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A MODEL SYSTEM FOR THE CONCENTRATION OF REOVIRUS FROM AQUEOUS ENVIRONMENTS

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University of New Hampshire PH.D. 1980

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A MODEL SYSTEM FOR THE CONCENTRATION OF REOVIRUS FROM AQUEOUS ENVIRONMENTS

by

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AMDG and to my Family

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ABSTRACT

A MODEL SYSTEM FOR THE CONCENTRATION
OF REOVIRUS FROM AQUEOUS ENVIRONMENTS

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A filter adsorption-elution method for the routine isolation of reovirus from model aqueous systems was developed. Reovirus was found to be stable within a pH range of 3 to 10, with a minimum loss of viability. A slight hydrogen ion excess minimized virus aggregation phenomena and promoted efficient adsorption of virus to filter surfaces. Adsorption of virus at pH 6.0 to all filters examined was enhanced by 50 mM $MgCl_2$. The use of $AlCl_3$ as an enhancing salt was contraindicated since reovirus was not recoverable from 0.5 mM $AlCl_3$ solutions. Elution of virus was most effective when a 3% beef extract eluent was used. In the model system, reconcentration of eluates was achieved by flocculation of beef extract proteins at pH 3.5. Reconcentration of reovirus and several representative enteroviruses was also achieved by means of a hemocyanin-enriched fraction derived from Limulus polyphemus hemolymph.

INTRODUCTION

The ability of animal viruses potentially pathogenic for humans to persist in water, coupled with the inability of bacterial indicators of water quality to accurately predict the presence of virus, underscores the need for virus monitoring of waters likely to be polluted with these pathogens. The growing need for reuse of water throughout the world, calling for renovation of virus polluted water is a special example of the danger associated with the persistence of virus pathogens in the aqueous environment. The increasing attractiveness of land disposal of sewage combined with the persistence of virus in the soil and on crops at such sites is a further indication of the need for virus monitoring.

Current methodology emphasizes the recovery of enteroviruses. As a result, many methods exclude the possibility of recovery of other viruses which might be inactivated by the conditions imposed by these procedures.

Reoviruses, like enteroviruses, are relatively resistant to chlorine treatment and various environmental conditions and are known to be present in wastewaters. Reovirus also has the capability to survive in digested wastes beyond the point at which enteroviruses would be detectable.

The purpose of this research was to develop a system for the efficient recovery of reovirus from model aqueous systems. Reovirus could be recovered routinely by appropriate modification of currently used enterovirus recovery methods. The

improved reovirus recovery extended the usefulness of virus monitoring procedures for the detection of virus pathogens in water.

LITERATURE REVIEW

The need for an efficient means of virus recovery from natural and wastewater systems has been emphasized by the number of potentially pathogenic viruses found to be associated with heavily contaminated and finished waters (Akin et al., 1971). Unfortunately no one system is capable of recovering all of the virus types which could occur in these water systems. The methods for virus concentration developed thus far are directed primarily toward the recovery of the culturable enteroviruses, and are dependent upon the enteroviruses' resistance to inactivation during extreme pH changes. Although a number of different viruses may actually be isolated by existing technology, certain deficiencies or extremes in recovery procedures may eliminate these viruses during the execution of these procedures. The extremes of pH used in enterovirus concentration would eliminate, for example, adenoviruses from water samples processed for enteroviruses. Fields and Metcalf (1975) determined that conventional filter systems could be utilized efficiently to isolate the adenoviruses if the extremes in pH were minimized. Therefore, re-evaluation of current methodology may result in more diverse virus recovery which would more accurately reflect the virus content of the medium in question.

Background

Historically, the need to monitor virus in the environ-

ment became apparent in the late 1940's and early 1950's with the increased incidence of poliomyelitis. Many attempts were made to correlate contaiminated sewage and drinking water as the source of local poliomyelitis outbreaks, but in the majority of the cases definitive evidence was lacking (Mosley, 1966). Melnick (1947) conducted studies of the occurrence of poliovirus in metropolitan wastewater in both epidemic and non-epidemic periods. In this study the seasonal occurrence of poliovirus-related disease episodes coinciding with the increased incidence of the virus in the environment was determined. The recovery of poliovirus in these grab samples was directly related to the seasonal fluctuations and, hence, their concentration in the environment.

