ was given to a preparation containing either viral aggregates or membranes with viral antigens in less than 25 percent of the fields, and a 4+ was given to preparations with both viral aggregates and membranes with antigens in over 75 percent of the fields.

All tests were read on a Zeiss model GFL fluorescence microscope equipped with an IV F vertical illuminator and a 2 FL reflector containing a chromatic reflector LP 520, excitor filters KP 490 and KP 500, and optical barrier filter LP 520.

Comparison with other tests

The solid phase fluorescent antibody test was compared with the fluorescent virus precipitin test (Foster, Peterson, and Spendlove 1975) and immunoelectron microscopy (Vassel and Ray 1974) in detecting rotaviruses in stool specimens. Four positive and four negative specimens were examined by each method.

Results

Plastic surfaces for adsorbing proteins

Table 1 shows the results when various concentrations of immune globulin at different pHs were adsorbed onto Immulon plastic surfaces. Serum protein adsorbed effectively when placed on the plastic at concentrations between 0.1 and 10.0 µg of protein per milliliter. Sensitivity of the test was less when 100 µg of protein per milliliter were used. Good results were obtained at adsorption pHs between 8.5 and 10.

The most interesting observation of this experiment was that viral antigen adsorbed as readily to the plastic surfaces that were not coated with antibody (a control preparation) as it did to antibody coated surfaces. This finding is not surprising since Immulon plastic is used in immunologic procedures because of its protein sorbing properties. As expected, viral antigen did not bind as well if the plastic that had not been coated with serum proteins were exposed to a PBS-Tween 20 wash before exposure to the fecal extract.

Immulon plastic appeared to be as efficient as the attached antibody in adsorbing viral aggregates and as efficient, if not more so, at adsorbing virus

Table 1. Adsorption of IgG to Immulon plastic.

pH	Experiment	Immune gamma globulin concentration (µg/ml) 0.0* 1	0.0* 2	0.0 1	0.0 2	0.1 1	0.1 2	1.0 1	1.0 2	5.0 1	5.0 2	10.0 1	10.0 2	100.0 1	100.0 2
8.5	A	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.0	A	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.5	A	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.0	A	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.5	A	+	+	+	+	+	+	+	-	+	-	+	-	+	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* = No Tween 20 used prior to addition of fecal suspension
A = Fecal suspension containing rotavirus
B = Fecal suspension without rotavirus
+ = Positive Test; - = Negative Test
1 and 2 = Experiments 1 and 2 respectively

containing membranes. Since there was no advantage to adsorbing the antibody to the plastic, and because its elimination saves time and reagents, this part of the procedure was eliminated.

Compounds used to block protein adsorption sites

Originally albumin, Tween 20, and Tween 80 were intended for the blocking of unreacted sites after the adsorption of unlabeled antibody to plastic surfaces. Since the adsorption of unlabeled antibody was determined to be unnecessary, the blocking of the sites before the addition of the fecal suspension would serve only to decrease adsorption of the virus. These sites must not remain unreacted after the addition of fecal suspensions, however, or the fluorescein labeled antibody will adsorb and give false positive tests.

In all but one case, the fecal suspensions tested contained enough adsorbable material that no further blocking of the sites was required. On the sample that required the additional blocking of some unreacted sites, all three compounds tested, i.e., albumin, Tween 20, and Tween 80, gave satisfactory results at the concentrations tested.

A PBS-Tween 20 mixture was used to wash the plates after the incubation of the fecal suspensions in subsequent experiments.

Adsorption of viral antigens to plastics

Various plastics were tested for their capacity to adsorb rotavirus antigens from fecal extracts (Table 2). Polystyrene and Immulon plastic microtiter plates both adsorbed more of the viral aggregates and virus containing cellular membranes from the feces than the polyethylene film adsorbed. No observable difference in performance was noted between the polystyrene and Immulon microtiter plates. The polyvinyl autofluoresced so intensely that it could not be used in the test.

Preparation of fecal specimens

The pH of aliquots of a fecal suspension was adjusted between 7.4 and 9.5 respectively. These preparations were used to determine the effect of pH on the adsorption of viral aggregates and membranes from infected intestinal cells to Immulon plastic. Optimal adsorption occurred at pHs of 8.5 to 9.0 (Table 3). Membranes adsorbed to the plastic at pH 9.5 contained little viral antigen; presumably the antigen was extracted by the high alkalinity.

Table 2. Adsorption of rotavirus to various plastics.

Fecal Samples	Relative Amount of Virus Adsorbed			
	Immulon	Polyethylene	Polystyrene	Polyvinyl
Rotavirus Positive				
#1	4+	2+	4+	NR
#2	4+	2+	4+	NR
Rotavirus Negative				
#1	-	-	-	-
#2	-	-	-	-

NR = No results
\- = Negative results
1+ = Viral aggregates or membranes containing viral antigens in <25 percent of the fields
2+ = Aggregates or membranes in <50 percent of the fields
3+ = Aggregates or membranes in <75 percent of the fields
4+ = Aggregates or membranes in >75 percent of the fields

Table 3. Effect of pH on the adsorption of rotavirus to Immulon plastic.

Fecal Samples	pH				
	7.4	8.0	8.5	9.0	9.5
Rotavirus Positive					
#1	2+	2+	3+	3+	1+
#2	2+	2+	4+	3+	2+
#3	2+	2+	3+	3+	1+
Rotavirus Negative					
#1	-	-	-	-	-
#2	-	-	-	-	-
#3	-	-	-	-	-

See Table 2 for explanation of -, 1+ through 4+ evaluations.

When several different procedures were used to extract viral antigens from fecal suspensions, the best results were obtained with the least pure extracts (Table 4), for example, when the feces were suspended in PBS, allowed to settle for five minutes and the fluids above the sediment were used in the test. With centrifugation and freon extraction, similar numbers of viral aggregates were observed, but there were fewer cellular membranes. Filtration of freon extracted suspensions further reduced the sensitivity of the test.

Stool specimens were suspended in PBS, adjusted to pH 8.5, allowed to settle for five minutes and the fluids above the sediment were exposed to Immulon plastic at different temperatures for different periods of time (Table 5). This procedure was followed to determine the optimal conditions for adsorbing the viral antigens to the plastic. Antigen

Table 4. Extraction of viral antigens from fecal specimens.

Extraction Procedure	Relative Amount of Viral Antigen Detected					
	Rotavirus in Fecal Samples			Rotavirus not in Fecal Samples		
	1	2	3	1	2	3
Settling (Minutes)						
5	4+	4+	4+	-	-	-
10	4+	4+	4+	-	-	-
15	4+	4+	4+	-	-	-
30	3+	3+	4+	-	-	-
Centrifugation	2+	2+	2+	-	-	-
Freon Extraction	2+	2+	2+	-	-	-
Freon Extraction with Filtration	-	1+	1+	-	-	-

See Table 2 for explanation of -, 1+ through 4+ evaluations.

Table 5. Optimal temperature for adsorbing fecal rotaviruses to Immulon plastic.

Incubation Temperature and Time	Relative Amount of Viral Antigen Detected			
	Rotavirus Positive Samples		Rotavirus Negative Samples	
	1	2	1	2
37°C				
1 hr	4+	4+	-	-
2 hrs	4+	4+	-	-
4 hrs	4+	4+	-	-
Overnight	2+	2+	-	-
22°C				
1 hr	2+	±	-	-
2 hrs	2+	±	-	-
4 hrs	3+	1+	-	-
Overnight	2+	2+	-	-
4°C				
1 hr	1+	-	-	-
2 hrs	1+	±	-	-
4 hrs	2+	±	-	-
Overnight	4+	2+	-	-

See Table 2 for explanation of -, 1+ through 4+ evaluations.
± = Inconclusive results

adsorption at 37°C was complete in one hour. Comparable results were not observed at 22°C or 4°C even with overnight adsorption.

Fluorescent antibody staining

The fluorescent antibody conjugate used in this investigation was diluted 1:60. Maximum staining of viral antigen adsorbed to the plastic was reached after 30 minutes of staining at 37°C and 22°C, or 60 minutes at 4°C (Table 6).

Table 6. Optimal time and temperature for fluorescent antibody staining.

	Relative Amount of Viral Antigen Detected					
	Rotavirus pos	neg	Rotavirus pos	neg	Rotavirus pos	neg
	4°C		22°C		37°C	
15 min.	1+	-	2+	-	3+	-
30 min.	2+	-	4+	-	4+	-
60 min.	4+	-	4+	-	4+	-
120 min.	4+	-	4+	-	4+	-
240 min.	4+	-	4+	-	4+	-

See Table 2 for explanation of -, 1+ through 4+ evaluations.

Recommended procedure for the solid phase immunofluorescence test

The following is a brief summary of the steps of the test that was developed.

1. Suspend stool specimens in PBS and adjust the pH to 8.5 with 0.5 N NaOH.
2. Allow the suspension to settle for five minutes.
3. Remove the fluid from above the sediment and transfer 30 µl to the upper surface of a flat well of an inverted Immulon microtiter plate.
4. Incubate the plate for one hour at 37°C.
5. Wash three times with a PBS-Tween 20 (0.05 percent) mixture agitating the plate for 30 seconds before removing each wash fluid.
6. Add the appropriate dilution of fluorescent antiviral antibody to each test surface.
7. Place the cover over the inverted microtiter plate and incubate at 37°C for 30 minutes.
8. Wash the test surface three times with the PBS-Tween 20 solution and examine by epi-fluorescence (incident-light) microscopy at a 1250 x magnification.

Specificity testing

No false positive results were observed when anti-reovirus and antiherpesvirus conjugates were reacted with positive rotavirus fecal specimens. When positive rotavirus preparations were "blocked" with unlabeled rotavirus antibody prior to fluorescent antibody staining, no fluorescence was observed.

Indirect test

Results with the indirect test were comparable to those observed using the direct test, i.e., rabbit antirotavirus antibody bound to rotavirus antigen adsorbed to plastic could be stained with goat anti-rabbit globulin fluorescent antibody, but not with goat anti-bovine globulin fluorescent antibody.

Comparison with other tests

Four positive and four negative fecal specimens were examined by the solid-phase fluorescence immunoassay, the fluorescent virus precipitive test and immune electron microscopy; the results obtained using the three methods were in agreement.

Discussion

The short range goal of this study was to develop a test for the detection of viral antigens in stool material that would be fast, simple, inexpensive to run, require a minimum of equipment, and utilize supplies readily available in virology laboratories. The ultimate goal is the application of the method in a procedure for the continuous monitoring of polluted waters for viruses. The new test utilized the protein binding properties of plastic to attach viruses, viral antigens, and infected cells to the upper surface of an inverted microtiter plate. Viral stool extracts were incubated on this sensitized surface to allow the viral aggregates and virus containing membranes to bind to the attached antibody. After rinsing the bound material, bound viral antigen was reacted with fluorescein labeled antiviral antibody so that it could be identified microscopically.

The attachment of the antibody to the plastic was the first aspect of the test to be examined. It was found that the number of viral aggregates and virus-containing membranes adsorbed was not increased by the attachment of the antibody, i.e., viral antigen adsorbed as well to the plastic as it did to antiviral antibody that was adsorbed to the plastic. When Tween 20 was used to block the sites not coated with antibody there was a decrease in the number of virus-containing membranes that adsorbed to the plastic test surface. Because there was no advantage in adsorbing antibody to the plastic plate this step was eliminated to save time, reduce cost, and decrease the overall complexity of the assay. The elimination of this step may enable a single plastic plate to be used to screen for more than one virus. A study needs to be conducted to determine if all known fecal viruses will adsorb to polystyrene surfaces.

Various plastics were examined, of these, a polystyrene microtiter plate (Falcon, Div. Becton, Dickinson and Co.) and Immulon flat bottom substrate plates, a polystyrene derivative (Dynatech Laboratories), were found to adsorb more of the viral aggregates and virus-containing membranes without apparent loss of specificity than polyethylene or polypropylene surfaces.

The best results were obtained when the fecal suspensions were adjusted to pHs between 8.5 and 9.0. There was a decrease in adsorption at lower pH levels and the virus was apparently extracted from the membranes at pHs above 9.0. Since the detection of membrane bound virus is important in this procedure, the removal of virus from the membranes is undesirable. Optimal conditions for adsorbing fecal virus antigens to the plastic surfaces were determined. The antigens adsorbed more readily at higher temperatures. The time required was 1 hour at 37°C, 4 hours at 22°C, or overnight at 4°C. The shorter incubation, i.e., 1 hour at 37°C, would best suit the goal of expediency. However, the other time and temperature combinations may be advantageous under special conditions, e.g., where incubators are not present or when samples are received at the end of the day. After adsorption and three washings, the plates can be stained or dried and stained at a later time.

Washing the plate consisted of aspirating the liquid, placing a drop of PBS-Tween 20 on the upper surface of each of the inverted wells being used, and agitating for 30 seconds. The Tween 20 is added to the wash mixture so the fluorescein labeled antibody

that is subsequently added will not adsorb nonspecifically to the plastic.

The dilution of the fluorescein labeled antibody to be used in staining is determined by testing serial dilutions of each lot of conjugated antibody prepared. Incubation at 37°C or 22°C for 30 minutes during staining with the conjugate gives satisfactory results. Including the counter stain (Evan's Blue) the fluorescein labeled antibody was found to significantly reduce background fluorescence while making both positive and negative samples easier to identify.

Examination of stained preparations involved the identification of both viral aggregates, and virus containing membranes from infected intestinal cells. Viral antigens associated with membranes are an important asset to this procedure and greatly enhance the sensitivity of the test. With most other assay procedures for fecal viruses, these membranes and the larger viral aggregates are removed in the virus extraction and purification procedures.

