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ENVIRONMENTAL PARAMETERS INFLUENCING THE RECOVERY OF ADENOVIRUS FROM SURFACE WATERS

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Dedicated to my wife Eileen whose patience, durability and devotion are immeasurable.

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ABSTRACT

ENVIRONMENTAL PARAMETERS INFLUENCING THE RECOVERY OF ADENOVIRUS FROM SURFACE WATERS

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Environmental and virologic factors influencing adenovirus recovery from estuarine waters by means of virus membrane adsorption technology have been studied. Adenovirus 5 enumeration using textile and membrane filters for clarification and virus adsorption has been accomplished. The recoveries were made from 25 gallon samples and involved input multiplicities of 200 to 2000 $TCID_{50}$ per ml. Virus assays were conducted using MicroTest II plates with assignation of 10 wells which provided 10 replicate points per dilution examined. Unlike enteric viruses, special pretreatment of clarifying filters was necessary to avoid loss of virus. Procedures were developed permitting removal of particles larger than 1 micron in size without simultaneous removal of virus. Virus adsorption to fiberglass textile and Cox membrane filters was enhanced by acidification of sample to pH 6.0 and addition of $MgCl_2$ to a final concentration of 0.05 M. The extent of acidification necessary for optimum adsorption was found to be less than levels required

for enteric viruses. The spectrum of virus adsorbing capacity of various textile filters was examined and fiberglass filters were found most efficient. Substances interfering with virus adsorption to textile and membrane filters were sought in estuarine waters. No interference was found when sample volumes up to 18 liters were passed through 127 mm filters with a limiting porosity of 0.45 micron. Recovery of adenovirus from adsorbent filters was carried out using alkaline beef extract solution. Reconcentration of virus was accomplished through aqueous two phase polymer separation methods. The application of these procedures to sewage effluent resulted in 106 adenovirus isolates confirmed by complement fixation tests.

INTRODUCTION

The lack of a suitable method for detecting small numbers of enteric viruses potentially pathogenic for man in water represents a serious problem for environmental health officials. There are more than 100 enteric viruses known to occur in human feces whose periodic appearance might be anticipated in waters polluted by raw sewage or improperly treated waste water. Viruses excreted by man are continually subject to devitalization by natural factors or waste treatment plant procedures. As the number of viruses in water decreases, the problem of their detection increases. Because a single virion is considered infectious for man, public health considerations emphasize the importance of a method capable of successfully detecting the presence of a few viruses (Katz and Plotkin, 1967; Plotkin and Katz, 1967).

The virus-in-water enigma is related principally to lack of reliable methods to recover viruses from various types of water. Grab samples can only be used to demonstrate virus where large numbers occur in raw sewage or grossly polluted raw waters. Low numbers of virus usually encountered in polluted surface waters require methods for concentration of virus before their demonstration can reasonably be expected. Use of gauze pads to promote more effective recovery of virus was instituted by Gravelle and Chin (1961).

Considerable improvement in virus detecting capability resulted, but the method suffered from an inability to determine how much water passed through the pad. Restriction of recovery efficiency to a qualitative evaluation basis represented a serious methodology defect. The greatest need in virus recovery methods research is for a procedure making possible efficient concentration of virus from large volumes of surface water under adverse sampling conditions. A related aspect of the same problem concerns laboratory procedures for isolation and identification of viruses recovered from water. Such procedures must take into account differences in isolation requirements represented by different enteric viruses known to occur in surface waters polluted by domestic waste discharges.

Due to their ubiquitous nature and relatively simple assay systems, enteroviruses have been the most widely studied viruses in the development of virus in water methods. Enteroviruses studied include 3 poliovirus, 6 coxsackievirus B and 34 echovirus serotypes. The human alimentary tract is their natural habitat and the source from which they are excreted into the environment. They can be recovered from patients with a variety of illnesses including poliomyelitis, aseptic meningitis, myocarditis and pericarditis. Included among the viruses found in water and pathogenic for man are the adenoviruses. They are associated with a variety of clinical syndromes, the most widespread of which are pharyngoconjunctival fever, acute febrile pharyngitis, and epidemic keratoconjunctivitis. Thirty-one serotypes have been

isolated from humans. Development of methods for quantitative enumeration of adenoviruses in water has been delayed by serious virologic problems. Human type cell cultures are needed for isolation purposes. Such primary cultures are more difficult to obtain and more expensive to use than monkey kidney. Separation of slow from fast-growing viruses in primary human cultures offers special difficulties. Conditions permitting plaque development in primary human cultures do not apply equally to all enteric viruses. The ability to isolate viruses and resolve virus mixtures by plaquing methods is adversely influenced by the plaquing difficulties encountered.

The first isolations of adenovirus were made by Rowe et al. (1953) from human adenoid explants cultured in vitro. After prolonged incubation periods transmissible cytopathic changes appeared in these cultures. Hilleman and Werner (1954) described a new cytopathic agent isolated from throat washings of a patient with primary atypical pneumonia during an epidemic of acute respiratory illness. Upon further investigation isolates from the two studies were shown to be immunologically related (Huebner et al., 1954). Rowe et al. (1956) demonstrated a similar immunologic relationship for isolates from adenoids and tonsils removed during surgery, and from nasopharyngeal and conjunctival secretions of persons with acute respiratory disease. These and subsequent isolates were named according to isolation source or clinical syndromes. Isolates were described as adenoid degenerating agents (ADA), respiratory illness (RI), acute respiratory

disease (ARD), or adeno-pharyngeal-conjunctival (APC) viruses. In 1956, Enders et al. recognizing nomenclature difficulties agreed on a common name, "adenovirus," based on the initial adenoid tissue isolations. Adenoviruses subsequently have been found ubiquitously distributed throughout the world. Isolations have been made from latent infections of healthy persons (Evans, 1958) as well as people suffering from clinical illness.

Thirty-one serotypes of human adenoviruses have been described on the basis of type-specific antigen. Only 18 have been established as pathogenic for man. The remaining 13, although associated with clinical illness, have not been proven to be etiological agents.

Adenovirus types 1 to 8 account for more than 90 percent of all adenovirus-caused illness. Types 1, 2, 5 and 6 are frequently the cause of sporadic cases of mild infections such as pharyngitis and common respiratory disease. Types 3, 4, 7 and 8 more often are associated with epidemics of greater clinical severity.

Infants and young children frequently are exposed to types 1, 2, 5 and 6. Infections often are subclinical or limited to mild upper respiratory tract illness. In older children and adults pharyngitis is the most common disease and is usually associated with type 3 or 7. These two types also cause follicular conjunctivitis. While minor respiratory illness, pharyngitis, and conjunctivitis occur sporadically, three common infections are epidemic in nature. These are pharyngoconjunctival fever, acute respiratory

disease, and keratoconjunctivitis. Pharyngoconjunctival fever resulting from exposure to adenovirus type 3, and occasionally type 7, can vary from a mild to a serious illness. Adenovirus types 4 and 7 have been isolated consistently during outbreaks of acute respiratory disease, and a rise in type specific antibody occurs in convalescent sera. Keratoconjunctivitis is a highly communicable disease which often appears in epidemic form in shipyards and heavy industrial plants.

While adenovirus infection often is followed by a mild illness, serious complications such as pneumonitis and otitis media can occur. There also is considerable evidence that adenoviruses, in addition to causing infections of the upper respiratory tract and eye, cause gastroenteritis, mesenteric adenitis, and rubelliform exanthem. Recently, adenoviruses have been associated with whooping-cough (Conner, 1970; Pereira and Caudeias, 1971). Adenoviruses have been isolated more often than any other virus from patients with typical whooping cough. While Hemophilus pertussis vaccination led to a reduced incidence of whooping cough in Great Britain in years past, recent experiences have revealed continuing infections in vaccinated children. Adenoviruses have been isolated from patients hospitalized with a diagnosis of acute infectious hepatitis (Rhodes and van Rooyan, 1968). Although no proof for an adenovirus etiology of infectious hepatitis in humans has been established, a canine species of adenovirus has been directly implicated in infectious canine hepatitis (Rowe and Hartley, 1962).

Although adenoviruses are primarily considered associated with respiratory disease, they are commonly isolated from feces. During the acute phase of a respiratory illness, concentrations reaching as high as 10^6 infectious doses per gram of stool are found (Jawetz et al., 1972). Rosen et al. (1962) demonstrated that many serotypes were more frequently isolated from anal specimens than from throat specimens.

Person to person transmission of adenoviruses occur by respiratory and ocular secretions, contaminated soap, towels, and ophthalmological instruments. Conjunctivitis often is known to be transmitted by swimming pool exposure.

The usual portal of entry is the upper respiratory tract and in most cases the infection appears to be limited to the mucous membranes and lymphoid tissues of this system. Infection of the conjunctiva may be by direct contact or extension from the respiratory tract along the lacrimal duct. Involvement of the middle ear probably occurs by transmission via the Eustachian tube.

For a more complete account of the clinical features and epidemiology of adenovirus diseases, the excellent text by Rhodes and van Rooyen (1968) is recommended.

It is of considerable import, especially in view of their ubiquitous distribution that certain adenoviruses were the first human viruses shown to possess oncogenic properties. Trentin et al. (1962) after injecting several neonatal hamsters with a number of different viruses found that human adenovirus type 12 induced the formation of

sarcomas. Subsequent investigators found that type 18 (Huebner et al., 1962), type 7 (Girardi et al., 1964) and type 31 (Pereira, 1965) could also produce tumors. These researchers also reported that once a tumor had been induced, infectious virus could not be recovered from either tumor or other tissues of the host animal. On the basis of oncogenicity the 31 serotypes have been arranged into three groups: the highly oncogenic (types 12, 18, and 31), the weakly oncogenic (types 3, 7, 11, 16, and 21), and the nononcogenic. This grouping is based on virus ability to induce tumors in neonatal hamsters (Green, 1970). Highly oncogenic adenoviruses induce tumor formation in a large proportion of newborn hamsters within two months after inoculation; weakly oncogenic adenoviruses produce tumors in only a small proportion of inoculated animals, and then only after four to eighteen months. This classification refers only to the time required and the frequency with which inoculated hamsters develop tumors, and does not imply that the tumors produced are fundamentally different. In addition to the induction of tumors in vivo, oncogenic adenoviruses also can cause transformation of cells in vitro (McBride and Weiner, 1964; Pope and Rowe, 1964). After transformation, infectious virus cannot be recovered; however, transformed cells can cause tumor formation upon injection into animals. Nononcogenic adenoviruses, able to cause in vitro transformation, cannot induce tumors upon introduction into animals (Freeman et al., 1967; McAllister et al., 1969).

Although there is no direct evidence that adeno-

viruses induce tumors in man, observations using animals and in vitro systems as models contribute to the growing interest in an adenovirus role in human oncogenesis. However, Gilden et al. (1970) were unsuccessful in attempts to demonstrate antibody to an adenovirus antigen in human cancer patients.

Simultaneous infection of rat embryonic fibroblast with adenovirus type 12 and Kilham rat virus (KRV) resulting in potentiation of KRV growth was reported by Chaney and Brailovsky (1966). A protein named "Stimulon" found in the supernate of adenovirus type 12 infected human embryonic kidney cells was able to potentiate the growth of KRV.

In addition to human types, simian, bovine, canine, murine, and avian subgroups are recognized (Pereira et al., 1963). All of these subgroups, with the exception of the avian Gal (Gallus, adeno-like) virus, have been shown to share a common group-specific antigen detectable by complement fixation. The Gal virus is included in the group because it satisfies all other taxonomic criteria. Adenoviruses from lower animals ordinarily are not pathogenic for man; conversely, human types ordinarily are not pathogenic for animals. A notable exception to the latter is the tumor inducing capability of human types for laboratory animals. Only a few adenoviruses of lower animals are associated with disease in their natural hosts and like the human types frequently are found as latent viruses. Some simian, bovine, canine and avian types have been reported to induce tumors under appropriate experimental conditions (Hull et al., 1965; Sarma et al., 1965; Darbyshire, 1966).

Adenoviruses are not inactivated by lipid solvents, an indication they do not contain a lipid membrane (Heubner et al., 1954; Feldman and Wang, 1961). Adenoviruses also are resistant to fluorocarbon compounds used for preliminary purification (Wilcox and Ginsberg, 1963). Adenoviruses are not affected by treatment with trypsin, deoxyribonuclease and ribonuclease. Types 1 to 4 are stable at 37°C for at least 7 days; at 23°C for 14 days; and at 4°C for 70 days (Ginsberg, 1956). Types 1 to 4 are also stable between pH 6.0 and 9.5 at room temperature, and at 36°C virus infectivity is not reduced at pH 6.5 for at least 7 days. Adenoviruses are more resistant to the affects of extreme acidity than extreme alkalinity. For example virus is not completely inactivated when incubated at room temperature for 30 min at pH 2.0. Complete inactivation occurs at pH 10.0 however, and in the case of type 5 virus is completely disrupted with dissociation of DNA from coat protein at pH 10.5 (Wilcox and Ginsberg, 1963).

Horne et al. (1959) were the first to describe adenovirus morphology by electron micrographs of negatively stained type 5 virus. These micrographs revealed virus particles 70 namometers (nm) in diameter and composed of 252 capsomers of 7 nm diameter arranged in icosahedral geometry. Two hundred and forty identical capsomers called hexons were distributed along the edges and faces of the twenty triangles. Other capsomers called pentons were located at the 12 vertices of the capsid. Each penton consisted of a base capsomer and an outward projection called a fiber, with a

terminal knob (Ginsberg et al., 1966). All adenovirus particles appear to have identical morphology regardless of the species of animal they infect (Ginsberg and Dingle, 1965).