One virus particle has been reported capable of causing infection in humans and other animals (Beard, 1965; Plotkin and Katz, 1965; Westwood and Sattar, 1976). The inefficiency of grab sampling to detect virus except in instances of extremely high numbers underscored the need for a method which would detect low numbers of virus in large volumes of water. One of the initial attempts to monitor virus in large volumes other than by grab sample was initiated by Gravelle and Chin (1961) using the gauze pad method. Although this appeared to be a significant improvement over simple grab samples, the method was non-quantitative since the actual volume of water passing through pads was unknown and the number of viruses recovered could not be related to the volume of water tested.

Liu et al. (1971) tested the efficiency of a flow-through gauze sampler to determine on a semi-quantitative basis the virus adsorption and elution capability of a gauze-pad system. The method developed originally was capable of recovering up to 47% of input virus numbers. Repetition of this study showed an inverse relationship between volume tested and virus recovered, however, and the method was considered unsuitable for quantitation of virus (Katzenelson, 1974).

Since these early studies the entire range of enteroviruses has been examined in aqueous systems. The enteroviruses would be one of the virus groups of choice because of their ubiquitous nature, resistance to pH and environmental conditions, and their survival after chlorination (Joyce and Weiser, 1967; Akin et al., 1971). The natural reservoir for the enteroviruses is the mammalian gut and, therefore, the wastes associated with populated areas introduce these viruses into the environment. Included in the enterovirus group are three polioviruses, thirty coxsackieviruses (types A and B), thirty-two echoviruses and additional enteroviruses with properties intermediate between typical coxsackie- and echovirus groups. Many of these viruses are associated with various clinical syndromes in the human including myocarditis, pericarditis, aseptic meningitis, generalized paralysis and gasteroenteritis.

Properties of the Reoviruses

Originally included in the enterovirus group was a class of ubiquitous enteropathogenic viruses. These viruses

were first isolated from the stools of healthy children and classified by Sabin as closely related to or identical with the echoviruses by virtue of their origin, isolation in primary monkey kidney and their lack of pathogenicity for newborn mice, rabbits, monkeys and guinea pigs (Sabin, 1959). One of the echoviruses (type 10) was reclassified and placed within this new grouping when it was determined that it was considerably larger than the typical enteroviruses (75 nm vs. 25 nm) and consistently exhibited different cytopathic effects in monkey kidney tissue culture. In addition, periodate treatment removed the receptors for human group-O erythrocytes from echovirus 10 but was without effect on other echoviruses or influenza virus (Sabin, 1959). At this time a new name and classification was suggested: Reovirus i.e., respiratory enteric orphan, emphasizing their sources of isolation and their unknown association with human disease states.

Although it was known that the genome of the newly classified reovirus was RNA, it was not until 1963 that Gomatos and Tamm reported that it was actually a double-stranded RNA molecule occurring in 10 distinct segments. The chemical composition of the reoviruses was established through studies of types 1 and 3. The virion is estimated to have a molecular weight of 70 million daltons (Gomatos and Tamm, 1963) of which approximately 11 million daltons (13 - 14%) is RNA and the remaining attributed to protein (Mayor et al., 1965). Although early reports indicated that periodate treatment adversely affected reovirus

agglutination of human-O erythrocytes, no polysaccharides were detected by periodic acid-Schiff techniques (Drouhet, 1960). This, however, may have been a reflection of the low amount of polysaccharide present. Other workers (Lerner et al., 1966) demonstrated that treatment of the virus with periodate or β-glucosidase inhibited the reovirus agglutination of human-O erythrocytes. Additionally N-acetyl-D-glucosamine was shown to inhibit reovirus type 1 agglutination with erythrocytes by binding directly to the red blood cells (Lerner, 1966; Gelb and Lerner, 1965). Evidence for glycoprotein was provided by radioactive labeling and polyacrylamide electrophoresis of purified virus fractions. In this study a modified alkaline ferricyanide method detected reducing sugars in the μ-class of polypeptides from the outer coat protein of reovirus (Krystal et al., 1976). The sugar moieties were determined to be present as a trimer or tetramer with a terminal N-acetylneuraminic acid linked via N-acetylgalactosamine to serine or threonine. It was estimated that this repeating unit occurred ten to twenty times on the surface of the virion. No evidence for lipid existed in the reovirus group since the virus was refractile to ethyl ether and other lipid solvents (Sabin, 1959; Rosen et al., 1960).