In the solid-phase fluorescence immunoassay and the fluorescent virus precipitin test, the "positive" events which are enumerated are fluorescent aggregates, infected cells, or pieces of fluorescently labeled membranes. Similar fluorescent objects are also found in the negative controls, although usually less frequently. Nevertheless, there is often some question as to whether a small fluorescent particle is viral associated or just nonspecific debris. The immunofluorescent cell counting assay (Barnett et al. 1975) does not suffer this ambiguity as the fluorescent cells are very characteristic and are never seen in negative controls. Thus when working with infectious virus the immunofluorescent cell count assay is a more reliable and a more sensitive assay than either the solid-phase fluorescence immunoassay or the fluorescent virus precipitin test. The shortcomings of the immunofluorescent cell count assay are that infectious virus and cell cultures are required, and the results are not read until at least 16 hours after beginning the assay. The advantages and disadvantages of immunofluorescent viral assays that detect infectious virus or viral antigen dictate which is the preferred assay on the basis of the nature of the sample, the facilities available, and time constraints.

For a laboratory with cell culture capabilities, the immunofluorescent cell count procedure is generally the preferred procedure if an assay for infectious virus is suitable. For laboratories without cell culture facilities, the solid-phase fluorescence immunoassay would be the test of choice. In either type of laboratory when test results must be available within the working day or even within two hours the solid-phase fluorescence immunoassay would be the test to use.

The solid-phase immunofluorescence test developed in our study is ideally suited for the large scale screening of fecal samples as well as for smaller scale diagnostic work. It can be easily completed in two hours at a minimal cost. It does not require highly trained personnel and utilizes readily available reagents. The main disadvantages to the test are that it requires the use of a fluorescence microscope equipped with epi-illumination, and it is not easily quantified.

A future goal for continuing study is to develop a procedure that has the capacity to continuously and simultaneously monitor water for all viruses that are considered to be important contaminants of water, viz., hepatitis viruses, rotaviruses, reoviruses, polioviruses, coxsackieviruses, echoviruses, adenoviruses, coronaviruses, etc. The design goal is to develop a device that continuously collects viruses in a series of channels through which water flows. Each channel will contain a plastic insert for adsorbing virus. At an appropriate time interval, the device in the water will be replaced. The plastic inserts from different channels of the device that has been in water will be stained with different antiviral fluorescent antibody conjugates. Some of the advantages and problems encountered in applying the test developed in this study in continuously monitoring water for viruses are discussed below.

Viral antigens adhering to plastic surfaces are examined by incident light fluorescence microscopy using a 12.5X eye piece and a 100X objective. At this magnification, a fluorescing aggregate containing as few as 10 small virus particles on an edge can be seen. This test is many orders of magnitude more sensitive than radioimmunoassay. A problem with a test of this sensitivity is nonspecific staining. In polluted water, there is undoubtedly debris that will take up fluorescein conjugates nonspecifically. Fortunately most of this debris is nonprotein, so relatively little should adsorb to the plastic surface used in the test. Therefore, water containing a lot of debris can be examined, since the debris can be removed by washing. This is important since prefiltration used in procedures to detect viruses in water removes large amounts of virus. In our studies with rotavirus, adsorption of infected cells and cell membranes containing viral antigen to the plastic were a valuable asset in the test.

Some of the potential problems that might be encountered in applying the test to detect viruses in water are: 1) bacteria might grow on the plastic and interfere with virus adsorption; 2) the water might require conditioning in order for the viruses to adhere well to the plastic; and 3) the water may impinge on the plastic too fast for the viruses to adsorb.

Conclusions

1. Viral aggregates and virus-containing membranes readily adsorb to the plastic surface of inverted microtiter plates.

2. The number of viral aggregates and virus containing membranes adsorbed to the surface is not increased by sensitizing the plastic surfaces with specific antiviral antibody.

3. The adsorbed viral antigen can be detected by staining with fluorescein labeled antiviral antibody and subsequent fluorescence microscopy.

4. Polystyrene surfaces are superior to polypropylene for adsorption of viral antigens.

5. The pH for maximum adsorption of viral aggregates and virus containing membranes is between pH 8.5 and 9.0 with a 1 hour adsorption period at 37°C.

This test has the potential of providing rapid, ultrasensitive testing with continuous sampling to assure virological safety of public water supplies.

B. Concentration of Infectious and Potentially Infectious Reovirus Particles from Polluted Waters by Protamine Sulfate

Introduction

Increased direct and indirect water reuse is inevitable. Therefore, development of better quality control test procedures for viruses is becoming increasingly important. Since different methods used for concentrating and detecting viruses in water are somewhat specific for certain groups of viruses, it is important that the viruses most consistently and abundantly present in fecally polluted waters be recovered and identified.

Reoviruses have been reported in various parts of the world to occur as frequently as picornaviruses in wastewaters and sewage (England 1972; Jopkiewics, Krzeminska, and Stachowska 1968; Knocke, Pittler, and Hoepken 1967; Malherbe and Strickland-Cholmely 1967; Nupen 1970; Sattar and Westwood 1978). Reoviruses can be recovered from lower animals as well as man (Rosen 1968).

In 1972, England developed a protamine sulfate concentration method that effectively precipitates reovirus and adenovirus from sewage effluents. She reported reoviruses to be the most frequently present and abundant animal virus occurring in sewage.

Infected cells release two types of reovirus particles: 1) infections virus (IV), that has a loose coat or has no outer coat; and 2) potentially infectious virus (PIV), that has a complete outer coat, and is not infectious (Spendlove, McClain, and Lennette 1970). The PIV can be converted to IV by removing its outer coat with proteolytic enzymes. This enzyme enhanceable reovirus fraction often accounts for over 90 percent of the total reovirus particles in infectious culture fluids (Spendlove et al. 1966). The phenomenon of enzyme enhancement of infectivity has also been demonstrated with rotavirus (Babiuk et al. 1977; Matsuno, Inouye, and Kono 1977; Theil, Bohl, and Agnes 1977).

Reoviruses have been demonstrated to be resistant to inactivation by heat (Spendlove and Schaffer 1965), U.V. irradiation (McClain and Spendlove 1966), and to some viricidal agents present in wastewaters (Cliver 1975; Ward and Ashley 1977). The intact outer coat of the PIV makes it more resistant to heat inactivation than IV (Spendlove and Schaffer 1965). This observation suggests that PIV may generally be highly resistant to inactivation by physical and chemical agents. Resistance to inactivation is a desirable characteristic for a virus to be used in water quality control testing. Regardless of these facts and numerous reports of isolations, reoviruses have received relatively little attention in water quality testing, and in none of these investigations have the PIV particles been studied.

Our study modifies England's (1972) protamine sulfate concentration method, so that IV and PIV particles are precipitated under conditions that permit both viral forms to be assayed by an immunofluorescent cell counting procedure. Immunofluorescent staining circumvents the problem of overgrowth of reoviruses by other enteric viruses as encountered in the plaque assay procedure. This assay technique also overcomes the problem of false plaque formation (Fannin et al. 1978; Leong, Barrett, and Trussell 1978). These modified procedures will enable a more precise evaluation of the potential usefulness of reoviruses in water quality testing.

Literature Review

Reoviruses in polluted waters

Wallis and Melnick (1967) in a study of virus recovery from water reported that all viruses except reoviruses were absorbed to $Al(OH)_3$, and all except reovirus and adenovirus were absorbed to $CaHPO_4$. Contrary to these results, Palfi (1971) employed $CaHPO_4$ as an absorbent and isolated reovirus from 31 percent of 527 samples of sewage in Budapest.

Malherbe and Strickland-Cholmley (1967) found reoviruses as frequently as enteroviruses in sewage in treatment plants, and showed that reoviruses and enteroviruses are not significantly reduced in number by trickling filters, while Malherbe, Strickland-Cholmley, and Geyer (1967) recovered significant amounts of reoviruses in slaughterhouse effluents. Knocke, Pittler, and Hoepken (1967) isolated reoviruses from wastewater.

Nupen (1970) pointed out a problem when attempting to detect reoviruses in water samples. She was unable to demonstrate plaque formation by reoviruses. Reovirus cytopathic effects did not appear in roller-tube cultures until seven days after inoculation and even then was only detectable in the higher dilutions, which had removed other enteric viruses that were present in lower concentrations. Berg (1971) recovered less than 20 percent of the reovirus in a 1 liter volume of distilled water by absorption to, and elution from PE 60 layers.

England (1972) developed a method for concentrating reovirus and adenovirus from sewage and effluents by protamine sulfate (salmine) treatment. She found reoviruses to be the most consistently recovered the year around. In a review article, England (1974) summarized the results she obtained when various absorbents were used to recover viruses from water. Her report shows that reovirus recoveries by salmine and by $AL(OH)_3$ were comparable, and that salmine was usually superior to PE 60.

Palfi (1974) isolated viruses from the different stages of sewage treatment. Of the total viruses isolated, 44 percent (60 isolates) were reovirus type 1. The next most common was echovirus type 7 (15 percent of the isolates).

Cliver (1975) studied survival of viruses during digestion of sewage sludge. Reoviruses appeared to resist inactivation in the sludge more than the other viruses (see Cliver's Table 2).

A study compared three methods used to free viruses embedded in solids of sludge, soils, mud, and surface waters (Wellings et al. 1975). Reoviruses were among the viruses isolated in the study.

Sweet, Ellender, and Leong (1974) used electro-osmosis to concentrate viruses from water. A reovirus was concentrated 25 to 30 fold with 67 to 75 percent recovery. In a system using activated charcoal-filtered tap water as a dilutent, considerably less reovirus was recoverable by electro-osmosis.

Berg (1970) and Berg, Dahling, and Berman (1971) adsorbed viruses in water onto filters, after which the viruses were eluted. Fifty-two to 78 percent of reovirus was recovered following sonication of the membrane in beef extract.

When 800 to 2000 plaque forming units of reovirus were added to water, the virus could not be recovered using reverse osmosis (Sweet, Ellender, and Leong 1974).

Infectious and potentially infectious reoviruses

Spendlove and Schaffer (1965) purified reovirus with a procedure that included exposure of the virus to chymotrypsin. The infectivity of the virus preparation increased dramatically. Subsequent work that is reviewed below showed that the enhanced infectivity resulted from the removal of capsid proteins from the virions. Evidently most host cells are not able to remove the outer reovirus capsid, so infections with intact virions are abortive. If the outer capsid is removed with proteolytic enzymes prior to inoculating reovirus onto cells, the virus is able to adsorb and cause infection.

Several lines of evidence show that enzyme treatment of reovirus does result in increased infectivity.

1. Chymotrypsin treatment was found in electron microscopic studies to convert essentially all virions to infectious subviral particles. The total infectious particle ratio upon treatment of the virus with enzyme changed from 35:1 to 1.1:1, 100:1 to 0.9:1, and 64:1 to 1.1:1 for reovirus serotypes 1, 2, and 3, respectively (Spendlove, McClain, and Lennette 1970).

2. Chymotrypsin treatment increased the infectivity without changing the hemagglutination titer of the virus (Spendlove and Schaffer 1965). Consequently, enhancement was not the result of disruption of aggregated virus.

3. Cliver (1968) observed about a 100-fold increase in infectivity when he treated a reovirus preparation with enzyme. Using a filter of 100 nm nominal porosity, he filtered enzyme treated virus and virus from the same preparation that had not been enzyme treated. About 10 percent of the infectivity in both preparations was lost. Thus, the enzyme did not enhance the infectivity by breaking up aggregates of virus.

4. Wallis, Melnick, and Rapp (1966) reported that incorporation of pancreatin into the agar overlay enhances reovirus plaque formation.

5. Heat removal of capsid proteins gave results similar to those obtained with enzymes (Spendlove and Schaffer 1965; Mayor and Jordan 1968; Engler 1968; Wallis, Smith, and Melnick 1964).

6. Mayor and Jordan (1968) found 5 to 10 percent of reovirus grown in tissue culture to lack an outer capsid. This concentration of capsidless virus is similar to the concentration of infectious virus in most viral preparations.

A factor to consider in preparing infectious subviral particles is the selection of appropriate cell lines and plaque purified virus strains. McClain, Spendlove, and Lennette (1967) assayed a number of reovirus strains in four different cells lines before and after enzyme treatment. The plating efficiency of the virus was affected profoundly by prior treatment of inocula with chymotrypsin, and was a function of the viral strain and the host cell used. In another study (Spendlove and McClain 1968), plaque isolates of Lang, D5 Jones, and Abney were enzyme treated and assayed for infectivity. The type 1 and 3 strains (Lang and Abney) were found to be heterogeneous. They contained some particles that increased in infectivity and others that lost infectivity. The former were referred to as chymotryspin^{+} (CT^{+}) and the latter as CT^{-} isolates. The CT^{-} isolates are not inactivated if treated with enzymes in the absence of calcium ions (Spendlove, McClain, and Lennette 1970).

The importance of enzymatic enhancement of reovirus infectivity in growth studies (Spendlove et al. 1966), and animal (Kundin et al. 1966) and insect inoculation (Spendlove, Whitcomb, and Jensen 1968) investigations have been described (Spendlove 1970). Other applications of proteolytic enzymes in reovirus studies include enhancement of reovirus plaque formation (Wallis, Melnick, and Rapp 1966) and isolation of viruses from clinical specimens.

Several conditions have been reported to cause a dramatic decrease in infectivity when purified reoviruses are exposed to chymotrypsin. The conditions under which the infectivity is lost have been reported by several groups (Joklik 1972; Shatkin and LaFiandra 1972; Borsa et al. 1973a, b, c).