Chemical analysis of highly purified preparations of human adenovirus showed them to consist entirely of DNA and protein (Pina and Green, 1969; Green et al., 1967; Schlesinger, 1969). Proteins constitute 86.5 to 88.4 percent of the dry weight of different adenovirus types. In addition to hexon, penton and fiber-proteins, there are other proteins associated with both capsid and nucleic acid structures. The DNA content varies between types from 11.6 to 13.5 percent of the dry weight of the virion. The highly oncogenic adenoviruses have less DNA than weakly oncogenic or nononcogenic types. The DNA is a double-stranded linear molecule with a molecular weight of $2 - 2.5 \times 10^7$ daltons. Adenovirus oncogenicity is related to the guanine-cytosine content. Nononcogenic adenoviruses have a high quanine-cytosine content of 57 - 60 percent. Highly oncogenic adenoviruses have a much lower quanine-cytosine content of 47 - 49 percent. Weakly oncogenic types are intermediate with 50 - 52 percent. Nucleic acid hybridization experiments have shown a similarity of base sequences within each group. Base composition of highly oncogenic adenoviruses is close to that of mammalian cells which have a quanine-cytosine content of 42 - 44 percent. This has led to speculation on the possibility of integration of oncogenic virus DNA into a host genome.

Each adenovirus possesses a group-specific antigen fixing guinea pig complement in the presence of homo- or heterotypic immune sera, and a type-specific antigen demonstrable by serum neutralization tests with homotypic immune serum (Huebner et al., 1954; Enders et al., 1956). Antigenic analysis reveals three kinds of antigens in adenoviruses. Pereira et al. (1959) separated three type 5 antigens by immunoelectrophoresis. They were designated A, B and C according to their migration in an electrophoretic field. Antigen A is group-specific and common to all adenoviruses except avian gal virus; B is mainly type-specific but has some group-reactive properties; C is strictly type specific (Klempera and Pereira, 1959). All three antigens have been shown to be structural proteins of the virus (Ginsberg et al., 1966). Antigen A is the hexon capsomer, B the penton capsomer, and C the fiber attached to the penton of the intact virus. Although human adenoviruses do not demonstrate hemadsorption properties, they can be divided into hemagglutinin groups on the basis of agglutination of monkey (rhesus) and rat erythrocytes (Rosen, 1960). Hemagglutination is a property of both the intact virion and antigens B and C separated from virus.

Adenoviruses grow in cells derived from tissues of their natural hosts (Pereira et al., 1963). Human types do not grow in monkey cells; nor do monkey types grow in human derived cells. Adenoviruses isolated from apes, which evolutionarily are intermediates between man and monkey, grow equally well in both human and monkey cells (Rowe et

al., 1955). Human adenoviruses, however, undergo an abortive infection in monkey cells. Infection occurs with synthesis of virus-specific RNA and DNA and early proteins. Production of late proteins is blocked however, and little or no production of mature virus particles occurs (Feldman et al., 1966; Friedman et al., 1970). Human adenoviruses can replicate in nonpermissive cells from heterologous species if a helper is present. For example, Rabson and coworkers (1964) reported simian virus 40 (SV40) growing in monkey cells acted as a helper for adenovirus 5 and 12 replication in African green monkey monolayers. Normally these cells are nonpermissive for replication of human adenoviruses. Adenovirus multiplication depended on prior or simultaneous infection of the cells with SV40. Although human adenoviruses do not replicate in cells from heterologous species, oncogenic types can transform nonpermissive cells to an oncogenic state.

Replication of adenoviruses is slow compared to many other animal viruses. The growth cycle of human adenoviruses takes at least 24 hours depending on virus type and cell culture used. Replication of poliovirus or influenza virus is complete in 6 or 7 hours. Highly oncogenic adenoviruses have a slower growth cycle than non-oncogenic types (Pina and Green, 1969).

A characteristic cytopathic effect is observed during growth of human adenoviruses in established cell lines. Examination of infected cultures shows intranuclear inclusions, which progress from an eosinophilic and Fuelgen-

negative to basophilic and Fuelgen-positive character (Boyer et al., 1959). Microscopic examination of unstained monolayers reveals distinctive rounding and aggregation of cells into grape-like clusters. Although infected cells demonstrate considerable cytopathic effect, they do not lyse; and progeny virus particles are not readily released. Investigations of adenovirus types 1 to 7 demonstrated less than 1 percent of the virus particles were found in culture fluids at the time of maximum virus production (Ginsberg, 1958; Denny and Ginsberg, 1961).

The occurrence and extent of adenovirus in polluted surface water is largely unknown. Lund et al. (1966) were successful in isolating adenovirus from sewage and fecal specimens by an aqueous polymer two-phase system. England was able to isolate a number of adenoviruses from an activated sludge-type waste treatment plant and from subsequent oxidation ponds (in G. Berg, 1967). Use of protamine sulfate (salmine) to facilitate recovery of natural adenovirus from raw sewage has been reported (England, 1972). These studies were limited by either lack of quantitative methods or restricted to conditions with high probability of adenovirus recovery.

A number of reports have appeared in the literature within the past several years on methods for concentrating viruses from water. Most of these methods share a common inadequacy. They are incapable of promoting quantitative enumeration of low numbers of virus in large volumes of polluted surface waters under real world conditions.

Methods for concentrating viruses from water are based on physiochemical properties of a virus particle. For example, viruses are nucleoproteins and behave as colloidal hydrophilic particles in aqueous suspension. Their physical and chemical properties are those common to proteins. Viruses display decreasing solubility with increasing concentrations of soluble salts such as ammonium sulphate. Viruses are amphoteric proteins with determinable isoelectric points. Since viruses exhibit polarity, they also are immiscible in organic solvents such as diethyl ether, n-butanol, chloroform, and fluorocarbons. Virus particles manifest unique surface properties represented primarily by an ability to adsorb readily to a number of substances such as celite, alumina gel, tricalcium phosphate, starch, and various resin and cellulose derivatives (Schwerdt, 1965). Viruses also have measurable molecular weights and sizes and therefore lend themselves to sedimentation from solutions by ultracentrifugation techniques.

Membrane-adsorption techniques for the concentration of viruses make use of the principle of electrostatic adsorption of virus to the surface of microporous membranes. These membranes usually are composed of cellulose derivatives. Epoxy-fiberglass filters (Cox Instruments Corp.) also have been used successfully. Concentration of viruses by membrane-adsorption techniques depends on alternate adsorption and elution of viruses from membrane surfaces. Substances used to elute viruses usually are proteinaceous in nature. Whole serum, albumin, beef extract, casein, veal

infusion broth, and nutrient broth may be used. All are used at alkaline pH values. Some of these substances have been used to pretreat filters to make them less capable of adsorbing virus. Many of the factors influencing filtration of viruses through the matrices of microporous membranes have been reported by Cliver (1965). Cliver (1965) indicated that penetration or retention of virus probably was the net result of several factors: (1) adsorption of virus to matrix surfaces of a membrane, (2) competition of macromolecules for adsorption sites on membranes; and (3) the presence or absence of viral aggregates. Cliver (1968) described adsorption phenomena to be influenced by the following: (1) chemical composition of filter membranes, (2) ratio of pore diameter to virion diameter, and (3) absence of substances interfering with virus adsorption. Cellulose triacetate filters were shown to adsorb virus poorly when membrane porosity exceeded the diameter of the virion by as much as 3 times. Conversely, cellulose nitrate filters were shown to adsorb virus very efficiently, even when membrane porosities exceeded virion diameter by as much as 285 times (Cliver, 1968).

Concentration on membrane filters of enteroviruses from crude virus-cell harvest suspensions was investigated by Wallis and Melnick (1967a). They reported enteroviruses could be made to adsorb or pass through membrane filters simply by manipulating the virus suspending medium. Addition of a divalent cation such as $MgCl_2$ significantly enhanced adsorption. In addition, adjustment of suspension pH

to 5.0 markedly enhanced adsorption. Conversely, the presence of organic or proteinaceous substances in the virus suspension interfered with adsorption, presumably by competing for membrane adsorption sites. Substances interfering with adsorption were identified as membrane-coating components (MCC). It was reported that MCC could be removed by passing samples through an anion-resin column, Dowex 1-X8 (Cl^-) 100-200 mesh. Other workers were less successful in removing MCC in natural waters by means of anion-resins (Borneff, 1970; Schafer, 1970). Wallis and Melnick (1967a) reported an 80 to 100-fold concentration of virus from crude virus-cell harvests using serum eluents. In a subsequent report the same investigators (1967b) were successful in applying membrane-adsorption techniques to recovery of viruses from 1 gallon samples of raw sewage.

Rao and Labzoffsky (1969) investigated the efficacy of membrane filters for detection of low multiplicities of viruses in what they considered to be large volumes of surface waters (500 ml quantities). By assembly of an AP 25 MF prefilter with an HA millipore membrane filter (0.45 um porosity), they were able to simultaneously clarify a sample and adsorb virus. Elution was accomplished by using 3 percent beef extract at pH 8. Virus adsorbed to the prefilter was eluted at the same time the membrane filter was eluted. They were able to recover 53 to greater than 100 percent of exogenously added virus. These investigators also reported enhancement of viral adsorption by addition of 200 ppm Ca^{2+} to the raw water before filtration.

Moore et al. (1970) compared various applications of a modified membrane-adsorption technique for concentration of viruses in waste-water. They observed an adverse affect of MCC on the efficiency of membrane filters (0.45 um porosity) to adsorb poliovirus. They were successful in removing MCC by the anion-resin pretreatment described by Wallis and Melnick (1967a). They failed to remove MCC with a preliminary protamine sulfate flocculation technique. They were able to recover 81 - 100 percent of input poliovirus by combination of aluminum hydroxide flocculation with membrane filtration. Virus adsorbed to alumina hydrates was recovered by elution of membrane filter.

Hill et al. (1972) using Millipore MF cartridge membrane filters were able to recover 97 percent or greater of input virus from 100 gal volumes of tap or estuarine water. In both instances Mg^{2+} cation was added to a final concentration of 1,200 ug/ml and the pH was adjusted to pH 4.5. Virus was effectively eluted from the membrane cartridge with 5 x nutrient broth in 0.05 M carbonate-bicarbonate buffer at pH 9.0.

Recovery of small numbers of virus from clean waters by cellulose nitrate membrane filters has been examined by Berg et al. (1971). The method consists of adsorbing viruses onto cellulose nitrate membrane filters (0.45 um pore size) from water containing sufficient Na_2HPO_4 to produce a final molarity of 0.05 and sufficient citric acid to produce a pH of 7. Adsorbed virus was recovered by elution with 3 percent beef extract combined with sonication of

filters. Complete recovery of poliovirus 1, echovirus 7 and coxsackie B3 resulted when less than 100 plaque-forming units were added to 1 liter quantities of water. Recoveries of reovirus 1 were almost as good. Preliminary studies showed this system could be applied to 25 gal quantities of high quality water relatively free from organic matter.

Wallis et al. (1972a) described the use of ten inch "Fulflo" textile filters (Commercial Filter Corp., Lebanon, Ind.) for removal of substances interfering with adsorption of enteroviruses to cellulose membrane filters. Organic material was removed by various anion resin treatments before samples were passed through adsorbent filters. By combining textile filters with anion resin, adsorbent filters were protected from naturally occurring substances in raw waters interfering with virus adsorption. Virus adsorption was enhanced by final concentrations of 0.05 M $MgCl_2$, and elution was accomplished by use of fetal calf serum in borate or Tris buffer at pH 9.0, or 1 percent polyethylene glycol containing 0.01 M NaCl in distilled water. Eighty-four percent of input virus was recovered from 150 gal of water and 61 percent from 300 gal of water.

A portable virus concentrator for testing water in the field was described by Wallis et al. (1972b). In this system, raw tap water containing virus is passed through clarifying textile filters with porosities of 5 to 1 um to remove particulate matter, and then through a 1 um cotton textile filter (Commercial Filter Corp., Lebanon, Ind.) to electrostatically remove submicron ferric and other heavy

metallic complexes. These clarifying filters do not, however, remove virus. Salts are then added to enhance the adsorption of virus to a fiberglass or cellulose acetate textile filter. Elution was achieved by passing 1000 ml of 0.05 M glycine adjusted to pH 11.5 through the adsorbent filter. The eluate could subsequently be readjusted to pH 5 - 6 and $MgCl_2$ added to a final concentration of 0.05 M. The initial eluate is then passed through a 90 mm membrane filter (0.45 um) to readsorb virus and eluted in 20 ml. In this manner 80 percent of poliovirus type 1 added to 300 gal of tap water could be recovered.

Homma et al. (1973) using the portable virus concentrator were able to recover 80 to 95 percent of poliovirus added exogenously to sewage. Orlon 10 in "Fulflo" filters in series of decreasing porosities (100, 10 and 1 um), and Tween 80 treated cellulose acetate textile filters were used to clarify raw sewage. Addition of $AlCl_3$ to a final concentration of 0.0005 M and adjustment of pH to 3.5 enhanced viral adsorption to a fiberglass textile filter. These investigators also reported virus adsorbents pretreated with as much as 35 gal of sewage could still adsorb 74 percent of added virus. Sixty-four percent of adsorbed virus was recovered following elution with 0.05 M glycine at pH 11.5. Combination of salt enhancement and acid pH proved capable largely of overcoming the harmful effects of MCC interference.

Sobsey et al. (1973) described an improved method for concentrating viruses from large volumes of clean water. Viruses in large volumes of water could be efficiently ad-

sorbed to epoxy-fiber-glass and nitrocellulose filters following adjustment of sample pH to 3.5 in the absence of exogenously added salts. Adsorbed viruses were eluted with 1 liter volumes of 0.05 M glycine eluent, pH 11.5, and reconcentrated by adsorption and elution from a small epoxy-fiber-glass filter. Small quantities of poliovirus in 100 gal of tap water were concentrated nearly 40,000-fold with an average virus recovery efficiency of 77 percent.