A unique aspect of reovirus morphology is the distinct double capsid arrangement (Loh et al., 1965; Jordan and Mayor, 1962; Gomatos et al., 1962; Vasquez and Tournier, 1962). The overall diameter of the virion was reported as approximately 75 nm (Luftig et al., 1972; Rhim et al., 1961)

with an inner core of approximately 45 - 50 nm (Luftig et al., 1972). The capsomeres of the outer layer were 10 nm long and 8 nm wide with a hollow core of 4 nm and appeared hexagonal or pentagonal (Vasquez and Tournier, 1962). They were arranged in an icosahedral symmetry (5:3:2) with the estimated total number of capsomeres being 92. Another interpretation of the arrangement of the capsomeres postulated by Vasquez and Tournier (1964) was that the columnar units were actually 180 structural units arranged in groups of 5 or 6 around 92 holes.

Removal of the outer capsid of reovirus is facilitated by proteolytic enzymes, notably chymotrypsin, and results in an enhancement of reovirus infectivity (Spendlove and Schaffer, 1965; Spendlove et al., 1970). Additionally, the low plaquing efficiency under agar media was resolved by the routine addition of pancreatin to the overlay (Wallis et al., 1966). Pancreatin is postulated to aid the cell-to-cell transmission of the virus under agar and results in visible plaques earlier than in monolayers without pancreatin. The plaques are larger and more numerous, showing an increase of 1.3 to 2.3 $\log_{10}$ units in titer.

Reovirus was also activated by 2 M $MgCl_2$ at 50°C (Wallis et al., 1964) with the resultant infective titer being increased 4 to 8 times. This is not unlike the stabilization of enteroviruses by high concentrations of divalent cations; however, enteroviruses were only stabilized at 50°C whereas reoviruses are activated (Wallis et al., 1964; Wallis and Melnick, 1962).

Reoviruses are found in a wide range of vertebrates including humans (Sabin, 1959; Rosen et al., 1963a), dogs (Lou and Wenner, 1963), mice (Hartley et al., 1961), cattle (Rosen et al., 1963b; Rosen and Abinanti, 1960) and monkeys (Hull et al., 1956; Hull et al., 1958). In many cases the reoviruses isolated in a given genus are indistinguishable from those isolated from man (Rosen, 1962). Although the ubiquity of the reoviruses in mammals has been well documented, their ability to cause overt disease in humans has not been established. Unlike reovirus infection in mice which can result in lesions in the liver, lungs and the nervous and pulmonary systems (Rosen, 1968), the connection to human disease is not so direct except in a few instances. Predominantly, the expression of reovirus disease in the human population is related to infantile diarrhea (Sabin, 1956; Rosen, 1960). Reoviruses have also been found associated with Burkitt's lymphoma and encephalitis but these conditions are exceptions (Rosen, 1968).

The widespread occurrence of the reovirus makes it and members of the group possible indicators of fecal pollution in the environment. Additionally, other members of the reovirus group such as the reovirus-like agents and the rotaviruses have now been established as gastroenteritis agents of significant epidemiological import. These double-stranded RNA viruses have now been implicated as the etiologic agents in episodes of non-bacterial gastroenteritis in human neonates (Kapikian et al., 1976; Murphy et al., 1977), juveniles (Elias, 1977), and adults (Bolivar et al., 1978).