The enzymatic removal of reovirus capsid polypeptides occurs in stages and, depending on conditions, proceeds through a definable series of reactions that lead to different digestion products. Several factors appear to be important in determining the sequence of polypeptides digested. 1) Level of virus purification. Inactivation usually occurs when the virus is highly purified, concentrated, and exposed to enzyme in buffers of low ionic strength. 2) Strain of virus used. Joklik (1972) compared three different virus strains and found substantial differences in their response to digestion. 3) Concentration of virus and enzyme. The three reovirus strains studied by Joklik (1972) were more resistant at low than at high virus/enzyme ratios. In general, at low enzyme concentrations, the virions were converted to noninfectious cores while at high enzyme concentrations the reaction products (referred to as paravirions) had lost about one-third of their capsid protein but were still infectious. At high virus concentrations, the viruses flocculated and lost infectivity. 4) The cations present. Shatkin and LaFiandra (1972) found that purified virus could be digested with chymotrypsin in the presence of 0.15 NaCl without infectivity being diminished significantly, while Borsa et al. (1973c) reported that enzyme treatment of purifed virus in the presence of 0.2 M Na or Li ions actually increase the infectivity 7.38 and 11.0 fold respectively. Replacing Na or Li with K (Shatkin and LaFiandra 1972; Borsa et al. 1973c) or Cs or Rb ions (Borsa et al. 1973b) results in loss of infectivity.

Stability of reoviruses

Sharp, Floyd, and Johnson (1975, 1976) have published articles describing reovirus-bromine inter-

actions. An important finding of these investigations was that aggregates strongly influence the bromine resistance of reoviruses in polluted water.

When singly dispersed reovirions were exposed to a 3 M concentration of bromine in turbulent flowing deionized water at pH 7, 20°C, the plaque titer fell at a rate of three log units/second (Sharp, Floyd, and Johnson 1976). Liu and McGowan (1973) determined the relative resistance of 25 human enteric viruses to inactivation by chlorine. Reoviruses were most rapidly inactivated while coxsackie A6 was most resistant. Aggregation could have played a role in the results obtained. In addition, potentially infectious reoviruses were not tested. Since potentially infectious reoviruses have a double coat they should be considerably more resistant than infectious reoviruses.

Potentially infectious reovirus (Lang strain) is stable at 56°C, but this temperature rapidly inactivates infectious reovirus particles (Spendlove and Schaffer 1965) and picornaviruses (Wallis, Melnick, and Rapp 1965).

Mayor and Jordan (1968) demonstrated that some reovirus particles are infectious after their capsid is removed by heat. The decapsidation of the entire population of reovirus by heating at 52 to 53°C for approximately three hours was accompanied by a 10-fold loss of infectivity. They concluded from their results that the residual infectious virus in the heated preparations does not contain a capsid, and consequently the RNA enclosed in a sub-capsid layer is all that is required for infectivity. They also found that heating viruses in $MgCl_2$ (Wallis, Smith, and Melnick 1964) removed the capsid from relatively few particles; this observation suggested that partial decapsidation or capsid loosening may be important in explaining the enhancement of infectivity when reovirus is heated in $MgCl_2$.

Engler (1968) heated reovirus in an attempt to release infectious RNA from the virions. The titer of heat-inactivated virus assayed with the facilitator kaolin, was as much as four log units higher than heated virus assayed without facilitator. He concluded that a complete loss of the viral capsid did not seem to occur up to a temperature of 80°C, as shown by RNase resistance and density gradient analysis of the heated virions.

Radiation has been considered as a means of inactivating viruses in water (Berg 1973). The double stranded RNA of the reoviruses makes them more resistant to inactivation by UV irradiation than viruses of the same genome size with single stranded nucleic acid. This fact was confirmed in a comparative study using reovirus and rous sarcoma virus (Spendlove and Levinson, unpublished results). McClain and Spendlove (1966) later studied the kinetics of reovirus inactivation by UV irradiation and found a portion of the population to resist inactivation. There are 10 pieces of RNA in each reovirus. When two reoviruses with UV damaged RNA infect the same cell, they share undamaged RNA segments and can produce infectious virus. This form of genetic recombination is called genetic reassortment, and accounts for reovirus multiplicity reactivation. An understanding of this phenomenon is very important in any attempts to inactivate viruses in water by radiation.

Materials and Methods

Virus and cells

Plaque purified type 1 (Lang strain) reovirus was used in all experiments. Virus stocks were prepared in roller bottle cultures of mouse L 929 cells. Dense cell layers were incubated for 1 hour at 37°C with approximately 20 viral particles per cell. Basal medium of Eagle containing antibiotics, 2 percent fetal bovine serum (FBS), and 5.6 percent sodium bicarbonate to adjust the pH of the medium to 6.8 to 7.0 was then added to the infected cells. After reincubation for 24 hours at 37°C, the cultures were frozen and thawed six times and centrifuged to remove cell debris. The supernatant fluids were then stored at -20°C in 2 ml aliquots.

Enzyme treatment and virus assay

The enhancement of viral preparations with chymotrypsin has been described (Spendlove and Schaffer 1965). Virus preparations were exposed to 20 to 200 µg of chymotrypsin per milliliter at 37°C for 1 hour. Viral assays were performed both before and after chymotrypsin treatment using Maden-Darby bovine kidney (MDBK) cells. To assay viral infectivity, confluent 15 mm coverslip cell cultures were inoculated with 0.02 ml of viral suspensions (Spendlove et al. 1964). After 20 to 22 hours, the cultures were dried, fixed with acetone and stained with an anti-reovirus fluorescent antibody. The fluorescing cells on each coverslip were counted and reported as immunofluorescent cell forming units (IFU)/ml Results of the assays were determined by averaging counts observed from three coverslip cultures at each dilution.

Concentration procedures

England's (1972) procedure for concentration of viruses from sewage and effluent was as follows. Prior to water or sewage filtration, Tween 80 was passed through all prefilters (Millipore Corp., no AP20) to prevent viral adsorption (Wallis and Melnick 1970). Tween 80 treated prefilters alone were used for filtration of the sewage samples and for collection of the protamine precipitate. Sample volumes of sewage ranging from 200 to 2,000 ml were filtered through a double AP20 prefilter by vacuum. Bovine serum albumin was then added to the filtrate to a final concentration of 0.25 percent. The albumin supplemented sample was adjusted to pH 7.5 to 7.8 with 1N HCl or 1N NaOH. A 1 percent stock solution of protamine sulfate (National Biochemicals Corp.) was then added to the sample to a final concentration of 0.05 percent for the sample's first precipitation, and 0.025 percent for the second. The flask containing protamine sulfate was stirred for 30 minutes at room temperature. The precipitate was collected by passing the sample through a Millipore prefilter AP20 disc under negative pressure. After a second precipitation and filtration, the precipitate on the upper surface of the AP20 disc was dissolved and collected in a test tube in the following manner. With the vacuum off, a small volume of 1M NaCl (usually 0.5 ml) was pipetted over the surface of the AP20 disc, allowed to soak for about 5 minutes to dissolve the precipitate, and then pulled into the receiving tube with vacuum. Sterile water, six times the volume of 1M NaCl, was pipetted over the surface of the prefilter disc and drawn into the test

tube to produce an isotonic filtrate. Fetal bovine serum, 10 percent by volume, was added to stabilize the virus. The concentrated filtrate was centrifuged at 2,500 rpm for 30 minutes to sediment bacteria. The supernatant was then inoculated onto cell cultures (England 1972).

Modifications of the above procedure, to concentrate and assay the IV and PIV were as follows. 1) Fetal bovine serum low in reovirus antibody was used, instead of bovine serum albumin, at the same (0.25 percent) concentration. 2) Reduction of the protamine sulfate concentration to 0.005 percent for the first precipitation and 0.0025 percent for the second. Higher protamine concentrations interfered with enzymatic enhancement of viral infectivity and with the fluorescent cell assay. 3) Elution of the protamine precipitated virus using two equal volumes of 1 N NaCl with the total volume equaling 0.5 to 1.5 ml. 4) After centrifuging to remove any bacteria present in the precipitated sample, the supernatant was assayed on coverslip cultures by a fluorescent antibody procedure (Spendlove et al. 1964) before and after chymotrypsin treatment of the virus concentrate. All results represent the average of two to four experiments, involving river water samples containing 500 to 4,000 ml.

Sonication

Several suspensions of reovirus in river water were sonicated to disrupt viral aggregates. One tenth to 1 ml of infectious culture fluids containing reovirus was diluted into 500 ml filtered river water, and was mixed for 5 minutes. A 15 ml sample of the viral suspension was withdrawn, placed in a glass tube in an ice bath, and sonicated with the small probe of the Biosonik III (Bronwill Scientific, Rochester, N.Y.). Samples were sonicated at maximum setting for 15 second intervals for a total of 3.5 minutes. After every 30 seconds of sonication, a 1 ml sample was removed and assayed for IV and PIV.

Viral aggregation

It has been shown that reoviruses aggregate when diluted less than 100-fold into distilled water (Floyd and Sharp 1977). This aggregation was concentration dependent, not happening at dilutions of 100-fold or greater. Therefore, 0.1 to 1.0 ml of stock virus was added to 50 to 4,000 ml of filtered river water containing 0.25 percent fetal bovine serum at a pH between 7.5 to 7.8. If unusual differences were observed between the infectivity titers of stock virus and river water inoculated with reovirus, the experimental results were discarded since it was assumed that the virus had aggregated.

River water

Class A[1] water was collected from the Logan River, in Logan, Utah, and filtered through a 0.22 µm pore size Millipore filter to remove suspended matter, and then inoculated with reovirus. If the water were not filtered, 10 to 30 percent of the virus adsorbed to the suspended solids and could not be recovered.

Sewage samples

Raw sewage samples were collected from Salt Lake City and Dugway, Utah. Salt Lake City sewage, collected at the north treatment facility, is greatly diluted with drainage and industrial wastewaters. Dugway sewage dilution is restricted to household water. Samples from both cities were collected at the point of inflow before treatment. Grab samples with volumes between 1,000 and 2,000 ml were taken for testing.

Results

Sonication studies of reovirus in river water

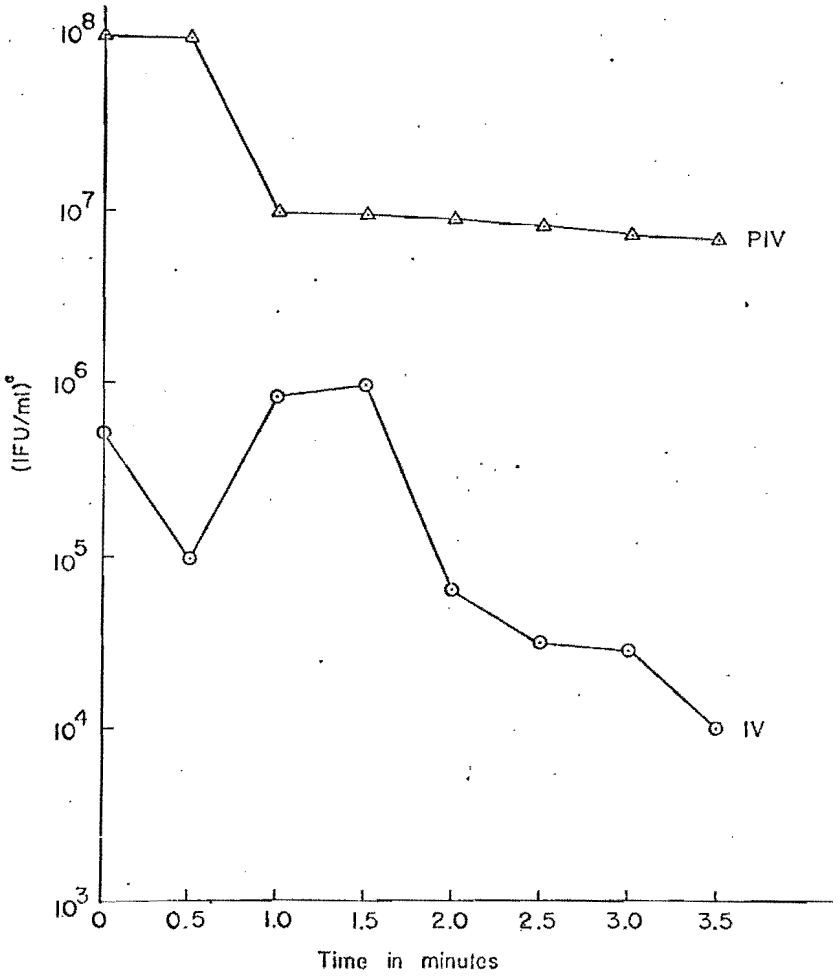
Suspensions of reovirus in river water were sonicated to disrupt viral aggregates. It appeared that the sonication was not breaking up aggregates, but was converting the PIV to IV and then inactivating the IV (Figure 2). If viral aggregates were being dispersed, infectivity assays of IV and PIV would both show initial increases. Only the IV showed an increase in virus titer (between 0.5 to 1.5 minutes of sonication). Concurrent with this increase was a decrease of PIV, which indicates conversion of PIV to IV followed by inactivation. The PIV tended to level off after 1 minute of sonication indicating that it is more resistant to sonication than the IV. Controls remained unchanged in titer throughout the experiments.

Protamine sulfate concentration of reovirus from river water

Experiments were conducted to precipitate IV and PIV from river water using England's (1972) protamine sulfate procedure. In preliminary experiments, a heavy protamine floc was produced which rapidly plugged the AP20 filters. An additional problem was encountered when coverslip cultures were inoculated with the dissolved protamine precipitate. A cloudy film formed and interfered with fluorescent antibody staining used in the viral assay procedure. Consequently, the concentrations of the reagents used in the protamine precipitation procedure were varied. Fetal bovine serum at 0.25 percent and protamine sulfate at 0.005 percent for the first precipitation, and 0.0025 percent for the second precipitation, resulted in clear cover slips for assay, and precipitated many of the IV particles. In these and other protamine experiments, a relationship between virus concentration and recovery efficiency was observed (Table 7). The best viral recoveries were obtained when virus concentrations were low. Controls for these experiments were the same samples added to filtered river water, mixed for the duration of the experiment, filtered, and then assayed before and after chymotrypsin treatment. The stock virus was also assayed before and after chymotrypsin treatment with each protamine precipitation experiment. Under the conditions of these experiments, no PIV was detectable in the protamine concentrate, and less than 1 percent of either fraction remained in the filtrate.