The efficacy of membrane and filter adsorption technology would seem to be predicated on the following: (1) proper selection of adsorbent materials, (2) addition of metal cations, (3) removal of MCC from raw water, (4) adjustment of water sample pH to 5 or below, and (5) selection of an eluant capable of efficient elution of virus.

Adsorption of viruses to precipitable salts, iron oxide, and polyelectrolytes relies on the ability of viruses to adsorb efficiently to a selected adsorbent. Stevenson et al. (1956) used an alum flocculation procedure for concentrating coxsackievirus A-2. Virus entrapped in the floc was eluted at an elevated pH. These investigators indicated the alum-floc method permitted virus concentrations of 100-fold or greater and was capable of detecting as little as 0.00625 LD_{50}/0.02 ml (volume inoculated per mouse).

Wallis and Melnick (1967c) concentrated a number of different viruses by adsorption on aluminum phosphate, aluminum hydroxide, and calcium hydrogen phosphate flocs. In each instance preformed precipitates (flocs) were added to aqueous suspensions containing virus. They observed that acid sensi-

tive types were concentrated on the aluminum phosphate floc while all the viruses tested, except reovirus and adenovirus, were concentrated on the aluminum hydroxide and calcium hydrogen phosphate floc. Recovery of virus from 1 liter volumes containing as few as 100 PFU/ml generally exceeded 80 percent. The aluminum hydroxide floc method was used to isolate naturally-occurring viruses in sewage (Wallis and Melnick, 1967c). Over a 4 week period 204 isolates from 1 gal samples were found. Unfortunately this method could not be used for samples greater than 2 gal.

Concentration of coxsackie virus A-9 by adsorption to iron oxide was examined by Rao et al. (1968). These investigators were successful in recovering 87 - 90 percent of input virus by passing a sample through 25 g of magnetic iron oxide (M.O. 2530, Magnetic Tape Division of Charles Pfizer Co.), followed by elution with 3 percent beef extract at pH 8. One of the major drawbacks to the use of iron oxide as an adsorbent was the clogging of the iron oxide bed which occurred following exposure to suspended particulates.

Concentration of viruses by adsorption to insoluble crosslinked copolymers of maleic anhydride (polyelectrolytes) was described by Johnson et al. (1967). They reported polymers based on divinyl benzene-crosslinked styrene/maleic anhydride could adsorb 100 percent of tobacco mosaic virus and more than 99.99 percent of poliovirus from aqueous suspensions. Elution of virus from the polymer structure was achieved by use of 1 M sodium chloride with a 52 percent recovery efficiency.

Wallis et al. (1969) reported concentration of viruses from sewage by adsorption to an insoluble crosslinked polymer of isobutylene: maleic anhydride designated as PE60 by the manufacturer (Monsanto Company, St. Louis, Mo.) In preliminary studies a batch method was developed which consisted of adding washed PE60 (400 mg/gal) to clarified sewage. The sewage was clarified by filtration through an MF fiberglass prefilter pad. Virus adsorption was enhanced following adjustment of pH to a 5 to 6 range. After addition of virus and mixing for 1 hr, the polyelectrolyte-virus suspension was filtered through a 47-mm fiberglass prefilter pad and the virus-laden polyelectrolyte complex was recovered from the filter pad with the aid of a spatula. Virus was eluted with 10 percent fetal calf serum adjusted to pH 8.0 or 9.0. In controlled laboratory experiments 93 percent of the input virus was recovered. The polyelectrolyte method was compared with membrane-adsorption and aluminum hydroxide-adsorption methods. While virus isolation differences among the three methods were not always statistically significant, the polyelectrolyte-adsorption method consistently gave higher isolation rates. In the same report Wallis et al. (1969) described a modified thin-layer PE60 procedure in which the PE60 was "sandwiched" between two fiberglass prefilter pads. This modification prevented polyelectrolyte displacement during the filtration process. The eluate obtained from the PE60 adsorbent was readsorbed onto PE52 (crosslinked copolymer of ethylene maleic ambydride). Virus was eluted from the PE52 adsorbent with physiological saline. This two

stage-adsorption-elution procedure resulted in reduction of 1 gal volumes of sewage to a final 3 ml volume, with efficient recovery of virus.

Grinstein et al. (1970) applied the polyelectrolyte-adsorption method (batch technique) to sewage and to a river stream receiving sewage effluents in Houston, Texas. An average of 45 to 286 PFU per gallon was obtained from a total of 76 one gallon samples tested over a 2 month period. The authors concluded it was possible to monitor virus in water by use of the polyelectrolyte adsorption method.

Application of the "sandwich" polyelectrolyte-adsorption technique to virus monitoring of volumes of tap water up to 100 gal or greater was studied by Wallis et al. (1970). Ten grams of PE60 were suspended in 200 to 300 ml of distilled water and filtered onto a 293 mm Millipore filtration unit containing a AP20 fiberglass prefilter pad. A second AP20 prefilter was then placed over the PE60 layer. By the use of this technique virus recoveries ranged from 65 to 80 percent.

Berg (1971) carried out experiments with the polyelectrolyte-adsorption "sandwich" technique using PE60. Recoveries of 51 to 53 percent were obtained from 1 liter volumes of distilled water containing 75 to 105 PFU per ml of poliovirus. Recovery effectiveness was considerably less with echovirus type 7 and reovirus type 1. Extension of PE60 techniques to the examination of field samples resulted in virus isolations despite fluctuations in method efficiency.

Another method for recovery of virus in water is the aqueous polymer two-phase separation method. The method involves liquid-liquid partitioning, a phenomenon closely related to adsorption. Partitioning occurs as a result of differences in particle surface properties and their distribution between two liquid phases. Adsorption occurs as a result of differences in particle surface properties and their distribution between a solid phase and a liquid phase (Schwerdt, 1965). Aqueous polymer two-phase separation methods consist of solubilizing two different polymers in water under specified conditions of ionic strength, pH, and polymer concentrations. Following adequate mixing and an 18 to 48 hr holding period in the cold, two phases are produced. The bottom phase, usually small, contains the concentrated virus. Partitioning of virus particles in aqueous polymer two-phase separation systems was reported by Philipson et al. (1960) and Wesslen et al. (1959).

Concentration of enteric viruses from water by a single-step and a two-step aqueous polymer two-phase separation system was described by Shuval et al. (1967). After addition of salt, sodium dextran sulfate, and polyethylene glycol, the mixture was shaken and transferred to a separatory funnel and held in the cold for 18 hr at $4^{o}C$. Following removal of a small bottom phase, potassium chloride to a final concentration of 1 M was added to precipitate the dextran sulfate. The mixture was then centrifuged at 2000 rev/min for 5 to 10 min and the supernatant fluid assayed for virus. In the two-step, two phase procedure, sodium

chloride was added to a final concentration of 1 M to the bottom phase. This did not precipitate dextran sulfate. After an additional holding period of 18 hr at 4°C, a new two-phase system developed in which virus was found concentrated in the small top phase. Shuval and coworkers reported a 52 to 200-fold concentration by the single-step procedure with virus recovery effectiveness ranging from 37 to 98 percent. The two-step, two phase procedure yielded a 274-fold virus concentration. Virus multiplicities as low as 0.066 PFU/ml were detected by this system, and they speculated that as little as 10 PFU/l could be detected. Shuval et al. (1969) reported further developments and refinements of the polymer two-phase separation system for concentrating enteroviruses in water. A median virus concentration of 520-fold was experimentally determined with recoveries ranging from 35 to greater than 100 percent. As few as 1 to 2 PFU/l of sample were detected about 85 percent of the time. The system was also tested in the field by examining raw sewage, water from wells, springs, streams, effluent from oxidation ponds, and effluent from a biofiltration plant. Viruses were detected in virtually all of the 1 liter volume field samples, with a maximum of 184 PFU/l found in raw sewage. Virus also was detected in 2 samples of drinking water from a shallow municipal well.

Limitations of the aqueous polymer two-phase separation technique for detecting viruses in dilute aqueous suspensions were reported by Grindrod and Cliver (1969). Seven enteroviruses were subjected to concentration. At high

input virus multiplicities all of the viruses tested were efficiently concentrated. However, at a low multiplicity virus input (1 - 10 PFU), recovery of poliovirus type 1 was considered satisfactory while the recovery of coxsackievirus A-9 was unsatisfactory, being less than 11 percent. It was observed that the sodium dextran sulfate was somewhat inhibitory to coxsackievirus A-9, and extremely inhibitory to coxsackievirus B-2 and echovirus type 6. The investigators concluded that the single-step two phase system was found to be a significant aid to detection of polio types 1, 2 and 3, coxsackievirus B-3, and coxsackievirus A-9. The method, however, was considered worse than no treatment at all for detecting coxsackievirus B-2 and echovirus type 6. In a subsequent publication (Grindrod and Cliver, 1970) these researchers indicated that there would be a 50 percent probability of detecting virus in a sample containing 1 - 2 PFU/l, depending on virus type and replacement of dextran sulfate with dextran in the two-phase separation system. Recoveries of enteroviruses were observed to range from 59 to 164 percent when tested with dextran as compared to 0.001 - 100 percent with dextran sulfate.

Nupen (1970) has applied the aqueous polymer two-phase separation technique to recovery of viruses from the Windhoek advanced waste-water reclamation plant at Windhoek, South Africa. Virus recoveries from 1 liter volumes in laboratory controlled studies averaged 40 percent. Nupen compared virus quantification from the aqueous polymer two-phase separation technique, by the $TCID_{50}$ assay method, and

the plaque technique. The $TCID_{50}$ method was considered to be superior to the plaque method.

In summary, the aqueous polymer two-phase separation technique for concentration of viruses from water offers a method of acceptable sensitivity for use with small sample volumes. A disadvantage related to an inhibitory effect of dextran sulfate on some enteroviruses (Grindrod and Cliver, 1969) can be overcome by removal of dextran through KCl precipitation.

Concentration of viruses onto soluble alginate filters represents a method by which viruses are either adsorbed, entrapped or otherwise retained on a filter that can be solubilized with sodium citrate. Gartner (1967) describes a method of preparing alginate filters and discusses their efficiency for concentration of virus from water. Using poliovirus alginate filters were found to completely retain input virus. Recovery of poliovirus from solubilized filters ranged from 25 to 100 percent. Comparison of direct inoculation and alginate filter methods was made with untreated sewage and effluents from waste treatment plants. The results reflected the need for virus concentration and the ability of the alginate filter method to serve this purpose.

Witt (1965) reported sample volumes up to 10 liters could be filtered through alginate filters. By solubilization of filters in 1 ml of isotonic citrate solution concentration factors of 10,000 could be achieved. He was able to detect 1 PFU of poliovirus 3 in one liter of water by this means.

Nupen (1970) studied virus recoveries made with alginate filters from samples collected at the Windhoek advanced waste-water reclamation plant. One liter samples were passed through 50 mm filters at negative pressures of 500 mm Hg. A virus-containing-alginate film was floated off the filter with 0.9 percent sodium chloride and then dissolved in 3 ml of 3.8 percent sodium citrate solution. Laboratory evaluation of the alginate filter technique showed an average recovery of 40.05 percent and a coefficient of variation of 41.69 percent.

Evaluation of the alginate filter technique in laboratory and field examinations indicated acceptable recovery capabilities from small volumes of clean water. A major disadvantage was the clogging of filters experienced with turbid water samples which placed serious constraints on sample volumes possible to test.

Continuous-flow ultracentrifugation using isopycnic zonal or rate zonal methods has been proposed (Anderson et al., 1967). Small viruses having similar densities to that of poliovirus can be completely sedimented within 1 to 1.5 hr from an aqueous suspension by a centrifugal field of 120,000 times gravity (Schwerdt, 1965). Depending on size, shape, density of virus particles, centrifugal force, viscosity, and density of the suspending medium, it is possible to fractionate different classes of particles by these methods. In rate-zonal centrifugation, particles are separated on the basis of differences in sedimentation rate in a density gradient which provides gravitational stability to

the system. In isopycnic-zonal centrifugation, particles which have the same sedimentation rate are separated on the basis of differing buoyant densities.

Cliver and Yeatman (1965) applied ultracentrifugation methods for the concentration and detection of viruses from water. By use of a Spinco Model L Preparative Ultracentrifuge, quantitative recovery of both poliovirus type 1 and coxsackievirus B-2 was achieved. Sample volumes less than 10 ml could be concentrated in 2 hr. It was reported initial virus input multiplicities as low as 0.025 PFU/ml could be detected 50 percent of the time.

Anderson et al. (1967) was able to remove 95 percent of suspended poliovirus with continuous-flow ultracentrifugation at a flow rate of 2 to 3 liters per hr. The virus was sedimented directly onto the wall of the rotor and removed by resuspending the pellet. It was observed, however, that certain types of viruses were inactivated after pelleting. This difficulty was overcome by trapping virus particles in stationary isopycnic density gradients. This system was tested successfully with both adenovirus type 2 and respiratory syncytial virus. High capacity ultracentrifuges with flow rates as high as 25 liters per hr have proven successful in recovering 80 - 90 percent of exogenously added influenza virus (Anderson, 1970).

Although isopycnic-zonal continuous flow centrifugation has yielded acceptable recoveries of virus from water, its routine application to viral assessment of natural waters offers diffuculties. Two major disadvantages are the initial

cost and maintenance of the equipemnt, and its inability to process large volumes of water in a reasonable period of time.