As with the reoviruses, rotaviruses have been consistently recovered from lower animals (Woode et al., 1976a) including calves (Flewett et al., 1974), piglets (Woode et al., 1976b) and lambs (McNulty et al., 1976). The rotaviruses are morphologically related to the reoviruses since they have a similar double-capsid arrangement and double-stranded RNA (Rodger et al., 1975; Schnagl et al., 1976; Newman et al., 1975; Todd and McNulty, 1976). However, antigenically the rotaviruses isolated from various vertebrate genera are closely related to themselves but only somewhat related to reovirus 1, 2 and 3 (Woode et al., 1976a; Flewett et al., 1974). A method able to consistently and efficiently recover reovirus may have, as an added advantage, the ability to recover rotavirus since many aspects of the reovirion are paralleled by these gastroenteritis agents. The presence of glycoproteins on the surface of both the reovirus (Krystal et al., 1976) and the rotavirus (Rodger et al., 1977) distinguishes them, along with the adenoviruses, as the only non-enveloped viruses that contain glycoproteins on their surface. The inability to recover both reoviruses and rotaviruses in aluminum hydroxide flocs (Farrah et al., 1978; Wallis and Melnick, 1967) demonstrated their similarity and may be directly related to the surface carbohydrate composition.

Virus Indicator Systems

Viruses associated with the alimentary tract of mammals such as the enteroviruses and reoviruses are likely candidates as indicators of fecal pollution because of their constant

input into the environment and their ability to withstand adverse conditions. The traditional coliform indicator system of monitoring fecal pollution is unreliable since it does not reflect the viral content of the water. Notably, the coliforms were found to be much more sensitive than poliovirus (Scarpino et al., 1972; Berg et al., 1978) and other enteroviruses (Englebrecht and Greening, 1978) to chlorine treatment. Reovirus 1 was also relatively insensitive to chlorine treatment with an inactivation time of 2.7 min when exposed to 0.5 mg/liter chlorine (Sproul, 1976). The difference in the effectiveness of chlorine inactivation of reovirus, other enteric viruses and Escherichia coli strongly suggests that the absence of coliforms does not necessarily indicate that the waters are virus-free. Ward and Ashley (1977) determined that poliovirus was extremely sensitive to uncharged ammonia in digested sludge (pH 8.0 or greater) whereas reovirus was much less sensitive and was readily recoverable. With the increased interest in the deposition of sewage sludge to soils and the persistence of virus in these soils and crops grown on sewage inoculated plots (Tierney et al., 1977), reovirus could serve as an indicator of potential virus transmission in the land disposal of sewage effluent.

Bacteriophage has also been considered as an indicator of viral pollution in various surface waters (Kott et al., 1974; Hilton and Stozky, 1973; Joyce and Weiser, 1967). Kott (1966) used a method similar to the most probable number (MPN) test for coliform bacteria to estimate low numbers of

bacteriophage in sewage and contaminated seawater. Good correlation was attained between MPN data and direct plate counts for values as low as 2 pfu/100 ml. However, contradictory results were reported regarding the usefulness of bacteriophage as an indicator of animal virus contamination. Kott reported that in all cases examined, both enteric viruses and coliphage could be shown to occur in a given sample. A subsequent study performed by Vaughn and Metcalf (1975) in estuarine waters indicated coliphage and enteric virus did not always occur jointly in the same sample. The presence (or absence) of bacteriophage would, therefore, seem to be a questionable method of monitoring human or animal associated virus in water.

Other Viruses in Water

Other viruses are shed in human feces and represent potential hazards when released into the environment. Adenovirus, hepatitis A and acute infectious non-bacterial gastroenteritis (AING) agent have been recovered in the environment and the latter two viruses have been directly implicated in disease episodes. Although the potential for respiratory or conjunctival infections involving adenovirus is recognized and members of the adenovirus group have been isolated from sewage (England et al., 1967; Lund and Hedstrom, 1966) and estuarine waters (Fields and Metcalf, 1975), evidence of a water transmitted disease route in natural waters is lacking.