Percentages of recovery of IV, shown in Table 1, were increased 5 to 15 percent by using two equal volumes of 1M NaCl to elute the protamine precipitate from the AP20 disc instead of one elution with the

[1]Class A water is that designated for protection of cold water species of game fish, waterfowl and agricultural use. In part, the quality restrictions require a BOD < 5 mg/l, NO_3 < 4 mg/l, and PO_4 < 0.25 mg/l.



[a]IFU (immunofluorescent cell forming units) determined by fluorescent cell counts.

Figure 2. Effects of sonication on reovirus. Virus that had not been enzyme treated was diluted in 500 ml of filtered river water which contained 0.25 percent fetal bovine serum, at pH between 7.5 to 7.8. A 15 ml sample was withdrawn and sonicated. IV--sonicated virus that was assayed before enzyme treatment. PIV--sonicated virus that was assayed after enzyme treatment. Results represent average of two experiments. Virus controls without sonication were assayed at each time interval and their titers remained unchanged throughout the experiments.

same total volume. Each volume of NaCl elutant was left on the filters that contained the protamine precipitate for 5 minutes before the sterile water was added.

Chymotrypsin treatment of reovirus before concentration with protamine

In attempts to determine why the PIV was not recovered in the protamine precipitation procedure, the reovirus stock was treated with 20 µg/ml chymotrypsin before adding it to the filtered river water. This procedure converted the PIV to IV. In this experiment, 79 to 95 percent of the total virus added was recovered (Table 8). The control in these experiments was chymotrypsin treated virus added to water, mixed for the duration of the experiment without protamine, filtered through an AP20 disc, then assayed before and after a second chymotrypsin treatment of 20 µg/ml. As expected, the PIV that was converted to IV with enzyme prior to being added to the river water was recovered. These results suggested that the protamine or the serum was interfering with the enzymatic enhancement of the viral infectivity.

Increasing the chymotrypsin concentration to detect PIV

Table 3 shows the results of varying the amounts of chymotrypsin used to enhance the infectivity of the viral preparations. The best recovery was obtained using 200 µg of chymotrypsin per milliliter, which is

Table 7. Protamine sulfate precipitation efficiency of reovirus from filtered river water as a function of the inoculated reovirus concentration.

IFU Added to[a] River Water	IFU Recovered in Concentrate	Recovery (Percent)	$\sigma^2 \pm \sigma$	Reovirus Particle Form
5.1×10^4_7 (w/o ct)	4.4×10^4_4 (w/o ct)	86	16.6 ± 4.10	IV
1.0×10^7 (ct)	4.4×10^4 (ct)	<1		PIV
5.2×10^5_8 (w/o ct)	3.9×10^5_5 (w/o ct)	75	38.0 ± 6.16	IV
1.1×10^8 (ct)	3.8×10^5 (ct)	<1		PIV
5.2×10^6_9 (w/o ct)	3.5×10^6_6 (w/o ct)	67	24.0 ± 4.89	IV
1.0×10^9 (ct)	3.2×10^6 (ct)	<1		PIV
5.3×10^7 (w/o ct)	3.4×10^7_7 (w/o ct)	64	56.9 ± 7.54	IV
1.2×10^{10}(ct)	3.3×10^7 (ct)	<1		PIV

IFU = Immunofluorescent cell forming units.
(w/o ct) = Virus precipitated with protamine and assayed without chymotrypsin treatment.
(ct) = Virus precipitate assayed after treatment with 20 µg/ml of chymotrypsin for 1 hour at 37°C. Four different virus concentrations were added to filtered river water and precipitated with protamine sulfate with subsequent assay by fluorescent cell counts.

[a]After reovirus was added to filtered river water, the water was mixed for the duration of the experiment, filtered, and then assayed before and after chymotrypsin treatment.

Table 8. Chymotrypsin treatment of reovirus prior to precipitation with protamine sulfate (conversion of PIV to IV).

IFU Added to[a] River Water	IFU Recovered in Concentrate	Recovery (Percent)	$\sigma^2 \pm \sigma$	Reovirus Particle Form
7.7×10^5_5 (ct-1)	7.3×10^5_5 (ct-1)	95	6.2 ± 2.49	IV
7.6×10^5 (ct-2)	7.3×10^5 (ct-2)	93	5.0 ± 2.23	IV
1.1×10^7_7 (ct-1)	8.7×10^6_6 (ct-1)	79	10.7 ± 3.26	IV
1.0×10^7 (ct-2)	8.6×10^6 (ct-2)	84	26.0 ± 5.09	IV

Virus was initially treated with 20 µg/ml of chymotrypsin for 1 hour at 37°C, added to filtered river water, and precipitated with protamine sulfate.

IFU = Immunofluorescent cell forming units.
ct-1 = Virus assayed after protamine precipitation before a second chymotrypsin treatment.
ct-2 = Virus assayed after protamine precipitation after a second chymotrypsin treatment.

[a]After chymotrypsin treated reovirus was added to filtered river water, the water was mixed for the duration of the experiment, filtered, and then assayed before and after a second chymotrypsin treatment.

ten times the concentration previously used. The efficiency of recovery seems to be related to the viral concentration (Tables 7 and 9). The control of these experiments was the same as in Table 7 except 200 µg/ml of chymotrypsin was used prior to assay of the PIV. Chymotrypsin was used at 200 µg/ml in all subsequent assays.

Stability of reovirus fractions in the protamine precipitate

The stability of reovirus in protamine precipitates was investigated to determine the feasibility of precipitating viruses from water in the field and transporting the precipitate to the laboratory. The viral concentrate, with 5 percent fetal bovine serum, was stored in glass tubes after elution from the prefilter. The stability of both IV and PIV in the protamine precipitates is shown in Table 10. The results show that the samples can be stored at -20°C or 4°C for at least 18 days with only a small loss of infectivity.

Concentration of reovirus from raw sewage

The modified protamine concentration procedure was used to concentrate and assay ten raw sewage samples from Utah sewage treatment facilities (Table 11). Five samples from Salt Lake City and five samples from Dugway, Utah, were collected. Three of the five samples collected at Salt Lake City contained IV. In two of these samples, virus was also detected after enzyme treatment, but at the same or lower concentration than without enzyme treatment. IV concentrations ranged from 2-5 x 10^3/l. Four out of five samples collected

Table 9. Detection of PIV in the protamine precipitate by increasing the chymotrypsin concentration.

Chymotrypsin Concentration (µg/ml)	IFU Added to[a] River Water	IFU Recovered in Concentrate	Recovery (Percent)	$\sigma^2 \pm \sigma$	Reovirus Particle Form
20	9.3×10^6 (w/o ct) 8.5×10^8 (ct)	6.3×10^6 (w/o ct) 6.1×10^6 (ct)	68 <1	28.2 ± 5.31	IV PIV
40	9.2×10^6 (w/o ct) 8.5×10^8 (ct)	6.4×10^6 (w/o ct) 6.2×10^6 (ct)	69 <1	30.9 ± 5.56	IV PIV
60	9.4×10^6 (w/o ct) 8.4×10^8 (ct)	4.4×10^6 (w/o ct) 4.3×10^6 (ct)	47 <1	122.0 ± 11.04	IV PIV
80	9.3×10^6 (w/o ct) 8.4×10^8 (ct)	2.9×10^6 (w/o ct) 2.7×10^6 (ct)	31 <1	142.9 ± 11.95	IV PIV
100	9.2×10^6 (w/o ct) 8.7×10^8 (ct)	3.8×10^6 (w/o ct) 3.5×10^6 (ct)	41 <1	88.2 ± 9.39	IV PIV
100[b]	4.6×10^4 (w/o ct) 4.3×10^5 (ct)	3.7×10^4 (w/o ct) 3.8×10^4 (ct)	80 9	13.55 ± 3.68 26.0 ± 5.09	IV PIV
150[c]	4.6×10^4 (w/o ct) 4.2×10^5 (ct)	3.9×10^4 (w/o ct) 4.3×10^4 (ct)	85 10	18.7 ± 4.32 23.2 ± 4.82	IV PIV
200	9.3×10^6 (w/o ct) 8.5×10^8 (ct)	7.4×10^6 (w/o ct) 8.4×10^8 (ct)	80 99	10.5 ± 3.24 1.19 ± 1.09	IV PIV
200[d]	4.5×10^4 (w/o ct) 4.3×10^5 (ct)	4.0×10^4 (w/o ct) 4.3×10^5 (ct)	89 100	17.68 ± 4.21 0.18 ± 0.43	IV PIV

IFU = Immunofluorescent cell forming units.
(w/o ct)= Virus precipitated with protamine assayed without chymotrypsin treatment.
(ct) = Virus precipitated with protamine and assayed after chymotrypsin treatment.

[a]After reovirus was added to filtered river water, the water was mixed for the duration of the experiment, filtered, and then assayed before and after chymotrypsin treatment.
b, c, d Observations taken from a second set of experiments using a different viral stock containing fewer IV and PIV.

at Dugway contained reovirus. IV ranging from 0.32-1.2 x 10^5/l was detected in three samples. PIV alone was detected in one sample at 2.1 x 10^5/l. The results indicate that different concentrations of chymotrypsin might be needed to accurately assay the PIV in different sewage samples.

Inactivation of reovirus in sewage

When the first Dugway sample (sample 1, Table 11) was collected, another 4 liter volume was collected, prefiltered, supplemented with 0.25 percent FBS, and stored for 3 weeks at 4°C. Before sampling, the stored sewage filtrate was vigorously mixed. A 1 liter sample was withdrawn and concentrated by protamine sulfate precipitation. Table 12 indicates that there was a conversion of PIV to IV followed by inactivation of the IV.

Discussion

Most investigators are in agreement that the absence of coliforms in water, particularly renovated wastewaters, is not adequate assurance that viruses have been removed or inactivated. Among the viruses that occur in sewage and polluted waters, the reoviruses, picornaviruses, and adenoviruses have been found most frequently. Since these sewage-borne viruses are the targets of water quality testing, it is important that waters be monitored for the most abundant and resistant among them.

It has been recommended in virological testing that no more than one infectious virus particle be present in a given volume of water. Of the proposed methods for concentrating viruses from water, each has specific limitations which precludes its successful application to all waters. Recovery and identification of infectious water-borne viruses are difficult, time consuming, and expensive. The procedures often use primary cell cultures and suckling mice. Plaquing with Buffalo green monkey (BGM) kidney cells has helped considerably, but there are still problems resulting from false plaques (Fannin 1978; Leong, Barrett, and Trussell 1978).

Reoviruses possess numerous attributes which recommend them as an indicator in environmental virology studies. These qualities include the following.
1) Abundance and consistent occurrence in sewage.
2) They are rarely known to cause disease in adult animals, so are relatively safe to handle in the lab-

Table 10. Stability of reovirus in the dissolved protamine precipitate stored at 4°C and -20°C.

Storage Conditions[a]	Storage Time (Days)	IFU Remaining in Concentrate	Percent of Initial Viral Fraction Remaining Infectious	Reovirus Particle Form
4°C	0	2.0×10^6 (w/o ct) 2.0×10^8 (ct)	100 100	IV PIV
	4	2.0×10^6 (w/o ct) 2.0×10^8 (ct)	100 100	IV PIV
	14	2.4×10^6 (w/o ct) 1.6×10^8 (ct)	120* 80	IV PIV
	18	2.6×10^6 (w/o ct) 1.6×10^8 (ct)	130* 80	IV PIV
20°C	0	2.0×10^6 (w/o ct) 2.0×10^8 (ct)	100 100	IV PIV
	4	2.0×10^6 (w/o ct) 2.0×10^8 (ct)	100 100	IV PIV
	14	2.1×10^6 (w/o ct) 1.9×10^8 (ct)	105* 95	IV PIV
	18	2.1×10^6 (w/o ct) 1.8×10^8 (ct)	105* 90	IV PIV

IFU = Immunofluorescent cell forming units.
(w/o ct) = Virus assayed without chymotrypsin treatment.
(ct) = Virus assayed with chymotrypsin treatment at 200 µg/ml for 1 hour at 37°C.

[a]Protamine precipitated samples were eluted from the prefilters, and stabilized with 5 percent fetal bovine serum low in reovirus antibody.
*Conversion of PIV to IV during storage.

Table 11. Protamine sulfate precipitation of reovirus naturally occurring in raw sewage.

Location	Sewage[a] Sample	(IFU/1) Recovered from Raw Sewage	Reovirus Particle Form
Dugway, Utah	1	<400 (w/o ct) 2.1×10^5 (ct)	IV PIV
	2	<400 (w/o ct) <400 (ct)	IV PIV
	3	1.2×10^5 (w/o ct) <400 (ct)	IV PIV
	4	1.5×10^5 (w/o ct) <400 (ct)	IV PIV
	5[b]	3.2×10^4 (w/o ct) 3.0×10^4 (ct)	IV PIV
Salt Lake City, Utah	1	<400 (w/o ct) <400 (ct)	IV PIV
	2	2.0×10^3 (w/o ct) 1.6×10^3 (ct)	IV PIV
	3	4.0×10^3 (w/o ct) 4.0×10^3 (ct)	IV PIV
	4	<400 (w/o ct) <400 (ct)	IV PIV
	5	5.0×10^3 (w/o ct) <400 (ct)	IV PIV

Table 11 (Continued)

IFU = Immunofluorescent cell forming units.
(w/o ct) = Virus precipitated with protamine and assayed before chymotrypsin treatment.
(ct) = Virus precipitated with protamine and assayed after chymotrypsin treatment at 200 µg/ml for 1 hour at 37°C.