Electrophoresis and electro-osmosis methods depend principally upon the amphoteric nature of viruses. As nucleoproteins viruses exhibit mobility at a given pH under a direct current electrical field. Application of forced flow electrophoresis to the concentration of virus in water was reported by Bier et al. (1967). Quantitative recoveries of coliphage T1 were made at flow rates of 60 to 240 ml per hour. The method emphasized selective adsorption of viruses by dialyzing membranes in fields of known force. In electro-osmosis, water diffuses from one side of a membrance to the other under the influence of electrical gradients. Virus suspended at a pH greater than its isoelectic point (anions) are firmly adsorbed during the process.

McHale et al. (1970) also described concentration of virus from water by forced-flow electrophoresis and electro-osmosis. The former procedure yielded 3-fold concentration of poliovirus at a flow rate of approximately 300 ml per hr. Poliovirus was concentrated 5-fold by electro-osmosis at a water removal rate of 0.8 ml per hr per cm^2 of membrane area. It was concluded that both procedures gave rapid concentration and under more gentle conditions than other methods for concentration of virus. Both methods possess the same disadvantage of an inability to process large volumes of polluted surface waters.

Cliver (1967) described the use of polyethylene glycol for concentration of virus from water by means of a hydroextraction procedure. In this method, a 100 ml sample of water containing virus is placed into a dialysis tube surrounded by 100 g of polyethylene glycol (Carbowax 20,000) dissolved in 100 ml of water. Water from a sample is extracted through the dialysis tube and provides a simple means of concentration. The extraction process was allowed to continue for 2 to 3 hr at room temperature at which time sample volume was reduced to 1 ml, giving a 100-fold concentration.

Liu et al. (1971) reported the results of a preliminary study in which a flow-through gauze sampler-device was used for recovery of virus from tap water and seawater. Recovery of 2 percent of poliovirus added to tap water was found. When seawater was examined, virus recovery increased to 19 percent. Addition of 3 percent NaCl to tap water increased virus recovery to 47 percent. Sampling flow rates in all experiments were set at 1 gal per min. Elution of virus was optimal when 5 percent calf serum in Tris buffer adjusted to pH 8.0 was used.

Selection of a method for concentration of viruses from water presupposes some knowledge of water quality criteria. Whenever very turbid waters are to be examined for virus, membrane-adsorption, alginate filter and iron-oxide or polyelectrolyte "sandwich" methods are unlikely candidates. Flow through equipment featuring clarifying and adsorbing filters offer the best chance of monitoring large volumes of surface waters for virus.

This study sought to determine whether such equipment could be used for recovery of adenovirus from polluted surface waters. Information was sought on parameters imposed on the virus collection process by properties possessed by and peculiar to the adenoviruses.

MATERIALS AND METHODS

Stock Virus Preparation

Adenovirus, type 5, obtained from the American Type Culture Collection, was used throughout this study. High titered stock virus was prepared in HeLa cells by two methods. A Spinner flask containing 150 ml of growth media was seeded with 6 - 8 x 10^6 cells and incubated at 37°C with constant mixing. After a concentration of 5 x 10^5 cells per ml was reached, the cells were centrifuged at low speed and the pellet resuspended in 150 ml of growth media. The cell suspension was infected with virus at a multiplicity of infection (MOI) of approximately 5 x 10^{-3} $TCID_{50}$ per cell. Incubation was continued until complete cytophathology occurred. Contents of a flask were frozen and thawed 3 times and cellular debris removed by centrifugation at 650 xg for 10 min. Supernates were dispensed in 5 ml aliquots and frozen at -70°C.

A second method of obtaining high titered stock virus was by infection of confluent HeLa cell monolayers in 1680 cm^2 roller bottles. After a 60 min adsorption period at 37°C with rotation at 0.25 revolutions per minute, 75 ml of maintenance media containing 20 mM of HEPES buffer was added and the roller bottle reincubated until advanced cytopathology occurred. The suspension was frozen and thawed 3 times, centrifuged, and dispensed in a similar manner as above.

Poliovirus 2, obtained from the American Type Culture Collection, was prepared in roller bottle cultures containing confluent monolayers of Vero cells. After complete CPE the virus-cell suspension was centrifuged at 650 xg for 10 min, the supernatant fluid dispensed in 5 ml aliquots and tubes stored at -70°C.

Cell Culture

HeLa (S-3 clone) cells were purchased from Flow Laboratories (Rockville, Maryland). The growth media consisted of Auto-Pow minimal essential medium (Auto-Pow MEM, Flow Laboratories) supplemented with 5 percent fetal calf serum (FCS; Baltimore Biological Laboratory, Cockeysville, Maryland) and containing 100 units potassium penicillin G, 100 mg streptomycin sulfate, and 1 mg amphotericin B per ml. Cultures were maintained in the same basal medium supplemented with 2 percent FCS. Subcultures were prepared by treating confluent cell monolayers for 10 min at 37°C with 10 ml of 0.25 percent trypsin solution (Appendix A) adjusted to approximately pH 7.8 with 1 N NaOH. The dislodged cells were centrifuged at 650 xg for 10 min, resuspended in 5 ml of growth media, and planted with 1 - 2 x 10^6 cells per Blake bottle.

A clone of porcine kidney cells designated Y-15 was used for propagation of adenovirus (Appendix C). The growth media consisted of equal volumes of Auto-Pow MEM and Liebowitz media (L-15) supplemented with 10 percent FCS. The growth media was changed every third day until the cells reached confluency at which time the cells were either placed

on maintenance media (2 percent FCS) or subcultured. Cells were removed from a glass surface following washing once with prewarmed (37°C) versene buffer (Appendix A) for 10 min at 37°C. Fifteen milliliters of prewarmed 0.5 percent trypsin solution adjusted to approximately pH 7.8 with 1 N NaOH was added. After 30 min incubation at 37°C a sterile magnetic stirring bar was added and the contents stirred slowly until all cells were removed from the glass. The cells were subjected to centrifugation at 650 xg for 10 min and the pellet resuspended in 5 ml of growth media. Monolayers were prepared by introducing 3×10^6 cells into a bottle containing 80 ml of growth media followed by incubation at 37°C.

Vero cells (Appendix C) were used for propagation of enteric viruses other than adenovirus. The growth medium and subculture procedures were the same as used for Y-15 cells.

Primary human embryonic kidney (HEK) cells purchased from a commercial source (Appendix C) were grown in 35 x 15 mm plastic petri dishes (Kimble) at 37°C in a humidified chamber at 5 percent CO_2 concentration. Cells were planted at a concentration of 6×10^5 cells per dish, and the growth media (Auto-Pow MEM supplemented with 10 percent FCS) was changed every third day. After confluency the cells were either used in a test or placed on maintenance media (Auto-Pow MEM with 2 percent FCS).

Virus Assay

Adenovirus assay was a modification of the method introduced by Rosenbaum et al. (1963). Each well of a sterile

MicroTest II plate was seeded with 1 to 2 x 10^4 HeLa cells per 0.2 ml of growth media and covered with a MicroTest II lid. The plate was incubated at 37°C in a humidified incubator at 5 percent CO_2 concentration until monolayers of 50 percent confluency formed. Serial tenfold dilutions of virus were made in maintenance media containing 20 mM HEPES buffer. Growth media was aspirated aseptically from each well, and 0.2 ml of a virus dilution introduced into each of ten wells. Maintenance media was replaced on three and six days post inoculation, and CPE determined on seven and eight days post inoculation. The 50 percent tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Muench (1938).

A plaque assay was developed for measurement of adenovirus plaque forming units (PFU) in Y-15 monolayers. Plastic tissue culture petri dishes (35 x 15 mm, Kimble) were planted with 2 ml of growth media containing 1 - 2 x 10^5 cells per ml and incubated at 37°C in a humidified chamber with flowing 5 percent CO_2. Serial tenfold dilutions were made of adenovirus in maintenance medium and duplicate monolayers inoculated with 0.1 ml of each dilution. After a 90 min adsorption period in the CO_2 incubator, cells were overlaid with 2 ml of an agar overlay medium cooled to 45°C (Appendix B). After a 45 minute interval the dishes were inverted and incubated at 37°C. Four days post infection an additional 1 ml of agar overlay medium was added, and seven days post-infection another 1 ml overlay with neutral red was

added. Plaques were counted on the seventh day and continued until plaque development ceased or monolayers disintegrated.

A plaque assay for poliovirus 2 in Vero monolayers formed in 1 oz white prescription bottles (Brockway Glass Co.) was used in this study. Serial tenfold dilutions were made in maintenance medium and 0.1 ml inoculated onto the monolayers. After a 1 hr adsorption period at 37°C monolayers were overlaid 5 ml of an agar overlay (Appendix B). Plaques were counted daily for 8 days or until no more appeared.

Sample Sterilization

Samples inoculated onto monolayers were freed from contaminants by ethyl ether treatment. One milliliter of anesthetic grade ether was added to 5 ml of sample to be sterilized. Samples were mixed 3 times on a vortex mixer and stored at 4°C for 18 - 24 hr. After this interval the aqueous phase was separated and transferred to a sterile glass petri dish. Evaporation of residual ether usually was complete after a 2 hr interval of standing (with petri dish cover slightly ajar) at room temperature. Samples were removed from the petri dish and stored in sterile glass test tubes. Storage at 4°C was made when assays were to be conducted within a week. Storage at -70°C was made when assays were not to be made within a week.

Adenovirus Stability to pH Change

Devitalization at acid and alkaline pH values

Adenovirus 5 in 0.5 ml volume was added to 45 ml aliquots of 0.05 M glycine, one at pH 3.5, the other at pH 7.5. Five milliliter amounts were removed from the aliquots at regular time intervals and added to an amount of 0.01 N NaOH predetermined to give a final pH of 7.5. Five milliliters also were removed from the 7.5 solution and added to a volume of 0.05 M glycine, pH 7.5, equal to the volume of 0.01 N NaOH used in the test.

Adenovirus 5 in amounts giving final concentration of 200 to 2000 $TCID_{50}$ per ml was added to 30 ml aliquots of 0.05 M glycine, one at pH 10.5 and another at pH 10.0. After a two minute interval, 5 ml was removed and added to an amount of 0.01 N HCl predetermined to return the pH to 7.2. Sample volume in each instance was equalized by addition of 0.05 M glycine, pH 7.5. The examinations of virus stability were continued at regular time intervals as outlined previously.

Devitalization using 0.1 N HCl or 0.1 N NaOH

Acid devitalization effects were examined following addition of adenovirus 5 to 25 gal of either estuarine or tap water in amounts giving final concentrations of 200 to 2000 $TCID_{50}$ per ml. Acid pH values of 6.0, 4.7 and 3.5 were obtained by addition of 0.1 N HCl by means of a peristaltic pump at rates not in excess of 80 ml per minute. Rapid mixing was accomplished with an industrial stirrer, VWR Cat #58963-109.

After equilibration at a given pH value, a 5 ml samples was removed, etherized and assayed.

Alkaline devitalization effects were examined following addition of adenovirus 5 to one liter aliquots of 3 percent beef extract (BE; Colab Laboratories Inc., Glenwood, Illinois), pH 7.0, in amounts giving final concentrations of 2000 to 20,000 $TCID_{50}$ per ml. Alkaline pH values of 8.0 and 9.0 were obtained by addition of 0.1 N NaOH accompanied by rapid mixing on a magnetic mixer. Five milliliter samples were removed, ether treated and assayed as described previously.

Virus Concentration

Clarification

Recovery and concentration of virus from aqueous samples by means of a portable virus concentrator were first described by Wallis et al. (1972a and 1972b). Samples prepared in Jackson Estuarine Laboratory (JEL) tap water (Artesian well, non-chlorinated), artificial sea water (Seven Seas, Utility Chemical Co.) and Great Bay (GB) estuary water to give final concentrations of 200 - 2000 $TCID_{50}$ per ml were mixed with an industrial stirrer and introduced into the virus concentrator under positive pressure (Flotec variable flow self-priming pump, Cole Parmer). Suspended solids were removed and samples clarified by passage through ten inch "Fulflo" textile filters (Commercial Filter Division, Carborundum Co.) at flow rates of 1 gal per min (GPM).

Filter characteristics and their use for clarification and concentration purposes have been described previously (Wallis et al., 1972a and 1972b). Orlon filters of 10 um (019) and 1 um (039) porosities, and a 1 um porosity cellulose acetate (W10A) filter were used to clarify water samples. Orlon filters were pretreated with 3 percent BE solution, pH 9.0. Cellulose acetate filters and orlon filters were pretreated with a non-ionic 0.1 percent Tween 80 solution (Tw80). Filters were immersed in a pretreatment solution 18 hr at 4°C. They were washed for a total of 15 min in filtered JEL tap water prior to use. Excess wash water was removed under positive pressure supplied by nitrogen gas. Filters were examined independently and in series.

Virus Adsorption

After clarification of a sample its pH was adjusted to 6.0 by addition of 0.1 N HCl with a Masterflex pump at rates less than 80 ml per min. Adsorption was enhanced by addition of magnesium chloride to give a final concentration of 0.05 M. Samples were passed through virus adsorbent filters at flow rates of 1 GPM. Fiberglass "Fulflo" textile filters of 1 um porosity (K-27), and 267 mm Cox membrane filter assemblies of 0.45 um filters preceded by 2 um filters were used to adsorb virus. Fiberglass filters were arranged in series when more than one was used. Membrane filters followed fiberglass filters and were in parallel arrangement when two were used.

Untreated orlon (039) and cellulose acetate (W10A) textile filters also were examined for viral adsorptive capabilities. The effect of different $MgCl_2$ molarities and pH values upon adsorption also was examined.