Hepatitis A virus has a long history of documented cases of transmission directly related to environmental conditions. Water-borne cases (Mosley, 1959) are known to occur

but the majority of the cases of infectious hepatitis are related to the consumption of raw (Mason and McLean, 1962; Dismukes et al., 1969; Ruddy et al., 1969) or incompletely cooked shellfish (Dienstag et al., 1976) harvested from contaminated waters. The problem of hepatitis A occurrence in shellfish and shellfish-growing waters remains a chronic one since areas designated for growth of virus-free shellfish have also been implicated in hepatitis disease outbreaks (Portnoy et al., 1975).

A parvovirus-like pathogen known as AING or Norwalk-type agent has been isolated in self-limiting cases of gastroenteritis (Alder and Zickl, 1969; Dolin et al., 1971). Epidemiological findings suggest the Norwalk-type viruses may be as widespread as the reoviruses and rotaviruses (Kapikian et al., 1972).

Virus Concentration Methods

Methods for the recovery of viruses from the aqueous environment deal primarily with enteroviruses and only secondarily with reoviruses and adenoviruses. A wide variety of techniques have been examined since the early studies which made use of grab samples and the gauze pad collection procedure: (i) Filter adsorption-elution; (ii) precipitation by adsorption to polyvalent cation salts, polyelectrolytes or minerals; (iii) co-precipitation with proteins; (iv) two-phase polymer systems; (v) ultrafiltration and (vi) hydro-extraction.

Techniques employed for virus isolation are dependent upon the behavior of viruses as amphoteric hydrophilic

colloids in aqueous suspension. Viruses are essentially polar macromolecules and are, therefore, miscible in water and will have a characteristic charge at a given pH depending upon the isoelectric point of the virus. The net charge is therefore dependent upon not only the pH, but also upon the ionic strength and the ionic species present in the solution.

One of the first to recognize the ability of viruses to adsorb or pass through filter substrates was Metcalf (1961) who used membrane filters to separate influenza virus from bacteria in experimentally prepared aqueous suspensions and clinical samples. In this study the retention of virus by filters with porosities many times greater than the virus was reported and the role of electrostatic forces in virus adsorption to filter substrates was suggested.

Cliver (1965) was able to adsorb and elute viruses from Millipore HA membranes with a resultant concentration of 1000-fold. Pre-treatment of filters with serum caused passage of virus through the filter. Competition of virus and other macromolecules dictated the degree of adsorption of virus to the filter; pre-treatment with protein exhausted available sites for subsequent virus attachment. Wallis and Melnick (1967a) noted that the presence of organic matter, acting as membrane coating components (MCC), interfered with virus adsorption to microporous filter surfaces. Removal of these substances was accomplished by passage of the sample through an anion exchange resin.

Cliver also described some of the many interactions occurring at the filter surface (1968). It was suggested that

not only was competition for adsorption sites an important factor but that pore size, viral aggregates and chemical composition of the filter also were contributing factors. Although direct entrapment was not a major premise, except in the case of aggregates, the ratio of pore size to virus diameter determined the intimacy with which virus and filter would come into contact. Mix (1974) has suggested several possible mechanisms for virus adsorption to filter surfaces including hydrogen bonding, hydrophobic interactions between nonpolar groups and cationic salt bridging between virus and filter. One or all of these mechanisms may ultimately contribute to virus adsorption to filter substrates.

Cationic enhancement of virus adsorption to a variety of filter substrates was studied by a number of workers. Wallis and Melnick (1967a) observed that at pH 5.0 addition of $MgCl_2$ markedly increased poliovirus adsorption to membrane filters. Addition of $CaCl_2$ to artificially contaminated seawater was reported to allow adsorption of poliovirus to a cellulose ester filter and subsequent elution with an alkaline solution of 3% beef extract resulted in an average recovery of 92% (Rao and Labzoffsky, 1969). A comparison of virus adsorption to cellulose nitrate filters in the presence of monovalent, divalent and trivalent cations indicated that aluminum chloride was 200 times more efficient in adsorbing virus than magnesium chloride and that monovalent cations were comparatively inefficient (Wallis et al., 1972).