[a]Two 1,000 ml samples were used for each determination. The average is recorded in the table.
[b]This sewage sample was stored for 3 weeks at -20°C before precipitation and assay.

oratory (Rosen 1968), and will probably not be reduced in numbers or eradicated by vaccine programs. (Therefore, standardized tests based on reovirus detection would not have to be changed in the future.) 3) They have a broad host range (Rosen 1968), so fecal contamination from man and lower animals could be detected. 4) They are amendable to low-cost isolation and identification procedures (McClain, Spendlove, and Lennette 1967). 5) Work with reoviruses may contribute to establishing controls for rotavirus infections; rotaviruses are a major cause of gastroenteritis in infants and young children (Kapikian et al. 1976). 6) An immunofluorescent cell assay technique is available which is capable of demonstrating reoviral anti-

Table 12. Stability of IV and PIV during storage of a prefiltered raw sewage sample at 4°C (Dugway sample no. 1)[a].

Storage Time (Days)	(IFU/1) in Concentrate (IV)[b]	(IFU/1) in Concentrate (PIV)[c]
0	<400	2.1×10^5
10	1.3×10^5	<400
14	4.1×10^3	<400
21	<400	<400

IFU = Immunofluorescent cell forming units.

[a]Virus from a 1 liter sample was precipitated for each determination.

[b](IV) Virus precipitated with protamine and assayed before chymotrypsin treatment.

[c](PIV) Virus precipitated with protamine and assayed after chymotrypsin treatment at 200 µg/ml for 1 hour at 37°C.

The four liter volume was filtered through a Millipore AP20 prefilter before storage at 4°C.

gen after 8 to 10 hours and yields quantitative data within 24 hours (McClain, Spendlove, and Lennette 1967). While this technique has the capability of demonstrating a single infectious particle, the dilutions necessary for this assay reduce its sensitivity to 50 particles per ml. 7) A single antiserum will detect reoviruses of all three serotypes. 8) Reoviruses are easily cultivated to high infectious titers for use in developmental studies. 9) Reovirus hemagglutination inhibition tests can be used to assay antibodies in epidemiologic studies. 10) PIV viruses have a double coat that makes them exceptionally thermo stable (Spendlove and Schaffer 1965). Consequently, if the PIV viruses are inactivated, most other viruses present in water should also be inactivated.

Reovirus infectivity is probably enhanced during infection by exposure to proteolytic enzymes in the intestinal tract. Reovirus produced in tissue culture have very little contact with proteolytic enzymes, hence most particles remain in the non-infectious PIV form. The existance and/or the importance of PIV in sewage and other polluted waters has not been determined.

In this report, a procedure was presented that efficiently recovers both IV and PIV from polluted waters. The protamine sulfate precipitation procedure described by England (1972), was modified and a fluorescent cell assay (Spendlove et al. 1964) was used to assay the IV and the PIV. Modifications were necessary to eliminate problems associated with interference of the chymotrypsin activity and with interference with the fluorescent antibody staining. The refinements included using fetal bovine serum and reduced protamine sulfate concentrations. Employing two washes of the precipitate with 1M NaCl resulted in better elution of the virus. These changes also resulted in clear coverslips for assay with fluorescent antibody staining. In developing these procedures, river water was filtered to prevent viral adsorption to suspended matter and permit quantitative assays of the concentrates. Increasing the chymotrypsin concentration to 200 µg/ml was necessary to enhance the infectivity of the PIV. The resultant procedures are capable of: 1) over 500-fold concentrations of reovirus from polluted waters; 2) recovery of between 80 to 100 percent of IV and PIV fractions; and 3) producing quantitative data within 24 hours after coverslip inoculation with the viral concentrate. In achieving these results, sonication experiments and appropriate dilutions were used to help circumvent viral aggregation problems.

During storage of the protamine concentrates there was an increase of 5 percent in the IV and a 10 percent loss of the PIV after 18 days at -20°C. Both fractions showed a more pronounced change when stored at 4°C; i.e., the IV increased 30 percent and the PIV decreased 20 percent. These changes might be due to a conversion of the PIV to IV or a combination of events including antibody reactions and disruption of aggregates. Regardless, the stability of reovirus in the protamine precipitates shows that it would be feasible to precipitate reoviruses from water and only bring the protamine-virus precipitate to the laboratory.

Application of these techniques to recovery and assay of naturally occurring reovirus fractions in raw sewage successfully recovered both the IV and PIV. Inconsistent results were sometimes obtained when sewage precipitates were treated with chymotrypsin. In experiments in which chymotrypsin is used to increase the infectivity of laboratory produced virus, the infectivity of IV is not lost upon enzyme treatment. In some of the sewage isolations, however, the IV and PIV if present, were lost upon treatment with 200 µg/ml of chymotrypsin. This loss could have been caused by several factors including heterogeneity in the components of raw sewage, the virus existing in a semi-weakened state (having altered outer and/or inner capsids), or the presence of chymotrypsin negative (CT^-) reovirus. Chymotrypsin treatment of the CT^- reoviruses causes inactivation of the virus rather than enhancement (Spendlove, McClain, and Lennette 1970).

A range of chymotrypsin from 50 to 300 µg/ml is often required to enhance the infectivity of reoviruses precipitated from sewage. In choosing the enzyme range to employ, one should consider both the method of concentration used and the system being examined. All samples should be assayed both with and without chymotrypsin treatment. This procedure will demonstrate the viral fraction (IV or PIV) being recovered and will prevent the loss of CT^- reovirus by inactivation with chymotrypsin.

The recoveries of over 10^5 infectious reoviruses per liter of some sewage (Table 5) suggests that the modified protamine precipitation and fluorescent antibody staining procedures can be useful in determining the potential roles of the IV and PIV in water quality testing. England (1972) pointed out that volumes which can be concentrated with her procedure fall far short of the volumes needed to detect minimal pollution such as in recreational waters, but would be effective if run parallel or in conjunction with other large volume concentration procedures. The protamine procedure can be used with large volumes of water by collecting the precipitates on pleated filters used in tangential flow filtration or by continuous flow centrifugation.

Reports have been made of incoming virus loads in raw sewage ranging from several thousand to several hundred thousand per liter (Katzenelson 1976). Without examination of the other virus populations at the locations checked in our study, reoviruses cannot be assumed to be the most abundant virus present. Nevertheless, their importance in these systems is evident. The results from Dugway, Utah, also indicate that the viral concentrations in undiluted raw sewage might be higher than previously expected. The fact that several concentration attempts recovered no reovirus may suggest the need for the development of a continuous monitoring system to detect intermittent viral pollution.

Loss of virus in the raw sewage sample stored at $4^{o}C$ might be explained by a number of factors: 1) reaction with antibody present in the fetal bovine serum added; 2) activation by naturally occurring proteolytic enzymes with subsequent inactivation; 3) adsorption to the suspended matter; 4) aggregation, or 5) a combination of the above factors. The conversion of PIV to IV shown in Table 12 does not necessarily indicate the same occurs under natural conditions in sewage.

It would be premature at this time to speculate about the overall presence or absence of the PIV in polluted waters; more extensive investigations of waters and wastewaters is needed. Currently (1979) studies are being conducted in which wastewaters are being examined for the presence of both the adsorbed and free IV and PIV fractions to further evaluate reoviruses as an indicator of viral pollution in environmental systems.

Conclusions

Using the procedure developed in this study it is possible to: 1) concentrate reovirus over 500-fold from river water and obtain a quantitative assay of both IV and PIV within 24 hours, with a virus recovery of 80 to 100 percent; 2) distinguish the PIV from IV thus allowing study of the role of each in water quality studies; and 3) observe reovirus at concentrations as high as 2×10^5 infectious units per liter in raw sewage samples.

A recent modification of the procedure has been used to detect viruses in sewage over a three month period. Fifteen one liter samples of sewage were tested; every sample contained approximately 10^5 reoviruses per liter. Therefore, the procedure appears to be the best available method for detecting viral pollution of public water supplies.

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SECTION II. INACTIVATION OF INFECTIOUS AND POTENTIALLY INFECTIOUS REOVIRUSES BY CHLORINATION

Introduction

A long range goal in facilitating virus control is to establish a reliable test virus for monitoring purposes. Tests so far suggest that reoviruses have the greatest potential of any enteric virus for use in water quality control testing. A very desirable characteristic of a test virus is a high resistance to inactivation by chlorination, i. e., if the test virus is inactivated by chlorination, all other viruses should also be inactivated.

Reoviruses are noninfectious in cell cultures when their outer capsid is intact (Spendlove, McClain, and Lennette 1970). Removal of the outer coat with proteolytic enzyme treatment converts potentially infectious virus (PIV) to an infectious virus (IV) particle form. Morphologically complete (enzymatically enhanceable) PIV particles are much more resistant to inactivation by heat than the IV particles (Spendlove and Schaffer 1965). This investigation was conducted to determine if the heat resistant PIV is more resistant to chlorination than IV.

Literature Review

This section discusses the literature relating to the resistance of reoviruses to chemical disinfection. A discussion of other aspects of reovirus stability can be found in the Literature Review of the previous section of this report under the title "Stability of Reoviruses." Unfortunately almost all of the work to date has excluded studies with PIV particles.

Liu and McGowan (1973) reported the relative chlorine resistance of 25 human enteric viruses added to Potomac River water. They found the reoviruses to be the most readily inactivated viruses. Their assay procedure could detect only the IV form of reoviruses.

Sattar and Westwood (1978) studied viral disinfection by chlorination in primary sewage plant effluents emptying into the Ottawa River. Of the viruses that were isolated and identified in field samples of raw sewage, 51.1 percent were reoviruses. In chlorination studies, viruses were detected in 80 percent of raw sewage samples, 72 percent of primary effluent samples, and 56 percent of chlorinated effluent samples. Thirteen of 23 raw sewage isolates (57 percent), 13 of 30 isolates from primary effluents before chlorination (43 percent), and 10 of 17 isolates from primary effluents after chlorination (59 percent) were reoviruses. These latter figures suggest that under natural conditions, reoviruses are more resistant to chlorination than most other viruses found in primary sewage effluents. The assay procedures used by these investigators would not detect the PIV form of the reoviruses.

A group at the University of North Carolina have published a number of papers showing the effect aggregation of viruses can have on viral disinfection (Sharp, Floyd, and Johnson 1975, 1976; Floyd, Johnson, and Sharp 1976; Young and Sharp 1977; Floyd and Sharp 1977; Young, Johnson, and Sharp 1977; Floyd and Sharp 1978a, 1978b). Their results are particularly pertinent since the chlorine resistant populations observed in our study were undoubtedly composed of viral aggregates. Floyd and Sharp (1978b) used a modified single particle analysis test to determine the effect of pH and dissolved salts on poliovirus and reovirus aggregation. Poduska and Hershey (1972) have also pointed out the importance of viral aggregates in chlorination resistance of viruses.

The rate of inactivation of reovirus by bromine (Sharp, Floyd, and Johnson 1976) was 25 times faster than the rate of inactivation of poliovirus (Floyd, Johnson, and Sharp 1976). Inactivation of the PIV form of reovirus was not studied by the North Carolina group.

Mahnel (1977) used heat and viricidal chemicals to decontaminate drinking and surface water. Infectious type 1 reovirus was found in water that had been exposed to a temperature of 56°C for 120 minutes. No viable poliovirus type 1 or ECBO virus was found after being exposed to a temperature of 56°C for 30 minutes; an adenovirus was inactivated by 60 minutes of heating at 56°C. Viable reovirus was observed after 30 minutes of heating at 60°C. ECBO virus was more resistant to inactivation by calcium hypochlorite than poliovirus and reovirus; the three viruses were equally susceptible to iodine; and poliovirus was more resistant to potassium permanganate than the other two viruses.

Ward and Ashley (1977a) found the amount of inactivation occurring in sludge through dewatering by evaporation is similar for poliovirus type 1 strain CHAT, coxsackie B1, and type 3 reovirus Dearing strain. These same workers (Ward and Ashley 1977b) reported an agent in wastewater sludge that reduces the heat required to inactivate reovirus. Heating at 45°C in the presence of the agent increased the density of the viral particles, i.e., the treatment probably released capsid polypeptides. The sludge factor that accelerates heat inactivation of reovirus also contains a substance that protects poliovirus against heat inactivation.

Materials and Methods

Cell Culture

Madin-Darby canine kidney (MDCK) and Madin-Darby bovine kidney (MDBK) cells obtained from American Type Culture, Rockville, Maryland, were propagated in Eagles' minimal essential medium supplemented with 10 percent fetal bovine serum. One percent of a stock solution of antibiotics was added to all media to give 250 units each of penicillin, streptomycin, and neomycin, and 2.5 units of bacitracin per milliliter. In

addition, 5 ml of 5.6 percent $NaHCO_3$ and 4 ml of a 3 percent stock solution of glutamine were added per 200 ml of medium.

Virus Propagation

Reovirus, type 1, Lang strain was treated with 20 µg of α-chymotrypsin per milliliter. Monolayers of MDCK and/or MDBK cells were infected at a multiplicity of infection of 1,000. The culture medium was changed daily and the removed medium that contained the virus was frozen at -20°C.

Purification and Concentration

The virus was prepared for use in the experiments by freezing and thawing infectious tissue culture fluids 4 to 6 times. An equal volume of Genetron 113 was added, the preparation was mixed for 5 minutes by shaking, allowed to stand 20 minutes at room temperature, and centrifuged for 30 minutes at 3,000 x g. Two liters of the aqueous phase fluids were placed in dialysis bags and covered with a slurry of polyethylene glycol (Carbowax) at 37°C for 6 to 8 hours. This procedure reduced the volume about 200-fold. The concentrated fluids were centrifuged in an IEC B-60 ultracentrifuge at 30,000 rpm for 1.5 hours in a SB 283 (swinging bucket) rotor. The pellet which contained the virus was resuspended in 0.001M EDTA in distilled water. The virus was then assayed for infectivity and used in the experiments.