Membrane-coating components interference with virus adsorption was examined by two procedures. In the first procedure virus was added to 12 liters of JEL tap water adjusted to pH 6.0 followed by enough $MgCl_2$ to give a final concentration of 0.05 M. Four liter samples were passed through fiberglass textile filters pretreated (1) with 50 gal of estuary water adjusted to pH 6.0 and containing 0.05 $MgCl_2$ (2) with 50 gal of estuary water clarified by passage through an orlon (039) filter (3) no pretreatment. Filtrates were examined for virus.

In the second procedure different volumes of estuary water containing added virus were adjusted to pH 6.0, and $MgCl_2$ added to give a final concentration of 0.05 M. Test suspensions were passed through 127 or 47 mm Cox 0.45 um filters preceded by 2.0 or 1 um filters. Five milliliter filtrate volumes were collected at periodic intervals and assayed for virus.

Virus Elution

Virus was recovered from adsorbent filters by elution using 3 percent BE solutions adjusted to pH 9.0. Elution was carried out with fiberglass and membrane filter holders connected to each other and to a peristaltic pump (Randolph Model 610, Houston, Texas) by tygon tubing fitted with quick connects (Flolok, Houston, Texas). Eluent was

added to filter holders and tubing, and circulated through the filters for 10 min. Fulflo filters were inverted several times during elution to improve elution efficiency. Eluate was collected, neutralized to pH 7.2 by addition of 0.1 N HCl, and stored at 4°C. Virus recovery also was attempted at differing pH values and with 0.05 M and 1 M glycine eluents.

Virus Reconcentration

Neutralized eluates were reconcentrated to reduce volume and further concentrate virus prior to inoculation of cell cultures. Two aqueous two phase polymer separation procedures were used for this purpose. The first procedure (System A, NaDxS-PEG-NaCl) was a modification of Shuval et al. (1969). Dry NaCl (0.5 M), sodium dextran sulfate 2000 (NaDxS-0.2 percent, Sigma Chemical Co.) and polyethylene glycol 4000 (PEG-6.43 percent, J. T. Baker Chemical Co.) were added to an eluate. The chemicals were added one at a time and each in its turn dissolved before the next one was added. After a final 1 hr mixing period at 4°C the solution was transferred to a 2 liter separatory funnel and kept at 4°C for 18 hr. Following development of two phases, bottom and interphase portions were removed. A second stage partitioning was carried out by addition of NaCl to these portions (final concentration of 1.0 M), mixing for 1 hr and an overnight standing interval (18 hr) at 4°C. The mixture was then centrifuged 10 min at 120 xg, and top and interphase portions withdrawn for assay.

A second procedure (System B, Dx-MC-PEG6000) followed a Wesslen et al. (1959) method. To each liter of eluate was added NaCl (0.15 M), 20 percent w/w dextran 2000 (26.2 g, Sigma Chemical Co.), methyl cellulose 4000 c.p.s. (4.8 g dissolved in 40 g of hot 5 M NaCl, Dow Chemical Co.) and 20 g of distilled water to rinse out the slurry. The chemicals were added sequentially with thorough mixing between each addition. After an additional hr of mixing, a solution was transferred to a 2 liter separatory funnel and kept at 4°C for 48 hr to allow full development of the two phases. Bottom and interphase portions were collected and an equal volume of 10 percent (w/w) polyethylene glycol 6000 added. Following a one hr mixing period at 4°C, a solution was transferred to a 250 ml separatory funnel and phases allowed to develop over a 24 hr period at 4°C. Bottom and interphase portions were withdrawn for assay.

Wesslen et al. (1959) reported System B did not appreciably concentrate particles with diameters as small as 25 nm. To verify these results adenovirus 5 and poliovirus 2 were added simultaneously to 3 percent BE solutions adjusted to pH 7.2. After phase separation and removal of the bottom phase of the second step a sample was etherized and assayed for poliovirus. Adenovirus assays were made following addition of 40 units of poliovirus 2 antiserum per 0.2 ml of sample. Assays were conducted in HeLa monolayers in Micro-Test II plates. Samples were diluted in HeLa maintenance medium containing 20 mM HEPES buffer and allowed to incubate 1 hr at 37°C to promote poliovirus 2 inactivation prior to inoculation of test monolayers.

Natural Virus Isolations

Fifty gallons of trickling-filter sewage effluent was collected from Otis Air Force Base, Falmouth, Massachusetts and transported to Woods Hole Oceanographic Institute for processing. Sample pH was adjusted to 8.0 with 0.1 N NaOH and passed through pretreated clarifying filters. A clarified sample then was adjusted to pH 6.0 with 0.1 N HCl and approximately 2 kg of $MgCl_2$ added to give a final concentration of 0.05 M. Samples were pumped at 1 GPM through two K-27 fiberglass textile filters and a 263 mm Cox filter assembly composed of 0.45 um and 2 um porosity filters. After processing of a sample, filters were refrigerated and returned to the Jackson Estuarine Laboratory, U. N. H. for elution. After reconcentration of a sample by aqueous polymer two phase separation (System B), the bottom phase was recovered, etherized and inoculated onto HEK monolayers. Removal of ether and sample volume used has been described previously. A 1 hr adsorption period at 37°C was followed by an Earle's balanced salt solution (Appendix A) wash of monolayers. Two washes were made. Monolayers were overlaid with nutrient agar overlay medium (Appendix B).

Identification of Adenovirus

Identification of natural virus isolates as adenovirus was made by means of complement fixation (CF) tests. All adenoviruses contain a soluble group antigen reactive with adenovirus antiserum. Detailed procedures of the CF method are described in a manual published by the Laboratory Branch, National Communicable Disease Center (1968).

Preparation of Reagents

Commercially prepared hemolysin (Microbiological Associates) was thawed and a 1:100 stock solution prepared by addition of 1 ml of hemolysin to 99 ml veronal buffer (VB, Appendix A). Stock solutions are stable for months when maintained at -20°C.

Sheep red blood cells (sRBC) were obtained by venous puncture and mixed with 3.8 percent sodium citrate, dihydrate (360 ml x 300 ml sRBC). The cells were centrifuged at low speed and supernatant fluid removed by aspiration. Cell pellets were washed three times. A 2 percent solution of sRBC in VB was prepared and stored at 4°C.

Lyophilized complement (Microbiological Associates) was restored in the restoring solution supplied on the day tests were to be performed. Restored complement (C') was prepared in chilled test tubes and kept at 4°C.

Known positive and negative adenovirus control antigens (Microbiological Associates) were thawed on the day of use and kept at 4°C. Adenovirus CF human reference antiserum (Grand Island Biological Company) was thawed on the day of use and stored at 4°C.

Hemolysin Titration

Dilutions were prepared from the stock 1:100 solution. A 0.05 ml volume was combined with 0.1 ml each of VB and a 1:30 dilution of C', and 0.05 ml of 2 percent sRBC suspension. Test mixtures were shaken gently and incubated at 37°C for 30 min. One hemolysin unit was defined as the high-

est dilution (least amount of hemolysin) giving complete lysis of sRBC (4+). Two units were used in all CF tests.

Complement Titration

Different volumes of a 1:30 C' dilution representing incremental increases of 0.025 ml, starting from an initial 0.025 ml volume and progressing up to a 0.2 ml volume were pipetted into eight Kahn serologic tubes. Each tube was adjusted to a 0.20 ml volume with VB. A 0.05 ml volume of hemolysin (2 units) and 0.05 ml of 2 percent sRBC suspension was added, the tubes shaken and incubated at 37°C for 30 min. One exact unit of C' was defined as the smallest amount giving 4+ hemolysis of sRBC. One full unit (FU) was represented by the next amount of C' (0.025 ml more) in the dilution series. Two FU of C' were used in all CF tests.

Antigen Titrations

Doubling dilutions of known positive and negative adenovirus antigen preparations (Microbiological Associates) beginning at 1:2 and extending through 1:16 were prepared. Intermediate dilutions of 1:6 and 1:10 were made also. A volume of 0.05 ml of each antigen dilution was added to a tube to which 0.05 ml of a 1:8 dilution of reference antisera and 0.1 ml of 2 FU of C' was added. Mixtures were shaken and incubated at 37°C for 30 min. Following primary incubation, 0.05 ml of 2 hemolysin units and 0.05 ml of 2 percent sRBC was added to each tube and secondary incubation carried out at 37°C for 30 min. One antigenic unit was defined as the greatest dilution (least amount of antigen)

giving complete fixation of complement (no hemolysis of sRBC). A minimum of 2 units (shown to be free from anti-complementary activity) was used in CF tests.

Antisera Titration

Doubling dilutions of human reference antiserum beginning at 1:2 and extending through 1:64 were prepared. A 0.05 ml volume was combined with an equal volume of known positive antigen (2 units) and 0.1 ml of C' (2 FU). Primary incubation at 37°C for 30 min was followed by addition of 0.05 ml volumes each of hemolysin (2 units) and sRBC suspension (2 percent). Secondary incubation was at 37°C for 30 min. One antibody unit was defined as the greatest dilution (least amount) of antiserum giving complete fixation of C' (no hemolysis). Four antibody units were used in CF tests.

Final Complement Fixation Test

Virus isolates were diluted 1:2 and 1:4 and 0.05 ml volumes of each placed in duplicate serologic tubes. A 0.05 ml volume of adenovirus reference antiserum (4 units) was added to one pair of virus dilution and an equal volume of VB (anticomplementary control) to the other pair. Complement (2 FU per 0.1 ml) was added to each tube followed by incubation at 37°C for 30 min. Controls included known positive and negative adenovirus antigens and 1:2 and 1:4 dilutions of maintenance media used for isolation of virus (antigen anticomplementary controls). Anticomplementary controls for known positive and negative adenovirus antigen were in-

cluded. Equal 0.05 ml volumes of hemolysin (2 units) and sRBC suspension (2 percent) were added, the tubes incubated at 37°C for 30 min and results of the test read. Fixation of C' by the test system (no hemolysis) indicated an isolate to be an adenovirus.

RESULTS

Adenovirus stocks were prepared from HeLa monolayers formed in Blake, Roller and Spinner bottles. Examination of stock titers varied somewhat depending on the method of propagation. Results of assays made on stocks prepared by each method are given in table 1. An increase in titer of one logarithm of roller and spinner culture prepared stocks over stationary cultures was recorded in each of four trials. The combination of greater titer and volume represented by the spinner flask procedure indicated it to be the method of choice for preparation of virus stocks.

Contemplation of attempts to demonstrate the presence of naturally occurring adenovirus in polluted surface waters made it necessary to consider methods for simultaneous separate isolation of enteric viruses with widely varying cell and plaquing requirements. For example isolation of adenovirus on monkey kidney monolayers would not prove possible due to the need for human cells by members of this group. A porcine kidney cell line, Y-15, capable of supporting adenovirus growth was examined for its ability to promote plaque formation by members of this group. Plaquing efficiency in Y-15 monolayers was compared with $TCID_{50}$ assay results using Y-15 and HeLa cultures. Results of the comparison are given in table 2.

HeLa monolayers and $TCID_{50}$ assay were most productive in promoting maximum virus recovery. Thirty-one times more

Table 1. Adenovirus titers obtained in stationary and rotated HeLa cultures

Trial	Culture method	Medium volume (ml)	Titer ($TCID_{50}$ per ml)
1	Stationary	80	2.11×10^6
2	Stationary	80	1.79×10^6
3	Stationary	80	2.59×10^6
4	Spinner flask	150	2.59×10^7
5	Spinner flask	150	2.11×10^7
6	Roller bottle	75	2.59×10^7
7	Roller bottle	75	1.79×10^7

Table 2. Adenovirus titers obtained in porcine kidney and HeLa monolayers

Host	Virus assay	
	PFU per ml	$TCID_{50}$ per ml
Porcine kidney	3×10^4	5.00×10^5
HeLa	NT	1.58×10^7

NT: Not tested

virus was produced in HeLa than Y-15 monolayers, measured by $TCID_{50}$ methods, and 16 times more virus by $TCID_{50}$ than plaquing procedures in Y-15 monolayers. The conclusion was inescapable, HeLa cells were the cells of choice for promoting maximum recovery of adenovirus, and cytopathic endpoints more effective than plaquing methods. The conclusion was not favorable to continued use of Y-15 monolayers for isolation of naturally occurring adenovirus. Use of Y-15 cells was discontinued due to their low sensitivity.

Samples had to be free from bacterial and fungal contaminants prior to inoculation onto cell culture monolayers. The success of ether treatment of water samples in removing such contaminants led to trial of ether for pre-inoculation treatment of adenovirus samples. Attention was directed toward detection of a devitalizing action. The results of 18 hour ether treatment are given in table 3. Little or no loss of infectiousness occurred, indicating ether could be used to "sterilize" adenovirus-containing samples prior to inoculation of cell culture monolayers. The data confirms studies by Huebner et al. (1954) indicating the ether resistance nature of adenovirus.

Adenovirus Stability to pH Change

Adsorption of adenovirus to textile and membrane filter surfaces depends upon electrostatic attraction of viruses to virus adsorption sites. Elution of viruses from these sites results from the reversal of the electrostatic attraction. In this regard, the pH of the aqueous suspension

Table 3. The effect of ether upon adenovirus infectivity

Trial	Sample volume (ml)	Virus assay ($TCID_{50}$ per ml) Before etherization	Virus assay ($TCID_{50}$ per ml) After etherization
1	5	1.39×10^2	1.58×10^2
2	5	9.65×10^2	7.30×10^2
3	5	2.11×10^7	2.11×10^7
4	50	5.00×10^3	3.40×10^3
5	50	5.00×10^3	5.00×10^3

Table 4. Devitalization of adenovirus 5 at pH 3.5 in 0.05 M glycine

Contact time (min)	Virus assay ($TCID_{50}$ per ml)	Virus inactivation (percent)
0	2590	--
2	1800	30
6	1800	30
10	1800	30
30	1800	30
60	1800	30
90	1800	30
120	1800	30
180	1800	30

plays a major role in determining the relative strength of the attraction, and the avidity of adsorption. In order to optimize the effectiveness of this phenomenon the effect of pH upon adenovirus stability had to be determined.