Berg and co-workers (1971) seeded poliovirus and reovirus into 0.05 M Na_2HPO_4 buffered to pH 7.0 with citric acid

(McIlvaine's buffer) and adsorbed virus to cellulose nitrate membrane filters. Virus in filtrates was not reported but eluates were monitored. Elution with 3% beef extract with 20 min of sonic treatment resulted in recoveries of 100% for poliovirus 1, echovirus 7 and coxsackievirus B3. Recovery of reovirus required extended sonication times (40 - 60 min) and addition of pancreatin resulted in recoveries of 20 - 100%. In this instance, filter pore size had a noticeable effect on reovirus recovery with smaller porosities (less than 1 μm) resulting in higher recovery of reovirus.

The need for the addition of cations was eliminated when acidification of clean water samples to pH 5.0 or lower allowed efficient adsorption of several enteroviruses to epoxy-fiberglass and nitrocellulose filters (Sobsey et al., 1973).

Recovery of virus from raw sewage was attained by Wallis and Melnick (1967b) by clarification through a fiberglass prefilter (Millipore AP 20) and a 0.22 μm porosity cellulose nitrate membrane (Millipore GS). Filtrates were then treated with an anion exchange resin to remove MCC. Clarified samples were then passaged through cellulose ester membranes with added $MgCl_2$ and virus was eluted by grinding membranes in Melnicks B medium containing 10% fetal calf serum. Recoveries of 80% were attained in experiments using poliovirus-seeded, natural sewage samples.

Concentration of virus from sewage was attempted by clarification of samples by centrifuging poliovirus-infected raw sewage at 1800 x g to remove gross particulates, blending

the supernatant fluid at pH 3.0 and an additional centrifugation at 9230 x g to further clarify the sample. The final supernatant was collected with no reported loss of virus. Samples were then adsorbed to cellulose ester filters at pH 3.0 in the presence of 0.05 M $MgCl_2$. Elution was performed with 3% beef extract, pH 8.0, and resulted in recovery of poliovirus with efficiencies in the range of 88 - 97% (Rao et al., 1972).

Virus recovery from estuarine and seawater environments has met with variable success due to the problem of MCC as was found in recovery from sewage samples. Addition of diatomaceous earth (Celite) to a final concentration of 0.01% to turbid estuarine waters seeded with large amounts of poliovirus and adsorbed to cellulose nitrate or epoxy-fiberglass-asbestos filters resulted in 56% recovery of input virus upon elution with 5x nutrient broth, pH 9.0. With smaller virus inputs, recoveries were reduced to a range of 0.4 - 2.2% (Hill et al., 1974). A subsequent study by Farrah et al., (1977b) found that poliovirus could be concentrated from 400 liters of artificially infected estuary water without previous clarification with an efficiency of 70%. Pleated cartridge filters of 0.45 μm porosity (Filterite Corp.) were protected by fiberglass depth filters (Carborundum K-27); both filters were considered adsorbing filters and elution was performed with 0.06 M glycine, pH 11.5. Further concentration was done on aluminum hydroxide flocs. Application of the system to natural field sampling resulted in the recovery of predominantly polioviruses but echoviruses were also isolated.

Wallis and Melnick (1972) developed a portable virus concentrator for field use. Orlon and Tween 80-treated cotton depth filters (Carborundum Co.) were used for clarifying water samples of particulates and complexed metals respectively. Adsorbent filters used were fiberglass (Carborundum K-27) or cellulose-acetate (Carborundum W10A). Clarifying filters allowed passage of virus while retaining materials which might interfere and foul adsorbing filters. Adsorption included the use of $MgCl_2$ at a final concentration of 0.04 M. Virus was eluted from K-27 and W10A adsorbent filters with Ca-Mg-free phosphate buffered saline, pH 11.5. The eluent was then neutralized, the $MgCl_2$ concentration adjusted to 0.05 M, and reconcentrated on a cellulose filter. Final elution in a small volume of phosphate buffered saline, pH 11.5, from the terminal concentrating filter resulted in recoveries of 78%.