Infectivity Assays

To assay PIV, the outer protein coat of the virus was removed. This was accomplished by treating the virus with 20 µg of α-chymotrypsin/ml of virus suspension, and incubating the mixture at 37°C for 30 minutes. Coverslip cultures of MDCK or MDBK cells were used for infectious virus assay after complete monolayers of cells had formed. The outgrowth medium was completely removed by aspiration from petri dishes (60 mm) containing three circular coverslip cultures 15 mm in diameter. A 0.02 ml aliquot of appropriate virus dilution was placed on each of three coverslip cultures using a 0.2 ml serological pipette. The inoculated cultures were incubated in a 37°C, CO_2 incubator for 1.5 to 2.0 hours to allow for viral adsorption. Four milliliters of medium containing 2.5 percent fetal bovine serum were added to the infected cultures, and the infection was allowed to progress for 16 to 18 hours before the cells were fixed and stained with fluorescent anti-viral antibody. The cultures were examined under a fluorescence microscope, and the number of fluorescing (infected) cells on each coverslip was counted.

Chlorination Studies

The procedure for exposing reoviruses (IV and PIV) to chlorine was as follows:

1. All reagents were brought to room temperature (26°C).
2. Using Purex and distilled water containing 10^{-5} M EDTA hypochlorite solutions were prepared, so the final volumes (after pH adjustment and reovirus addition) had concentrations of 0.625, 1.25, and 2.50 ppm of free available chlorine (FAC) respectively.
3. The pH of each chlorine solution was adjusted to 6.8 to 7.2.
4. Reovirus was added to each chlorine solution and mixed.
5. At intervals of 1, 2, 4, and 8 minutes samples were removed. a) The pH was determined on part of sample. b) Part of the sample was placed in tubes containing 0.00625 M sodium thiosulfate solution to neutralize combined and/or free chlorine, and assayed for IV and PIV as described above.

Evaluation of Reovirus Resistance to Chlorination

Baumann and Ludwig (1962) reviewed the chlorine disinfection literature and graphically presented results showing the relative resistance of various microorganisms to inactivation by chlorine. Each virus was assigned a Ct number (C=concentration of FAC and t=time of contact required for a 99.6 to 100 percent kill). For example, if 10 minutes were required to kill 99.6 percent of a virus population exposed to 1 ppm FAC the virus would be assigned a Ct envelope value of 10. If 6 minutes were required, the Ct value would be 6.

Results

Kinetics of inactivation of reoviruses (IV and PIV) by chlorine were studied. Table 1 shows the initial pH, temperature, and chlorine concentration of each sample. Figures 1 through 3 show the kinetics of inactivation of IV and PIV during exposure to different concentrations of chlorine. There was a relatively rapid inactivation for 1 to 2 minutes at all chlorine concentrations after which the rates of reaction greatly decreased due to the presence of viral particles that were resistant to inactivation.

As expected, the morphologically complete PIV was somewhat more resistant to inactivation than IV. This resistance was evident in both the susceptible and resistant viral fractions.

If the zero to 1 minute curves are extrapolated to minimize the contribution of the resistant fraction, the time required for a 99 percent kill at concentrations of 0.625, 1.25, and 2.50 ppm FAC with IV is 7, 6, and 1.5 minutes respectively. With PIV at the same respective concentrations, the time required to inactivate 99 percent of the virus is 12, 7, and 2.25 minutes. When these results are plotted the IV has a concentration-temperature (Ct) envelope between 3.8 and 7.3 while PIV has a Ct envelope between 6.1 and 9.0 (Baumann and Ludwig 1962). Extrapolation of the IV and PIV resistant fraction curves would give extremely high Ct envelope values.

In test number 5 of Table 1, samples taken at 0, 1, 2, 4, and 8 minutes were tested by the orthotolidine-arsenite (OTA) method for residual FAC concentrations (Table 2). During 8 minutes, the FAC concentration dropped from 2.50 to 1.87 ppm.

Discussion

The main purpose of this work was to determine if PIV is more resistant to disinfection by chlorine than IV. In no other reovirus chlorination study have PIV particles been included in the investigation. This fact is surprising since PIV particles probably comprise the majority of viruses in reovirus preparations and they are much more resistant to thermal inactiva-

Table 1. Chlorine concentrations used in tests.

Test No.	Initial pH	Temp. (°C)	Chlorine, ppm Dose	Total, OTA	Free, OTA
1*	7.4	26	0.0	0.0	0.0
2	7.0	26	0.625	0.61	0.60
3	7.05	26	1.25	1.26	1.24
4*	7.3	26	0.0	0.0	0.0
5	7.0	26	2.5	2.5	2.35

*Zero-time control tests to verify initial titer.

Inactivation results obtained with test numbers 1 and 2, 1 and 3, shown respectively in Figures 1 and 2. Results obtained with test numbers 4 and 5 are shown in Figure 3.

Note: Initial chlorine dosage shown as both the amount added and the amount analyzed by the Orthotolidine-Arsenite (OTA) method. "Purex" was used as the chlorine source.

Table 2. Free available chlorine (FAC) residuals versus time.

Time, Minutes	FAC Residual, ppm
0	2.5
1	2.4
2	2.21
4	2.16
8	1.87

Test number 5 of Table 1.

tion than IV particles (Spendlove and Schaffer 1965). In every comparative assay in which the viruses were exposed to chlorine in our current study, the PIV titers were higher than the IV titers.

To get an estimate of the relative resistance to chlorine of PIV and IV, and to compare these reovirus particles to other microorganisms discussed in the report of Baumann and Ludwig (1962), we extrapolated the zero time and 1 minute time points of Figures 1 through 3 to a 99 percent kill. This extrapolation was done to better approximate the results obtained if we had examined singly dispersed particles. The potential error in this extrapolation is evident, i.e., a 30 second time point might fall on a line with a much steeper slope than the 0 to 1 minute slope.

The extrapolation results with the 0.625 mg/l chlorine dosage are probably most accurate because the 0, 1, and 2 minute points fall on a relatively straight line. The Ct envelope values for IV and PIV respectively using Figure 1 results are 5.0 and 9.0. Stated differently, it would require 6.6 minutes exposure to 1 ppm of FAC to kill 99.6 percent of the PIV, and only 3.8 minutes for the same kill of IV.

The report of Baumann and Ludwig (1962) showed that under conditions of pH and temperature similar to those used in our reovirus study, Escherichia coli, Shigella dysenteriae, poliovirus, and coxsackievirus A2 are all inactivated to at least 99.6 percent kill by a Ct envelope of 6. PIV appears to be more resistant to inactivation by chlorine than poliovirus and coxsackievirus A2, and considerably more resistant than E. coli and S. dysenteriae.

Much of the early work did not take into consideration the importance of rate changes during disinfection. For example, the Baumann and Ludwig (1962) report gives hepatitis virus a Ct envelope value of about 12. The original work by Neefe et al. (1947) showed that 12 volunteers were protected when they were exposed to hepatitis virus that had been treated with 3.25 ppm initial FAC for 30 minutes at room temperature and a final pH of 6.7 to 6.8. A chlorine exposure time much less than 30 minutes might have been sufficient to protect the volunteers, so the Ct envelope value might have been considerably less than 12.

In our current study, if the results were based on only the 0 and 9 minute exposures, and these were extrapolated to a 99.6 percent kill, the Ct envelope values for both IV and PIV particles would have been very large.

An interesting observation in this study is the inactivation rates for the aggregates (the resistant fraction). The results in Figures 1 and 2 were ob-

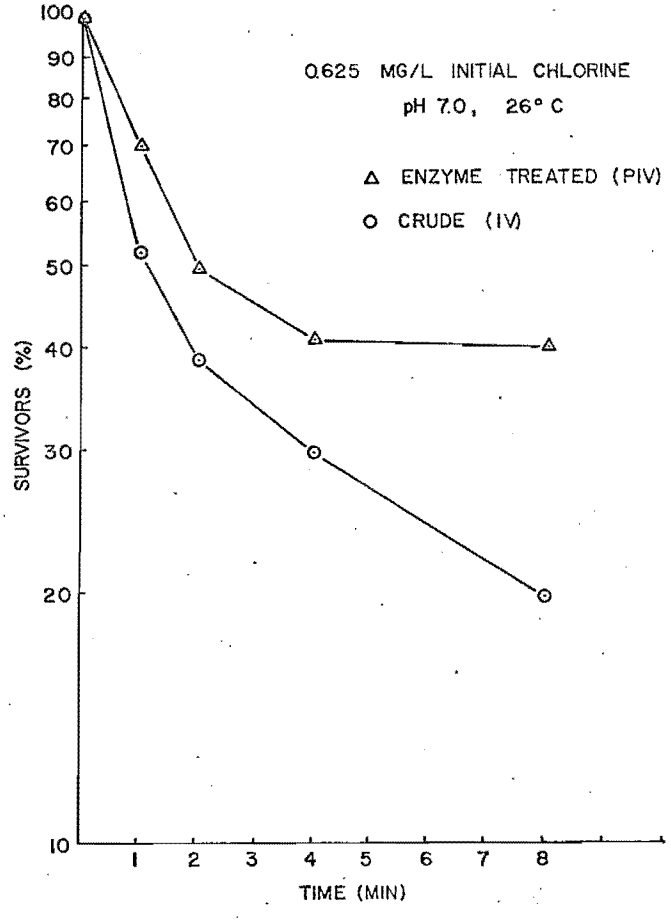

Figure 1. Inactivation of IV and PIV by 0.625 mg chlorine per liter.

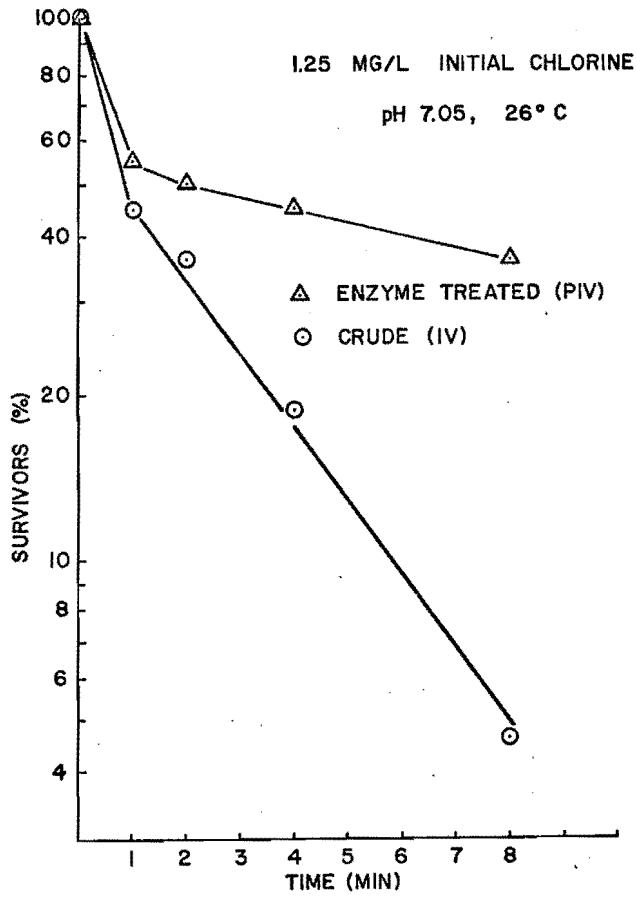

Figure 2. Inactivation of IV and PIV by 1.25 mg chlorine per liter.

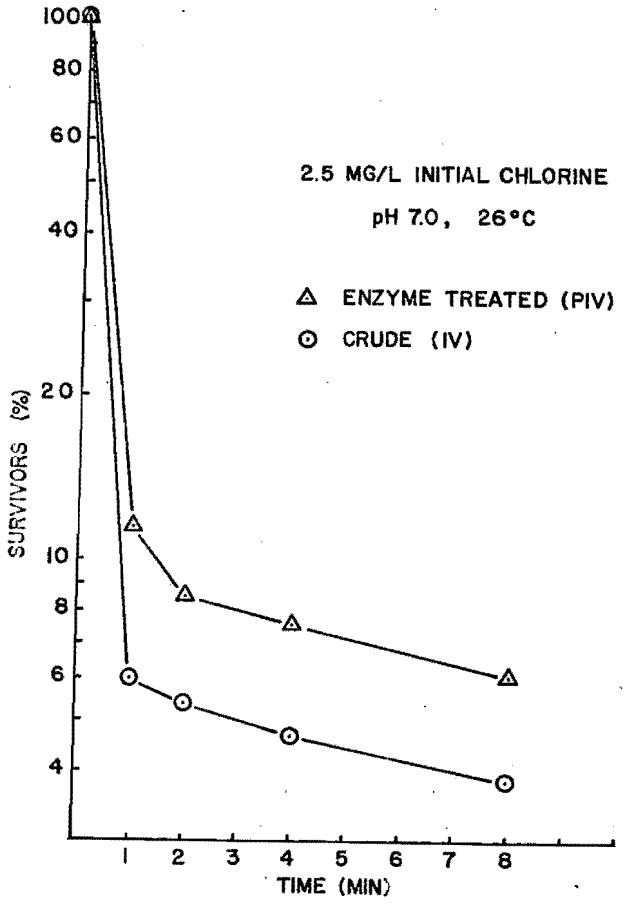

Figure 3. Inactivation of IV and PIV by 2.5 mg chlorine per liter.

tained using samples from the same virus preparation. Therefore, the aggregates should have been composed of the same mixture of IV and PIV. Assay of the stock preparation indicated that 90 percent of the population was composed of PIV, i.e., there was a 10-fold increase in infectivity when the infectious culture fluids used in the investigation were treated with chymotrypsin. If the assumptions regarding the composition of the aggregates are true, the rates of inactivation of the resistant IV fractions in Figures 1 and 2 should be greater than the inactivation rates for the PIV resistant fractions, which is exactly what was observed.