Table 4 presents data on the devitalization of adenovirus at pH 3.5. There was an initial loss of 30 percent viability due to the effect of introducing 0.5 ml of virus into 45 ml of 0.05 M glycine adjusted to pH 3.5. After this initial inactivation adenovirus remained stable at this pH through 180 min.

In order to determine the optimum pH of adenovirus contaminated water which would favor adsorption of virus to filters, the effect of acidification of the water sample had to be examined. Either JEL tap water or GB estuarine water was acidified to the desired pH with 0.1 N HCl. The results of the effect of this acid adjustment are exhibited in table 5. Adenovirus was inactivated to an appreciable extent after pH adjustment to pH 3.5 was realized, to a lesser extent after pH adjustment to pH 4.7, and not at all after pH adjustment to pH 6.0. The data indicate that adenovirus adsorption to filters at pH 6.0 would avert the deleterious effects of acidification.

Virus was exposed to selected pH values for 2 and 5 min to determine the stability of adenovirus in various alkaline solutions of 0.05 M glycine. Samples were subsequently neutralized and assayed. The results of this examination are presented in table 6. Adenovirus remained stable at pH 10.0 after 5 min contact; however, after only 2 min

Table 5. Adjustment of adenovirus 5 seeded water to various pH values with 0.1N HCl[1]

Trial	Water	pH	Virus assay ($TCID_{50}$ per ml) Before acidification	After acidification	Virus inactivation (percent)
1	JEL	3.5	1120	500	55
2	JEL	3.5	259	158	39
3	JEL	4.7	2110	1340	37
4	JEL	4.7	259	179	31
5	JEL	6.0	2590	2590	0
6	JEL	6.0	397	397	0
7	GB	6.0	2590	2590	0
8	GB	6.0	1100	1340	0
9	GB	6.0	965	1130	0

1. pH adjustment made at constant flow rate and with constant stirring.

Table 6. Inactivation of adenovirus 5 at various alkaline pH values after 2 minutes and 5 minutes

Contact time (min)	pH	Virus assay ($TCID_{50}$ per ml) Control	Test	Virus inactivation (percent)
2	8.5	259	259	0
	9.0		259	0
	9.5		259	0
	10.0		259	0
	10.5		81	69
	11.0		32	88
5	8.5	259	259	0
	9.0		259	0
	9.5		259	0
	10.0		259	0
	10.5		31	88
	11.0		21	92

contact at pH 10.5 there was a 69 percent loss of viability and after 5 min at pH 10.5, 88 percent was lost. Devitalization at pH 11.0 was more severe resulting in an 88 percent loss of virus after 2 min and 92 percent lost after 5 min.

The amount of time required to inactivate adenovirus at pH 10.0 and 10.5 was studied by introducing a small volume of a virus suspension into a large volume of 0.05 M glycine adjusted to the desired pH value. Tables 7 and 8 indicate the percent devitalization of adenovirus after the specified contact time the virus remained at the pH under test. Adenovirus remained stable at pH 10.0 for 6 min after which there was a gradual decrease of viability. However, at pH 10.5 adenovirus devitalization was extensive after 3 min of contact time and almost complete after 10 min.

The effect of alkaline pH adjustment on the viability of adenovirus under conditions of high virus input multiplicities was examined by slowly adding 0.1 N NaOH to 1 liter of 3 percent beef extract containing virus. The results are presented in table 9. No loss of virus activity was observed at any of the selected pH values. In addition, virus remained stable at pH 9.0 for 40 min.

Virus Concentration

Clarification

Before water suspected of containing adenovirus could be processed through adsorbent filters, suspended material greater than 1 um in size had to be removed by a clarification process which would not concomitantly remove virus. The pH

Table 7. Devitalization of adenovirus 5 at pH 10.0 in 0.05 M glycine

Contact time (min)	Virus assay ($TCID_{50}$ per ml)	Virus inactivation (percent)
0	1120	--
2	1120	0
4	1120	0
6	1120	0
8	870	22
10	870	22
15	340	70
30	305	73
45	260	77

Table 8. Devitalization of adenovirus 5 at pH 10.5 in 0.05 M glycine

Contact time (min)	Virus assay ($TCID_{50}$ per ml)	Virus inactivation (percent)
0	2120	--
3	670	68
6	159	93
8	26	99
10	0	100
15	0	100
30	0	100
45	0	100

Table 9. Effect of pH adjustment with 0.1 N NaOH on adenovirus 5 viability in 3 percent beef extract

Trial	pH	Contact time (min)	Virus assay ($TCID_{50}$ per ml) Before adjustment	After adjustment	Virus inactivation (percent)
1	7.0	--	25,900	--	--
2	8.0	5		25,900	0
3	9.0	5		25,900	0
4	9.0	40		25,900	0

Table 10. Effect of pH on adenovirus 5 adsorption to clarifying filters[1]

Trial	pH	Volume (gal)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus adsorption (percent)
1	7.4	1	870	870	0
2	7.8	1	870	870	0
3	6.5	5	650	0	100
4	7.5	5	211	211	0

1. Untreated 019, 039, Tw80-W10A.

of raw waters effected successful clarifications. An examination of table 10 suggests that when the pH of JEL tap water containing virus was adjusted to pH 6.0 and filtered, all virus was lost to the clarifying filters (untreated 019, 039, Tw80-W10A). Conversely, when the pH was adjusted to a value greater than 7.4, all virus penetrated the same clarifying filters. This observation held true for 1 gal samples and 5 gal samples.

The effect of salinity also affected the success of clarification. Five gallons of artificial sea water were prepared at various salinities and adjusted to pH 7.7. After the addition of virus the samples were passed through similar clarifying filters. The results of the experiment are illustrated in table 11. Even at a pH value previously shown (table 10) to promote virus penetration of the clarifying filters, salinities as low as 10 ppt resulted in considerable retention of virus. At salinities of 30 and 39 ppt, adenovirus was removed almost totally from suspension.

To identify filters responsible for the retention of adenovirus suspended in artificial sea water, orlon and cellulose acetate filters were examined independently. Artificial sea water (29 ppt) adjusted to pH 7.8 and containing virus was filtered through the various filters and assayed at 5 gal increments as table 12 indicates. The data reveal that untreated orlon filters were responsible for removal of adenovirus. As more water passed through these filters more virus was retained by them, whereas virus freely penetrated the cellulose acetate filter.

Table 11. Retention of adenovirus 5 by clarifying filters[1] following passage of 5 gallon samples of artificial sea water of varying salinities[2]

Trial	Salinity (ppt)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus retention (percent)
1	10	2800	500	82
2	21	1800	500	72
3	30	7400	120	98
4	39	1100	21	98

1. Untreated 019, 039, Tw80-W10A.

2. Sample adjusted to pH 7.7 before filtration.

Table 12. Retention of adenovirus 5 by orlon and cellulose acetate textile filters during passage of 25 gallon samples of artificial sea water[1]

Filter	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus retention (percent)
Orlon[2]	0	260		
	5		95	64
	10		50	81
	15		18	93
	20		0	100
	25		0	100
Cellulose acetate[3]	0	340		
	5		340	0
	10		340	0
	15		340	0
	20		340	0
	25		340	0

1. Salinity 29 ppt; pH 7.8.

2. Untreated 019 and 039 filters of 10 and 1um porosities respectively washed with filtered tap water.

3. Tw80-W10A filter of 1um porosity washed with filtered tap water.

To preserve the clarifying function of the orlon filters, they were pretreated to render them no longer capable of removing virus. In this regard, the orlon filters were presoaked in 0.1 percent Tw80 or 3 percent beef extract (pH 9.0) overnight and washed with filtered tap water before use. The data in table 13 clearly indicate that both kinds of pretreatment altered the properties of the orlon filters such that they no longer removed virus.

Table 14 compares the clarification of adenovirus seeded GB estuarine water through treated and untreated clarifying filters. The data demonstrate that GB estuarine water was no different than artificial sea water in the need to pretreat clarifying filters. Only after pretreatment did adenovirus completely penetrate the filters.

The effect of turbidity influencing adenovirus penetration of treated clarifying filters was studied by adding 300 mg of GB sediment per liter of GB estuarine water (salinity 23 ppt; pH 7.5). After adding virus and mixing well the 25 gal sample was pumped through the clarifying filters. The virus assay of before and after filtration remained unchanged. It was thus apparent that virus found in extremely turbid water can be successfully clarified without retention onto filters or entrapment by suspended material.

Virus Adsorption

The ability of the Cox membrane filters to adsorb adenovirus was examined by preparing two 5 gal samples of artificial sea water (salinity 20 ppt). One 5 gal sample

Table 13. Retention of adenovirus 5 by pretreated orlon filters during passage of 25 gallon samples of artificial sea water[1]

Pretreatment[2]	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus retention (percent)
3 percent beef extract	0	180		
	5		180	0
	10		180	0
	15		180	0
	20		180	0
	25		180	0
0.1 percent Tween 80	0	340		
	5		340	0
	10		340	0
	15		340	0
	20		340	0
	25		340	0

1. Salinity 29 ppt; pH 7.8.

2. 019 and 039 orlon filters, 10 um and 1 um respectively, pretreated as indicated overnight at 4°C and washed for 15 minutes in filtered tap water before use.

Table 14. Retention of adenovirus 5 by treated and untreated textile filters during passage of 25 gallon samples of Great Bay estuarine water

Filters	Salinity (ppt)	pH	Sample volume	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus retention (percent)
A[1]	11	7.8	0	615		
			5		0	100
			10		32	95
			15		0	100
			20		26	97
			25		50	92
B[2]	19	7.6	0	965		
			5		965	0
			10		965	0
			15		965	0
			20		965	0
			25		965	0

1. Untreated 019 and 039 washed filters followed by a Tw80-W10A.

2. Three percent beef extract, pH 9.0, pretreated 019 and 039 filters followed by a Tw80-W10A.

was adjusted to pH 3.5, the other to pH 6.0 and after adding $MgCl_2$ (0.05 M), virus and thorough mixing, the virus-sea water suspension was passed through 127 mm Cox filters of 0.45 um and 2.0 um porosities. Table 15 shows that complete adsorption of adenovirus onto 127 mm membrane filters was accomplished at both pH 3.5 and 6.0.

Jackson Estuarine Laboratory tap water and GB estuarine water were examined for the effect of pH on adenovirus adsorption to adsorbent filters. The adsorptive capabilities of the adsorbent filters were unaffected by the type of water examined. Moreover, efficient adsorption occurred for each pH value studied (table 16).

In addition to pH, the use of a metal cation (Mg^{2+}) was an important parameter in enhancing the adsorption of virus to adsorbent filters. Various concentrations of $MgCl_2$ were added to 25 gal of JEL tap water adjusted to pH 6.0. After the addition of virus and thorough mixing, the water was passed through a K-27 fiberglass textile filter followed by a 267 mm Cox filter assembly containing a 0.45 um and a 2.0 um filter. The filtrate of each adsorbent filter was assayed after various volumes had been filtered. The results are expressed in tables 17a and 17b. As the molarity of $MgCl_2$ was increased to 0.05, adenovirus adsorption to the fiberglass filter was enhanced. At 0.05 M all virus exogenously added to 25 gal of JEL tap water had been adsorbed onto the K-27 filter. In addition, the adsorption of adenovirus to Cox filters was unaffected by changes in salt concentrations.

Table 15. Effect of pH on adenovirus 5 adsorption to Cox membrane filters during passage of 5 gallons of artificial sea water[1]

pH	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus adsorption (percent)
6.0	730	0	100
3.5	500	0	100

1. Salinity 20 ppt.

Table 16. Adsorption of adenovirus 5 to adsorbent filters[1] at various pH values during passage of 10 gallons of water containing 0.05 M $MgCl_2$

Trial	Water	pH	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus adsorption (percent)
1	JEL[2]	3.5	500	0	100
2	JEL	3.5	158	0	100
3	JEL	4.7	1340	0	100
4	JEL	4.7	179	0	100
5	JEL	6.0	2590	0	100
6	JEL	6.0	397	0	100
7	GB[3]	6.0	2590	0	100
8	GB	6.0	1340	0	100
9	GB	6.0	1130	0	100

1. Fiberglass textile filter (K-27) followed by 267 mm Cox filters of 0.45 um and 2.0 um porosities.

2. Jackson Estuarine Laboratory.

3. Great Bay.

Table 17a. Adsorption of adenovirus 5 to adsorbent filters[1] at various molarities of $MgCl_2$ during passage of 25 gallons of Jackson Estuarine Laboratory tap water adjusted to pH 6.0

$MgCl_2$ (M)	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml)			Virus adsorption (percent)	
		Before filtration	K-27 filtrate	Cox filtrate	K-27 filtrate	Cox filtrate
0	0	116				
	5		0	0	100	100
	10		21	0	82	100
	15		78	0	33	100
	20		116	0	0	100
	25		116	0	0	100
0.005	0	119				
	5		0	0	100	100
	10		18	0	85	100
	15		0	0	100	100
	20		26	0	78	100
	25		119	0	0	100

1. Fiberglass textile filter (K-27) followed by 26[7] mm Cox filters of 0.45 um and 2.0 um porosities.

Table 17b. Adsorption of adenovirus 5 to adsorbent filters[1] at various molarities of $MgCl_2$ during passage of 25 gallons of Jackson Estuarine Laboratory tap water adjusted to pH 6.0

$MgCl_2$ (M)	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml)			Virus adsorption (percent)	
		Before filtration	K-27 filtrate	Cox filtrate	K-27 filtrate	Cox filtrate
0.01	0	134				
	5		0	0	100	100
	10		0	0	100	100
	15		0	0	100	100
	20		0	0	100	100
	25		21	0	84	100
0.05	0	211				
	5		0	0	100	100
	10		0	0	100	100
	15		0	0	100	100
	20		0	0	100	100
	25		0	0	100	100

1. Fiberglass textile filter (K-27) followed by 267 mm Cox filters of 0.45 and 2.0 um porosities.

The effect of turbidity on adenovirus adsorption to adsorbent filters was examined by adding GB sediment to 25 gal of GB water to a final concentration of 300 mg per liter. After clarification, pH acidification to 6.0, and $MgCl_2$ adjustment to 0.05 M, virus was added and passed through adsorbent filters. The filtrates of each filter were examined for presence of virus after 5 gal increments. The only virus detected was after 25 gal had passed through the K-27 filter. This penetration accounted for 13 percent of the input virus concentration. There was, however, no penetration of the Cox filters through the entire 25 gal. The results indicated that virus could be successfully adsorbed from extremely turbid water (after clarification) under conditions of pH 6.0 and 0.05 M $MgCl_2$.

Various textile filters were examined for their ability to adsorb adenovirus from GB estuarine water (tables 18 - 20). Twenty-five gallons of water were clarified through treated filters and the filtrate adjusted to pH 6.0 and 0.05 M $MgCl_2$. After the addition of virus and thorough mixing, the virus-water suspension was passed through various textile filters followed by a 267 mm Cox filter assembly. After 5 gal increments the filtrates were assayed for the presence of virus. All textile filters examined adsorbed virus to varying degrees; however, the K-27 fiberglass filter adsorbed virus best. The viral adsorptive capacities of the Cox membrane filters following the various textile filters were also examined (tables 18 - 20). The virus adsorptive integrity of the Cox membrane filters was preserved

Table 18. Adsorption of adenovirus 5 to fiberglass textile filters during passage of 25 gallons of Great Bay estuarine water[1]

Filter	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml)			Virus adsorption (percent)	
		Before filtration	Textile filtrate	Cox filtrate	Textile filtrate	Cox filtrate
Fiberglass[2]	0	1130				
	5		0	0	100	100
	10		413	0	63	100
	15		243	0	78	100
	20		212	0	81	100
	25		77	0	93	100

1. Salinity 15 ppt; pH 6.0; 0.05 M $MgCl_2$.

2. K-27 filter of 1 - 8 um porosity.

Table 19. Adsorption of adenovirus 5 to orlon textile filters during passage of 25 gallons of Great Bay estuarine water[1]

Filter	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml)			Virus adsorption (percent)	
		Before filtration	Textile filtrate	Cox filtrate	Textile filtrate	Cox filtrate
Orlon[2]	0	2590				
	5		1560	211	40	92
	10		1360	157	47	94
	15		500	152	81	94
	20		131	157	95	94
	25		187	259	93	90

1. Salinity 15 ppt; pH 6.0; 0.05 M $MgCl_2$.

2. 039 filter of 1 um porosity washed with filtered tap water.

Table 20. Adsorption of adenovirus 5 to cellulose acetate textile filters during passage of 25 gallons of Great Bay estuarine water[1]

Filter	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml)			Virus adsorption (percent)	
		Before filtration	Textile filtrate	Cox filtrate	Textile filtrate	Cox filtrate
Cellulose acetate[2]	0	1340				
	5		1340	18	0	99
	10		965	18	28	99
	15		765	21	43	98
	20		500	87	63	93
	25		211	211	84	84

1. Salinity 15 ppt; pH 6.0; 0.05 M $MgCl_2$.

2. W10A filter of 1 um porosity washed with filtered tap water.

only when the Cox filters were preceded by a fiberglass textile filter.

The influence of membrane coating components (MCC) on the virus adsorptive capacity of fiberglass textile filters was examined by challenging untreated and treated filters (table 21) with 1 gal samples of JEL tap water (pH 6.0; 0.05 M $MgCl_2$) containing virus. The water before and after filtration was assayed for virus. All filters studied remained capable of adsorbing 100 percent of the challenge virus. Table 22 presents data on the ability of various sizes of Cox membrane filters to remove virus. In every case virus was not detected in the filtrates. Thus, the smallest filter studied (47 mm) was capable of adsorbing 100 percent of adenovirus added to 6 liters of GB estuarine water.

The conditions described for the adsorption of adenovirus to adsorbent filters (pH 6.0; 0.05 M $MgCl_2$) were tested for the adsorption of poliovirus 2 from various types of water. Table 23 demonstrates that poliovirus 2 adsorbs efficiently to a fiberglass filter after virus suspended in 25 gal of JEL tap water had been filtered. However, poliovirus 2 adsorption to adsorbent filters after passage of virus suspended in 25 gal of GB water was much less efficient. The lowered poliovirus adsorption from GB water as compared to JEL tap water demonstrated MCC interference.

Virus Elution

Virus adsorbed to adsorbent filters had to be recovered in a small volume of eluate to achieve concentration.

Table 21. Influence of membrane coating components on the virus adsorptive capacity of fiberglass textile filters

Filter	Water	Sample volume (gal)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus adsorption (percent)
Fiberglass[1]	JEL[2] tap water	1	890	0	100
Fiberglass[3]	JEL tap water	1	890	0	100
Fiberglass[4]	JEL tap water	1	890	0	100

1. K-27 filter pretreated with 50 gallons of Great Bay water (pH 6.0; 0.05 M $MgCl_2$).

2. Jackson Estuarine Laboratory.

3. K-27 filter pretreated with 50 gallons of 039 clarified Great Bay water (pH 6.0; 0.05 M $MgCl_2$).

4. K-27 untreated.

Table 22. Influence of membrane coating components on the virus adsorptive capacity of Cox membrane filters[1]

Trial	Filter diameter (mm)	Sample volume[2] (ml)	Virus assay ($TCID_{50}$ per ml) Before filtration	After filtration	Virus adsorption (percent)
1	127	0	1120		
		125		0	100
		250		0	100
		500		0	100
		1000		0	100
		2000		0	100
		4000		0	100
2	127	0	2230		
		4000		0	100
		6000		0	100
		8000		0	100
		10,000		0	100
		12,000		0	100
		14,000		0	100
		18,000		0	100
3	47	0	3450		
		200		0	100
		375		0	100
		750		0	100
		1500		0	100
		3000		0	100
		5000		0	100
		6000		0	100

1. Cox filter assemblies consist of a 0.45 um filter preceded by a 2.0 um filter.

2. Great Bay estuarine water (salinity 11 ppt; pH 6.0; 0.05 M $MgCl_2$).

Table 23. Adsorption of poliovirus 2 to adsorbent filters[1] during passage of 25 gallons of sample water

Water[2]	Sample volume (gal)	Virus assay (PFU per ml)			Virus adsorption (percent)	
		Before filtration	K-27 filtrate	Cox filtrate	K-27 filtrate	Cox filtrate
Jackson Estuarine Laboratory tap	0	190				
	5		1	0	99	100
	10		2	0	99	100
	15		1	0	99	100
	20		2	0	100	100
	25		1	0	99	100
Great Bay estuarine	0	450				
	5		100	0	78	100
	10		170	0	62	100
	15		80	0	82	100
	20		170	10	62	98
	25		170	50	62	89

1. Fiberglass textile filter of 1 - 8 um porosity followed by 267 mm Cox filters of 0.45 um and 2.0 porosities.

2. pH 6.0; 0.05 M $MgCl_2$.

Various eluents were examined for their ability to recover virus (table 24). Neither 0.05 M glycine nor 1.0 M glycine at pH 9.5 were able to elute virus. The use of 3 percent beef extract at pH 9.0, however, eluted virus completely resulting in recoveries of greater than 100 percent in three separate trials. To test the efficiency of 3 percent beef extract at pH 9.0 on the elution of poliovirus 2 from adsorbent filters, virus was added to 10 gal of JEL tap water (pH 6.0; 0.05 M $MgCl_2$) and after adsorption the filters were subjected to elution. The recovery efficiency of poliovirus 2 was 100 percent. Thus, both adenovirus and poliovirus 2 could be efficiently eluted with 3 percent beef extract.

Virus Reconcentration

The 3 percent beef extract eluate had to be reconcentrated to a smaller volume when low multiplicites of virus were expected in the eluate. Two different aqueous two phase polymer separation techniques were examined for their ability to concentrate adenovirus. The relative efficiencies of the two systems studied is presented in table 25. Both two phase systems concentrate adenovirus; however, system B achieves a greater virus recovery than system A. Table 26 presents data on the comparative ability of system B to concentrate adenovirus and poliovirus 2. System B is only able to recover 3 percent of the input poliovirus; whereas, 48 percent of the input adenovirus was recovered.

Sensitivity of Virus Concentration

Twenty-five gallons of JEL tap water adjusted to pH 6.0 and 0.05 M $MgCl_2$ were seeded with 976 PFU of adeno-

Table 24. Recovery of adenovirus 5 from adsorbent filters[1] with various eluents

Trial	Eluent	Virus assay ($TCID_{50}$ per ml)		Eluate volume (ml)	Virus recovery	
		Before filtration[2]	After elution x 10^4		Total $TCID_{50}$ x 10^7	Percent
1	A[3]	2590	0	3300	0	0
2	A	397	0	2985	0	0
3	A	50	0	3300	0	0
4	B[4]	220	0	5200	0	0
5	C[5]	158	2.11	2600	5.46	>100
6	C	243	2.22	1150	2.55	>100
7	C	500	7.30	1200	8.76	>100

1. Fiberglass textile filter (K-27) followed by 267 mm Cox filters of 0.45 um and 2.0 um porosities.

2. Twenty-five gallons of Jackson Estuarine Laboratory tap water (pH 6.0; 0.05 M $MgCl_2$).

3. 0.05 M glycine; pH 9.5.

4. 1.0 M glycine; pH 9.5.

5. Three percent beef extract; pH 9.0.

Table 25. Reconcentration of adenovirus 5 by aqueous polymer two phase separation systems in 3 percent beef extract[1]

System[2]	Trial	Virus assay ($TCID_{50}$ per ml)		Virus recovery	
		Before reconcentration	After reconcentration	Total $TCID_{50}$	Percent
A	1	119	2.59×10^4	4.14×10^4	70
	2	9650	3.75×10^6	3.75×10^6	78
B	1	9650	1.81×10^6	5.07×10^6	>100
	2	9650	2.11×10^6	4.85×10^6	>100

1. 500 ml; pH 7.2.

2. A: NaDxS-PEG4000-NaCl
   B: Dx-MC-PEG6000

Table 26. Reconcentration of adenovirus 5 and poliovirus 2 by system B aqueous polymer two phase separation in 3 percent beef extract[1]

	Virus assay		Virus recovery	
Virus	Before reconcentration	After reconcentration	Total	Percent
Adeno 5	286 $TCID_{50}$ per ml	1.36×10^4 $TCID_{50}$ per ml	6.8×10^4 $TCID_{50}$	48[2]
Polio 2	1100 PFU per ml	3800 PFU per ml	1.9×10^4 PFU	3

1. 500 ml; pH 7.2.

2. Antipolio type 2 rabbit hyperimmune sera was added to each serial log dilution at a concentration of 40 antibody units per 0.2 ml.

virus assayed in HEK monolayers. After the virus-water suspension was passed through adsorbent filters, the filters were eluted with 3 percent beef extract. The eluate was reconcentrated by system B and the reconcentrate assayed for virus in HEK monolayers. Ninety-four percent of the adenovirus added to 25 gal was recovered in the reconcentrate. Low input multiplicities of 0.015 PFU of adenovirus per milliliter could be successfully detected and recovered from water. Cytotoxicity to HeLa cell monolayers was observed upon assay for adenovirus $TCID_{50}$ in MicroTest II plates. This cytotoxicity perhaps was due to the high concentration of dextran found in the bottom phase of system B.

Natural Virus Isolations

Adenovirus concentration methodology was employed in the examination for the presence of adenovirus from different sources of raw water. One-hundred and sixteen plaques were isolated on HEK cell monolayers from the examination of 50 gal of Otis Air Force Base sewage effluent. Each plaque was picked and inoculated into stationary tubes of HEK cell monolayers. After complete CPE the isolates were subjected to CF tests. The negative control antigen, the maintenance media control, and anticomplementary controls resulted in complete hemolysis of sRBC. The positive antigen control and 106 of the 116 natural virus isolates did not result in any hemolysis of sRBC. Thus, these 106 isolates were confirmed as belonging to the adenovirus group.

An examination of 50 gal of GB estuarine water and 50 gal of Cocheco River water did not yield any virus plaques

on HEK cell monolayers. The examination of 50 gal of Durham, New Hampshire, raw sewage resulted in the complete destruction of HEK cell monolayers with large enteric plaques thereby masking adenovirus plaques if present.

DISCUSSION

To evaluate the potential public health hazard of adenovirus in water, methods had to be developed for the quantitative enumeration of small numbers of virus particles in large volumes of water. Various investigators have reported that viruses suspended in aqueous solutions could be concentrated on membrane and textile filter surfaces in the presence of metallic cations (Wallis and Melnick, 1967a; Rao and Labzoffsky, 1969; Moore et al., 1970; Hill et al., 1971; Sobsey et al., 1973). However, these studies were predicated on isolation and detection of enteroviruses. There is very little information concerning the relative contamination of water with adenovirus. This study represents an effort to adopt virus adsorption technology to the recovery of adenovirus from raw waters.