The changes that are needed in experimental design in future chlorination studies with reoviruses are the use of singly dispersed viruses and/or the use of lower chlorine levels with more frequent sampling during the early time periods.

In one experiment of this investigation, the FAC residual was determined at different time intervals. During an 8 minute period the FAC residual dropped from 2.5 to 1.87 ppm. This drop means that only 75 percent of the chlorine was available to act on the virus at 8 minutes, and suggests that part of the decrease in the rate of inactivation was a result of a loss of chlorine, and part due to viral aggregates.

It is interesting to speculate regarding the resistance of reoviruses to chlorine inactivation in sewage. If the great majority of reovirus is in the PIV form then the resistance to inactivation would be greater. Proteolytic enzymes in sewage and intestinal enzymes must convert some of the PIV to IV (Clive and Herrmann 1972). Regardless of this, there is some evidence (Sattar and Westwood 1978) that reoviruses are more resistant to chlorination than most other viruses found in sewage. Forty three percent of the isolates in primary effluents before chlorination were reoviruses. After chlorination, 59 percent of the isolates were reoviruses.

Conclusions

1. The PIV is more resistant than IV to inactivation by chlorine.

2. For both IV and PIV, a fraction of each population is relatively resistant to inactivation by chlorine.

3. The PIV is at least as resistant to inactivation by chlorine as poliovirus and coxsackievirus A-2, and is more resistant than E. coli and S. dysenteriae.

The highly chlorine resistant reoviruses (PIV) can be used in safety testing to further guarantee that the public will have safe water supplies.

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SECTION III. REMOVAL OF VIRUSES FROM WATER BY FILTRATION SYSTEMS

Introduction

General

Potential virus contamination of culinary water supplies is a persistent threat to municipalities. An objective of this project was to assess the ability of water treatment filtration processes to remove viruses. During the course of this research, a supplemental study concerning the effectiveness of in-line direct filtration systems as a means of treating public water supplies was funded by the State of Utah through the Utah Water Research Laboratory (Malek et al. 1979). To avoid duplication of research effort the principal investigators of both projects agreed that Part I (Detection of Infectious Viruses and Viral Antigens in Water) and Part II (Inactivation of Infectious and Potentially Infectious Reovirus Particles by Chlorination) would receive major emphasis in this report. The following text is a summary and analysis of the filtration results obtained by Malek et al. (1979).

Water Treatment Systems

Conventional treatment of drinking water consists of the addition of chemicals (coagulants) which enhance the agglomeration of dispersed colloidal particles. These agglomerated particles have increased mass and thereby greater settling characteristics. A conventional water treatment scheme is composed of a rapid mix basin, where chemical addition occurs; a flocculation basin, where destabilization of colloidal particles and cultivation of the chemical floc particles results; and sedimentation, where these large floc particles are gravimetrically removed.

Recently, for low turbidity waters (up to 60 NTU's[1]) interest has been focused on the development of low-cost water treatment methods. One such method is the use of direct filtration as a means of treating public water supplies. This method could eliminate the coagulation and sedimentation phases normally found in water treatment facilities. All direct filtration systems eliminate the sedimentation process from the treatment scheme. The effectiveness of such filtration processes in the removal of turbidity from source waters has been adequately demonstrated (Culp 1964; Tridgett 1974; Harbert 1976; and Montgomery Engineering 1976). Insufficient information, however, is available on the ability of direct filtration systems to remove viruses present in the source water. The objective of the study conducted by Malek et al. (1979) was to evaluate the ability of a particular in-line direct filtration system to remove viruses from potable water supplies.

The water treatment system investigated in this study (Malek et al. 1979) consisted of the addition of alum to the rapid mix influent followed directly by the filtration process. The filtration processes evaluated were rapid sand, dual-media (sand and anthracite), and multi-media (sand, anthracite, and garnet). The option of adding polyelectrolytes prior to filtration was also included in the system.

The research was divided into two distinct phases. Phase I used jar test and batch assays to determine effects of various filter media, coagulants, pH, and turbidity on virus levels in water. In Phase II of the study continuous filtration runs were designed and conducted based on the information obtained in Phase I.

The test agent used during the study was the bacteriophage MS2. MS2 phage is icosahedral androphagovirus (RNA-containing bacterial virus). The phage has a diameter of 25 nm and is similar to poliovirus in size, shape and type of nucleic acid.

Phase I

Batch viral assays were conducted to assess the effect of each granular media (sand, anthracite, and garnet) or the virus. One milliliter of a virus preparation with a titer of 1×10^3 PFU/ml was added per gram of each media used. The batch assays were agitated on Lab-Line shaker tables at constant temperature.

Jar tests were conducted with alum (aluminum sulfate) and polyelectrolytes. Alum concentrations investigated were from 1 to 10 mg/l alum. Polyelectrolytes used were provided by Calgon Chem Co. and Nalco Chemical Co. Concentrations of each polyelectrolyte used during the jar test ranged from 2 to 10 mg/l.

Phase II

Continuous filter runs were conducted on three filter columns. One filter was a rapid sand filter. The second filter contained dual media (anthracite and sand). The third column was a tri-media filter containing anthracite coal, sand, and garnet. Hydraulic loadings studied were 12.2 $m^3/hr/m^2$ (5 gpm/ft^2) and 7.3 $m^3/hr/m^2$ (3 gpm/ft^2). The optimum alum dosage was determined by classical jar tests (APHA 1975). The alum dosage at which a discernible floc was observed was chosen as the dosage for the continuous filter runs. The concentration of polyelectrolyte used during the run was based on jar test and the ability of the polyelectrolyte to reduce the virus levels.

[1] NTU is a Nephelometric Turbidity Unit which is quantitive value of a given concentration of a formazin suspension. Formazin polymer has gained acceptance as the turbidity standard reference suspension for water.

All experimental testing was repeated a minimum of two times in order to verify results.

Results

Phase I

Batch assays were conducted to determine the effects of each granular media on the virus, MS2. Anthracite coal adsorbed 99+ percent of the virus in 24 hours. With 1.5 hours into the batch assay, however, only 20 to 30 percent of the virus was observed. The hydraulic detention time through the anthracite was less than 5 minutes, therefore, anthracite was kinetically limited in its ability to remove the virus from water. Garnet and sand showed no potential for removing MS2.

Jar tests were conducted with alum (aluminum sulfate). At alum concentrations from 1 mg/l to 10 mg/l alum inactivitation of MS2 was observed. Alum concentrations of 20, 30, 40, and 50 mg/l indicated a significant removal of MS2 (80 to 98 percent). This removal is believed to be due to either inactivation or enmeshment of the virus by the aluminum precipitate.

The capability of polyelectrolytes to inactivate or remove MS2 was also investigated. Three polyelectrolytes from Nalco Chemical Co. and one from Calgon were tested. Up to 93 percent aggregation of MS2 was observed with the Nalco polyelectrolyte (Nalcolyte 8101).

Phase II

On the basis of jar tests an alum dosage of 6 mg/l was selected to treat the turbidity in the water. The influent turbidity to the rapid mix basin ranged from 10 to 15 NTU's. Figure 1 shows the removal of turbidity through the tri-media filtration system at a hydraulic loading of 12.2 $m^3/hr/m^2$ (5 gpm/ft^2) using 6 mg/l alum. Turbidity in the effluent was less than 1 NTU. However, there was no effective removal of MS2 through the system (Figure 2). Similar results were obtained for the rapid sand and dual-media filtration processes. At a hydraulic loading of 7.3 $m^3/hr/m^2$ (3 gpm/ft^2) the same turbidity and virus results were observed as obtained at the higher loading (Figures 3 and 4). MS2 was unaffected by the water treatment system.

The effect of Nalcolyte and alum was evaluated at 7.3 $m^3/hr/m^2$. The filter media in each filter were precoated with Nalcolyte 8101 prior to filter operation. Using 6 mg/l alum and 2 mg/l Nalcolyte 8101 the data shown in Figures 5 and 6 were obtained. Significant breakthrough of MS2 occurs after 1 hour into the continuous run with all filter systems.

Procedures followed for addition of polyelectrolyte to the filter were obtained from Nalco Chemical Co., Salt Lake City Utah. In theory polyelectrolytes adhered to the filter media will destabilize colloidal particles which are transported near the media. By this procedure, colloidal destabilization is achieved by attachment of the polyelectrolyte to the colloid and the filter media. Batch jar test indicated that removal of virus particles would be enhanced by addition of polyelectrolyte in the rapid (flash) mix basin of the water treatment system. Introduction of polyelectrolyte at this point in the treatment scheme would allow greater contact of the virus with the polyelectrolyte and therefore improved destabilization of the virus particle (i.e.,virus particle attached to other virus particles or other colloids).

Conclusions

1. Low concentrations of alum (< 10 mg/l) were not found to effectively destabilize MS2 virus particles (20 to 34 percent).

2. When turbidity was present in the solution, alum concentrations less than 10 mg/l did not remove MS2 particles.

3. Batch jar test simulating conventional water treatment practices indicated higher concentrations of alum (20 - 50 mg/l) either inactivated or enmeshed 80 - 98 percent of the virus after 1 hour settling.

4. Whereas in-line direct filtration is effective in the reduction of turbidity in water, it has been shown in this study ineffective in the removal of MS2 virus.

5. In batch jar test, Nalco 8101, a cationic polyelectrolyes, reduced phage concentration in the water by 93 percent.

Direct filtration water treatment systems are a viable means of removing colloidal particles in water. This research, however, indicates that without the addition of polyelectrolytes at the rapid mix basin the filtration system will be ineffective in removing viruses. Nalcolyte 8101 was the most effective cationic polyelectrolyte for removal of virus. Alum dosages utilized in conventional water treatment systems are capable of removing 98+ percent of the virus particles prior to chlorination.

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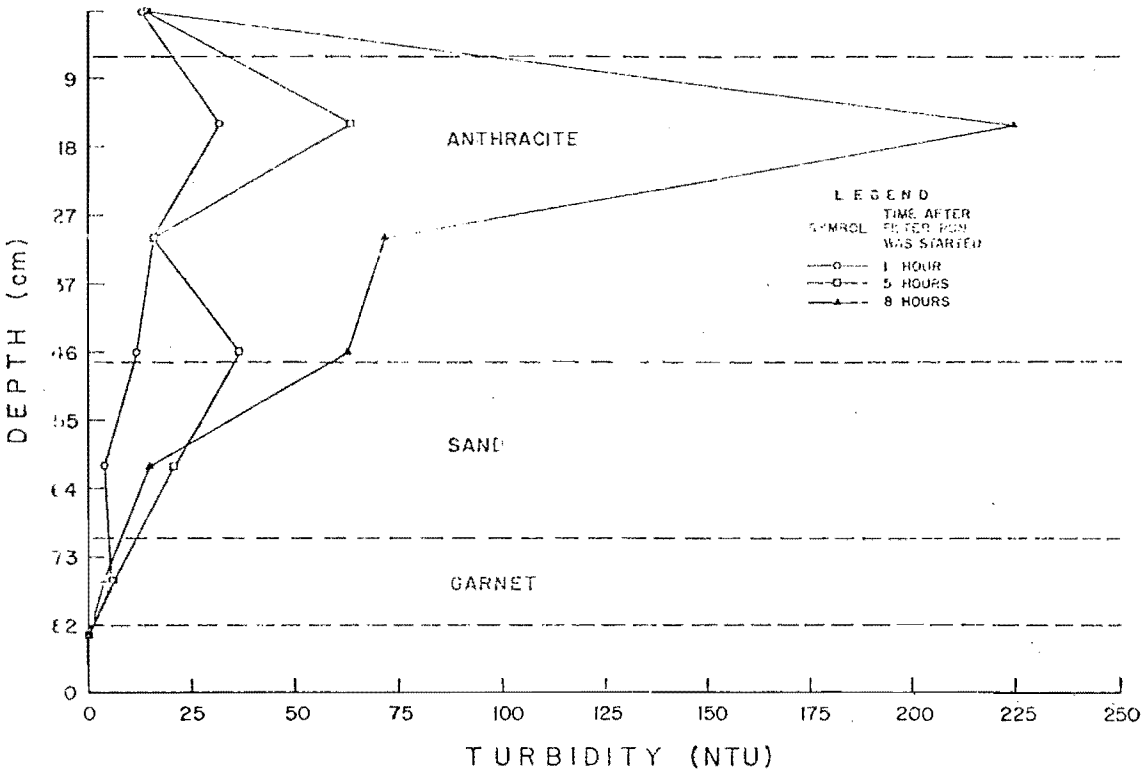

Figure 1. Turbidity of influent, effluent, and at various depths through the tri-media filter at 12.2 $m^3/hr/m^2$ (5 gpm/ft^2) with 6 mg/l alum added.