The microtiter assay system used for determining $TCID_{50}$ for adenovirus was simple, reliable, and economical, but not as sensitive as alternative methods. Adenovirus concentrations had to be greater than 17 $TCID_{50}$ per ml if virus was to be detected. Thus, when working with large volumes of adenovirus seeded water, enough virus had to be added to exceed the sensitivity level of the assay system. Use of roller bottle and Spinner culture propagation of adenovirus yielded high titers of stock virus such that large volume experiments could be examined. In addition, adenovirus resistance to ether was used to free samples of contaminating biotic forms of life before assay in cell cultures.

It was hoped that the established porcine kidney cell line designated Y-15 could be used in the primary isolation of natural adenovirus by a plaquing method. However, because of the low sensitivity of this cell line as determined in a comparison with HeLa cells, this idea was abandoned.

Virus adsorption phenomena were dependent on various parameters including the pH of the aqueous media, selection and concentration of a salt for enhancement of virus adsorption, porosity of adsorbent filters, chemical nature of adsorbent filters, flow rates, and type of water used for virus examination. These variables had to be known for successful concentration of viruses.

It has been reported (Wallis and Melnick, 1967a and 1967b) that virus adsorption to membrane surfaces was related to the pH of the water. At low pH levels viruses adsorbed to membranes more efficiently when organic compounds were present. These results were attributed to the fact that in the presence of excess hydrogen ions, interfering organic compounds (MCC) were not able to compete for membrane surface sites required by the virus. Alkaline eluents have been used successfully to recover adsorbed virus. Various pH devitalization experiments were performed to establish the lowest pH value permissible for adsorption and the highest value for elution. Although adenovirus remained stable at pH 3.5 for long periods of time, acidification of water samples from ambient pH to a pH of 3.5 resulted in up to 55 percent devitalization of virus. Acidification was achieved by adding 0.1 N HCl by a pump which delivered acid at a

constant rate to rapidly mixing water. Use of 0.1 N acid represented a reasonable compromise between the volume of acid needed and the time required to achieve the desired pH value. Nevertheless, acidification to pH 3.5 or 4.7 with 0.1 N HCl resulted in an unacceptable level of inactivation. Acidification to pH 6.0, however, showed no loss of virus. Relatively high multiplicities of virus were used in every pH stability experiment. If, however, the volume of water a virion occupied was large, as would be the case when low virus input multiplicities were examined, then perhaps the results of acidification would not be as pronounced. By the time the acid reached the virus particle it would be sufficiently diluted and no longer injurious. Acidifications to pH 6.0 posed no problems and consequently virus adsorption experiments were performed at this pH value.

The use of alkaline eluents to recover virus adsorbed to filters is well documented (Hill et al., 1971). Elution was achieved by a reversal of electrostatic attraction. Wallis et al. (1972b) used 0.05 M glycine at pH 11.5 as an eluent without detectable loss of infectivity. Other eluents such as beef extract, nutrient broth or serum exchanged for adsorbed virus. The combinations of proteinaceous solutions and high pH to elute virus have been used successfully by a number of investigators (Berg et al., 1971; Hill et al., 1972). To determine the highest pH that would effectively elute virus yet not result in loss of infectivity, pH devitalization studies at alkaline values were performed. Adenovirus was rapidly inactivated at pH 10.5,

whereas at pH 10.0 infectivity was maintained for at least 6 min. Elution with 3 percent beef extract, therefore, was conducted at a pH value of less than 10.

The function of clarification was to remove suspended material greater than 1 um without concomitant removal of virus. Orlon and cellulose acetate textile filters were used to achieve this function. These filters were examined for their virus retentive capacities under varying environmental conditions. It was observed that adenovirus was retained on clarifying filters when pH was adjusted to 6.5; however, at pH values of 7.4 or greater all the virus penetrated the filters and appeared in the filtrate. The retention by clarifying filters of adenovirus suspended in artificial sea water adjusted to various salinities was examined. Virus was retained to a very considerable degree despite pH adjustments to values exceeding 7.4. Each filter was examined to determine which was responsible for the retention of virus. The passage of adenovirus seeded artificial sea water through untreated orlon filters resulted in significant virus retention. Cellulose acetate filters pretreated with 0.1 percent Tw80 demonstrated complete virus penetration. Thus in order to preserve the clarifying function of the orlon filters, they must be pretreated in a manner which would eliminate their viral retentive capacities. Pretreatment with 0.1 percent Tw80 or pretreatment with 3 percent beef extract proved to be effective means of superposing viral retentive sites.

In addition to the effects of pH and salinity on successful clarification, the turbidity of the sample was

an important environmental parameter. Suspended material greater than 1 um in size were retained by the filters, and this material potentiably may entrap virus particles. However, virus added to heavily turbid GB water was successfully clarified with all virus found in the filtrate.

The adsorption of virus to adsorbent filters was related to pH (Wallis and Melnick, 1967a and 1967b). However, due to the significant devitalization of adenovirus during acidification from ambient pH to either 4.7 or 3.5, adsorption of adenovirus was carried out at pH 6.0. Adsorption to adsorbent filters, however, occurs equally efficiently at pH 3.5 and at pH 6.0.

In addition to the effect of pH on adsorption, the introduction of salt to water resulted in an enhancement of the adsorption phenomena. Adsorption to fiberglass textile filters (porosity 1 - 8 um) increased with increasing molarities of $MgCl_2$. In the absence of salt, adenovirus suspended in JEL tap water adjusted to pH 6.0 was completely adsorbed to Cox membrane filters. Thus, the combination of pH 6.0 and the smaller porosity of the Cox membrane filters (0.45 um) were sufficient to affect adsorption.

Membrane coating components found in natural waters are probably organic in nature. Various resins have been used to successfully remove these factors from water (Wallis and Melnick, 1967a). Other investigators have been successful in overcoming the deleterious effects of MCC on virus adsorption by acidification of the water sample to pH 3.5 and addition of $AlCl_3$ to a final concentration of 0.0005 M

(Sobsey et al., 1973; Homma et al., 1973). The effects of MCC interference on adenovirus adsorption to textile and membrane filters, therefore, represented an important environmental parameter. After pretreating a fiberglass textile filter with 50 gal of clarified or unclarified GB water and challenging that filter with 1 gal of water containing virus, it was determined that the adsorptive capabilities of filters remained unaltered. Additionally, small diameter membrane filters (47 mm) retained all exogenously added adenovirus suspended in 6 liters of GB water adjusted to pH 6.0 and 0.05 M $MgCl_2$. Adenovirus adsorption to adsorbent filters was not influenced by the presence of MCC in GB estuarine water. Perhaps the avidity of adsorption was sufficiently strong as to contradict MCC interference. Increased adsorptive avidity might be a function of the size of adenovirus being 3 times larger in diameter than enterovirus.

An examination of various textile filters for virus adsorptive capabilities demonstrated that fiberglass filters (K-27) were a better adsorbent than either orlon (039) or cellulose acetate filters (W10A). Moreover, the K-27 filter preceding a Cox filter assembly maintained the virus adsortive integrity of the Cox membrane filters. Both the orlon and cellulose acetate filtrates contained factors, perhaps MCC, which interfered with virus adsorption to a membrane filter; however, the K-27 filtrate was free from these factors as evidenced by complete adenovirus adsorption to the following membrane filter.

An examination of adsorption of poliovirus 2 to adsorbent filters during passage of 25 gal of sample water adjusted to pH 6.0 and 0.05 M $MgCl_2$ revealed that adsorption was much less efficient with GB estuarine water than with JEL tap water. This decreased adsorption probably resulted from MCC interference. At a pH value of 6.0, perhaps, the electrostatic attraction of poliovirus 2 to adsorbent filters was less than at lowered pH values resulting in a weak attraction easily broken. This diminished adsorption of poliovirus 2 under the conditions described for adenovirus adsorption resulted in a system which was discriminatory against enteroviruses. The discriminating nature of the adenovirus concentration system would preclude the use of enterovirus antisera. The presence of enterovirus would mask the detection of adenovirus in HEK cell monolayers. This became extremely important when isolating virus from raw waters contaminated with both entero and adenovirus types.

The use of 0.05 M glycine adjusted to pH 9.5 was unsuccessful in reversing the electrostatic attractive forces of virus to adsorbent filters. The use of 3 percent beef extract at pH 9.0 provided an eluent which would exchange for the virus at the virus adsorptive site as well as reverse electrostatic attractive forces. Recovery efficiencies of greater than 100 percent might be attributable to disassociation of virus aggregates.

The selection of an eluent was based not only on its efficiency to recover virus but also on whether it would lend itself to reconcentration methods. Adenovirus in 3 percent

beef extract eluates was successfully reconcentrated by aqueous polymer two phase separation techniques. Of the two systems studied, system B provided a greater degree of adenovirus concentration. In addition, system B did not significantly reconcentrate poliovirus 2. Thus, if enteroviruses were adsorbed to adsorbent filters and eluted with 3 percent beef extract, they would not be appreciably reconcentrated by system B.

In conclusion, environmental and virologic parameters of adenovirus adsorption to textile and membrane filters were studied. Small numbers of virus particles in large volumes of water could be recovered by (1) clarifying at a pH value greater than 7.4 through a series of pretreated textile filters, (2) adsorbing virus to adsorbent filters by acidifying the sample to pH 6.0 and adding $MgCl_2$ to a final concentration of 0.05 M, (3) eluting adsorbed virus from filters with 3 percent beef extract at pH 9.0, and (4) reconcentrating eluate by aqueous polymer two phase separation methods. These procedures were applied to 50 gal of trickling filter sewage effluent. From this field study 106 adenovirus isolates were obtained. The application of the methods described in this study will allow quantitative enumeration of adenovirus from water and waterways for the first time.

APPENDIX A

1. Earle's Balanced Salt Solution (EBSS), 10X Concentrated

Solution A	1 liter
NaCl	68 gm
KCl	4 gm
$MgSO_4 \cdot 7H_2O$	2 gm
$NaH_2PO_4 \cdot H_2O$	1.4 gm
Glucose	10 gm
Distilled water	800 ml

Solution B	
$CaCl_2$ (anhydrous)	2 gm
Distilled water	200 ml

Dissolve each salt in the order listed and autoclave solutions A and B separately at 15 psi for 15 min. When cool slowly stir solution B into solution A. Dispense in 100 ml aliquots and stopper tightly. Working solution is 1X concentration.

2. Versene Buffer, 1X Concentrated

Formula	1 liter
NaCl	8.0 gm
KCl	0.2 gm
KH_2PO_4	0.2 gm
$Na_2HPO_4 \cdot 7H_2O$	2.16 gm
Distilled water	1000 ml

Dissolve each salt in the order listed and filter sterilize through a 0.45 u porosity membrane filter.

3. Trypsin 0.25 Percent

Formula	1 liter
Difco trypsin (1:250)	2.5 gm
EBSS 10X	100.0 ml
Distilled water	900.0 ml

Add trypsin and EBSS to 900.0 ml of distilled water and mix for 2 hr. Prefilter solution through an AP20 Millipore prefilter. Filter sterilize through a 0.45 u membrane filter. Store in 200 ml aliquots at -20°C.

4. Veronal Buffer Solution (VB)

Solution A	
NaCl	83.80 gm
$NaHCO_3$	2.52 gm
Sodium barbital (sodium 5, 5-diethyl barbiturate)	3.00 gm
Distilled water	1000 ml

Solution B	
Barbital (5, 5-diethyl barbituric acid)	4.60 gm
$MgCl_2 \cdot 6H_2O$	1.00 gm
$CaCl_2 \cdot 2H_2O$	0.20 gm
Hot distilled water	500 ml

After cooling, add solution A to solution B and bring to 2000 ml (5X concentrated). Sterilize by filtration through a 0.45 membrane filter. Working solution is 1X concentration.

APPENDIX B

1. Agar Overlay for Y-15 Cells

Solution A	100 ml
MEM(2X) without phenol red	50.00
FCS	2.00
Glutamine (2.92 percent)	1.00
$NaHCO_3$ (7.5 percent)	5.25
$MgCl_2$ (5M)	0.50
Non-essential amino acids (100X)	1.00
Penicillin G (1000X)	0.10
Streptomycin sulfate (1000X)	0.10
Neutral red (1:300)	2.30

Solution B	
Ion agar no. 2	0.9 gm
Distilled water	40.0

Aseptically mix ingredients of solution A into 2X MEM. Mix and sterilize solution B by autoclaving at 15 psi for 15 min. When both solutions have equilibrated to 45°C, add solution A to B. Adjust pH to 7.3.

2. Agar Overlay for Vero Cells

Solution A	100 ml
MEM(2X) without phenol red	50
$NaHCO_3$ (7.5 percent)	5.25
$MgCl_2$ (5M)	0.50
Glutamine (2.92 percent)	1.00
Penicillin G (1000X)	0.10
Streptomycin (1000X)	0.10
Fungazone (1000X)	0.10
Neutral red (1:300)	0.60

Solution B	
Difco agar	1.5 gm
Distilled water	40

Prepare solutions in a similar manner as for Y-15 cells.

3. Agar Overlay for HEK Cells

Solution A	100 ml
MEM(2X) without phenol red	50.00
FCS	2.00
Glutamine (2.92 percent)	1.00
Penicillin G	0.10
Streptomycin sulfate	0.10
Neutral red (1:300)	2.30

Solution B	
Ion agar no. 2	0.9 gm
Distilled water	40.0

Prepare solution in a similar manner as for Y-15 cells.

APPENDIX C

1. Y-15 cells were obtained from Dr. Benjamin Sweet, Gulf South Research Institute, New Orleans, Louisiana.

2. Vero cells were obtained from Dr. Edward Larkin, Food and Drug Administration, National Environmental Center, Cincinnati, Ohio.

3. Primary HEK cells were purchased from Microbiological Associates, Bethesda, Maryland.

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