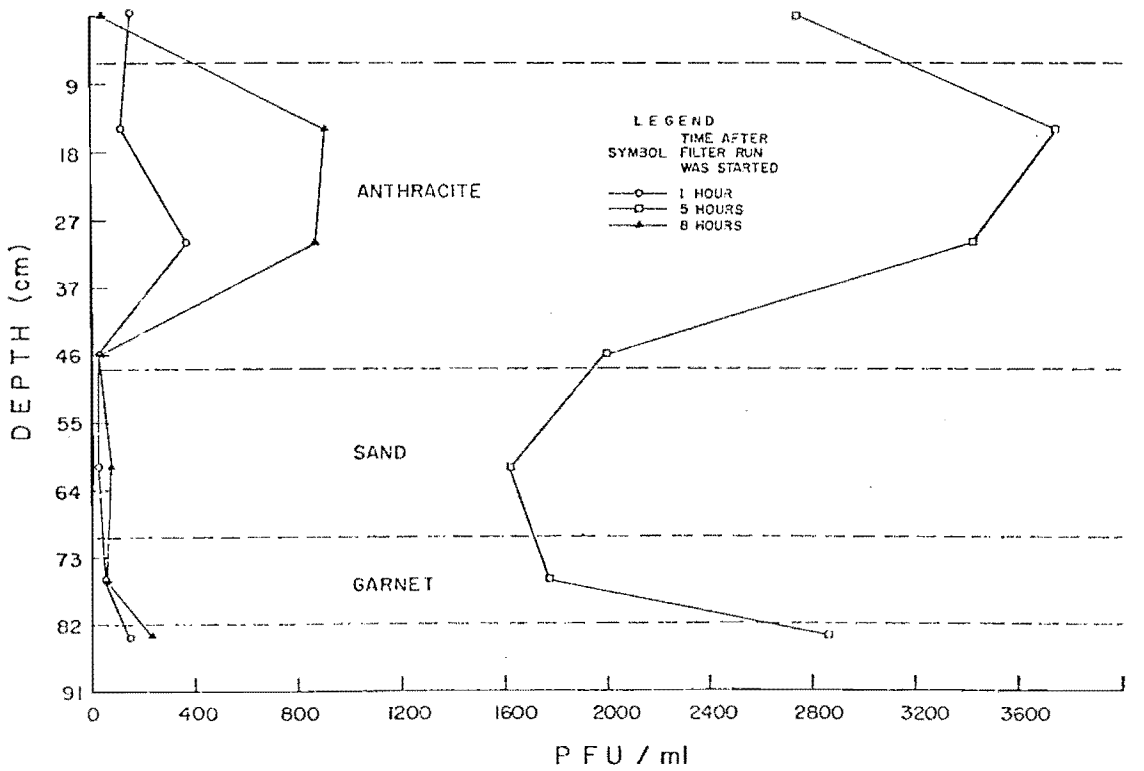

Figure 2. MS 2 virus concentration (PFU/ml) of influent, effluent, and at various depths through the tri-media filter at 12.2 $m^3/hr/m^2$ (5 gpm/ft^2) with 6 mg/l alum added.

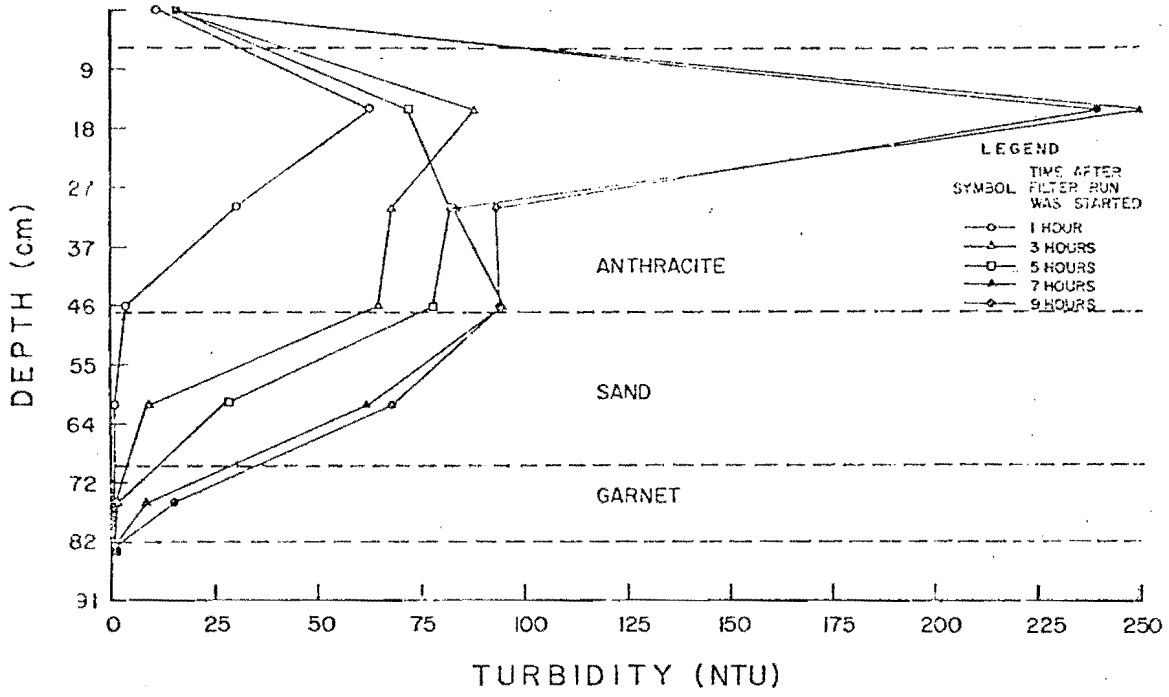

Figure 3. Turbidity of influent, effluent, and at various depths through the tri-media filter at 7.3 $m^3/hr/m^2$ (3 gpm/ft^2), with 6 mg/l alum added.

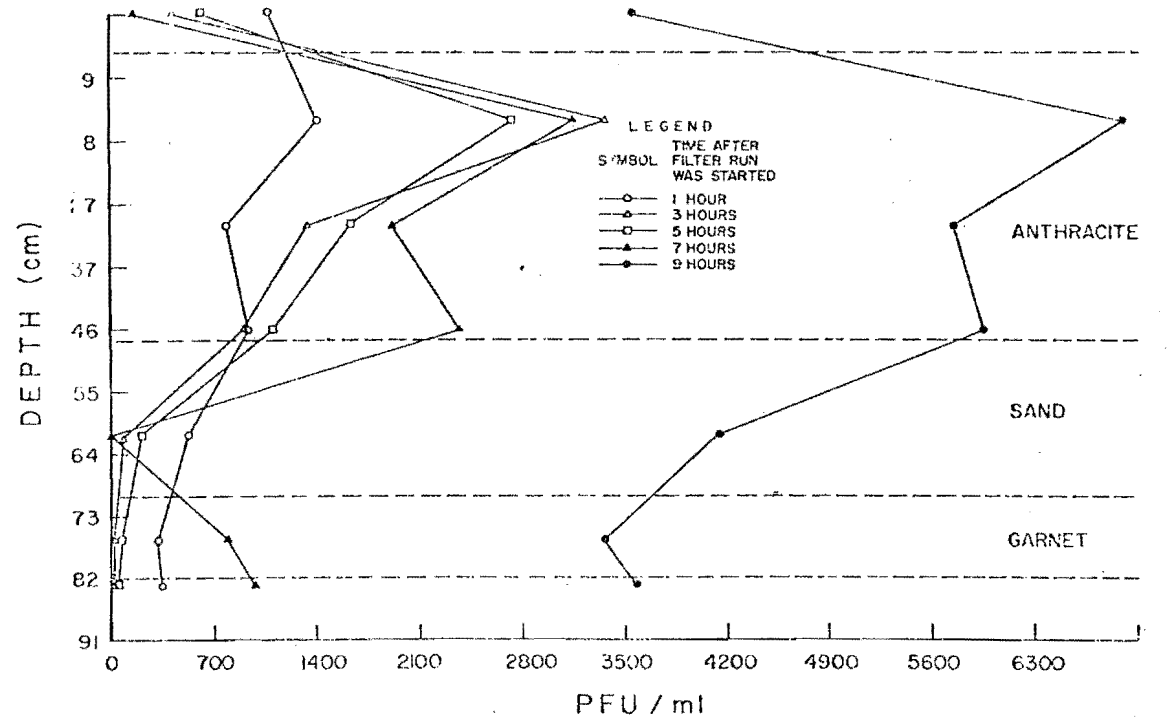

Figure 4. MS 2 virus concentration (PFU/ml) of influent, effluent, and at various depths through the tri-media filter at 7.2 $m^3/hr/m^2$ (3 gpm/ft^2) with 6 mg/l alum added.

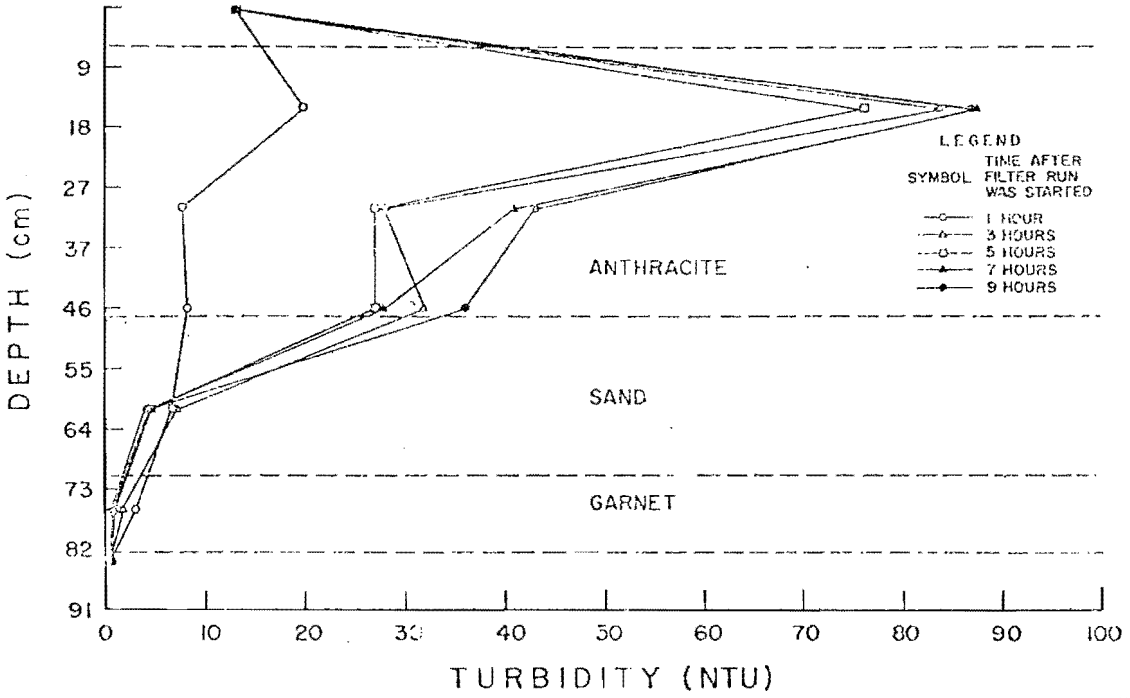

Figure 5. Turbidity of influent, effluent, and at various depths through the tri-media filter at 7.3 $m^3/hr/m^2$ (3 gpm/ft^2) with 6 mg/l alum and 2 mg/l Nalcolyte added.

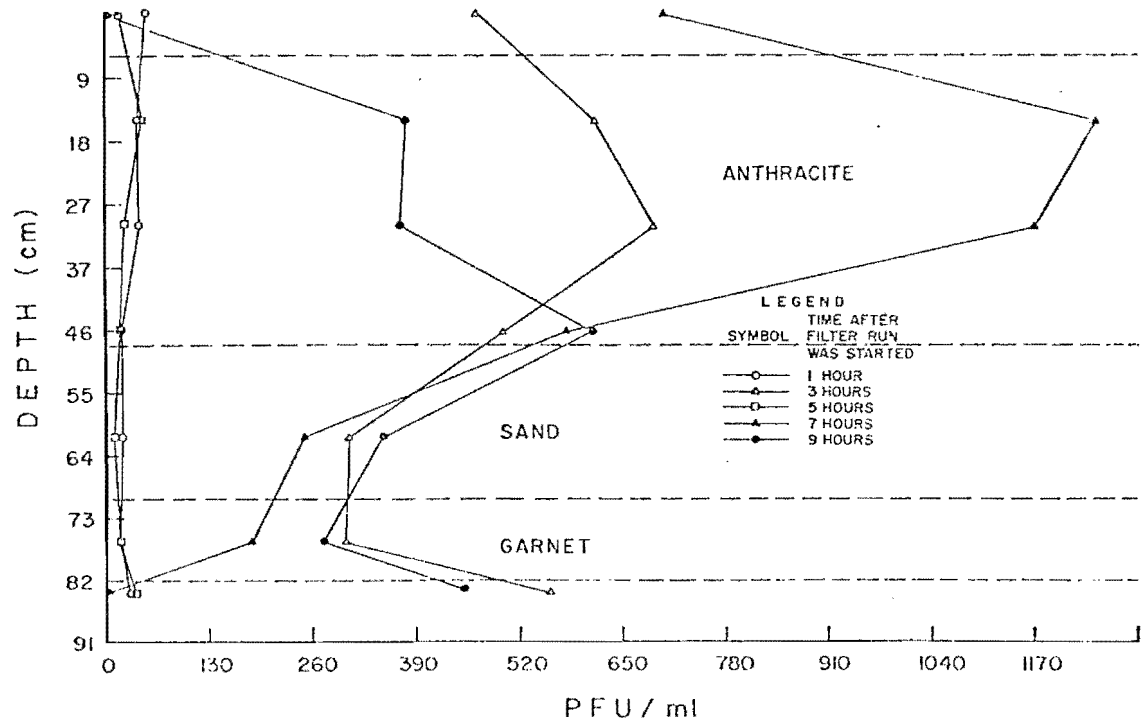

Figure 6. MS 2 virus concentration (PFU/ml) of influent, effluent, and at various depths through the tri-media filter at 7.3 $m^3/hr/m^2$ (3 gpm/ft^2) with 6 mg/l alum and 2 mg/l Nalcolyte added.