Later studies indicated that the portable virus concentrator could possibly be an efficient system for the recovery of natural virus. Metcalf et al. (1974) seeded polluted surface waters with poliovirus and, using the techniques described by Wallis and Melnick, were able to recover 56 - 71% of the input virus upon elution from the terminal Cox filter adsorbent.

Evaluation of nitrocellulose membrane filters, AA Cox M-780 epoxy-fiberglass-asbestos disks, Balston epoxy-fiberglass tubes and K-27 (Carborundum) fiberglass depth filters in conjunction with AA Cox M-780 indicated slight differences in virus recovery (Jakubowski et al., 1975). The Balston filter

was the most efficient in virus recovery with the single Cox filter the next most efficient. The K-27-Cox combination and the nitrocellulose filters were about equal in recovery capability but both systems were said to be slightly less efficient than either Balston or Cox filters alone. The study by Jakubowski also showed that effective elution with 0.05 M glycine could be accomplished at a pH lower than 11.5. A subsequent study in Jakubowski's laboratory using various combinations of the same filter substrates indicated no significant difference among the sensitivities of the various systems (Hill et al., 1976).

Farrah (1976) evaluated a fast-flow system using a pleated fiberglass-melamine-impregnated epoxy filter (Filterite) in conjunction with a K-27 fiberglass filter. Adsorption of 80 - 90% of the virus in acidified (pH 3.5) tap water was obtained at a flow rate of 26 liters/min. Recoveries from pH 10.5 glycine eluates by aluminum hydroxide flocculation ranged from 40 - 50%. A subsequent study using the same pleated filter (Farrah et al., 1977a) found that treatment with 0.1 N NaOH or autoclaving could successfully regenerate the filter for re-use.

Sobsey and Jones (1979) investigated more positively charged filters composed of cellulose, diatomaceous earth and charge-modified resin mixtures (Zeta Plus, AMF/CUNO). These filters were reported to have a net positive charge up to pH 6.0 and were able to efficiently adsorb virus at pH values around neutrality. Adsorbed virus was eluted with pH 9.5 - 11.5 glycine-NaOH and reconcentrated on smaller Zeta-Plus filters.

Standard methods using conventional less positively charged filters (K-27, W10A, etc.) resulted in recoveries of 5% whereas the positively charged substrates recovered 64 and 22% of added virus in one- and two-stage concentration procedures respectively.

Adsorption of viruses to precipitable salts, iron oxides and polyelectrolytes rather than filters has been examined as an alternative method of concentrating viruses. Wallis and Melnick (1967c) reported the ability of a variety of viruses to adsorb to aluminum phosphate, aluminum hydroxide and calcium phosphate precipitates. Enteroviruses including poliovirus, echovirus and coxsackievirus adsorbed efficiently to aluminum hydroxide and calcium phosphate precipitates whereas acid sensitive viruses such as herpesvirus, influenza and parainfluenza adsorbed only to aluminum phosphate. Adenoviruses adsorbed only to aluminum hydroxide and reovirus did not adsorb to any of the salts. Wallis and Melnick also found that small quantities of poliovirus (less than 1 pfu/ml) could be detected by their procedure. Prepared aluminum hydroxide was added to one-liter volumes of dilute virus. The suspension was then allowed to mix and the precipitate was trapped on a millipore HA filter (0.45 μm). The precipitate was removed with a spatula and suspended in saline for assay. Recoveries of poliovirus ranged from 80 to 84%.

Coxsackievirus A9 was also found to adsorb efficiently to iron oxide columns (Rao et al., 1968). Virus-contaminated samples were allowed to pass through columns containing 25 g iron oxide for adsorption. Elution was performed with 3%

beef extract, pH 8.0, and resulted in recoveries of up to 90% of the virus input. The major difficulty with the procedure was the rapid fouling of the column with suspended matter.

Polyelectrolytes have also been used for the adsorption and removal of viruses from water samples. A divinylbenzene-crosslinked styrene/maleic anhydride copolymer was found to adsorb tobacco mosaic virus with an efficiency of 100% (Johnson et al., 1967). The same study indicated that poliovirus was removed in excess of 99%. However, the experiments with poliovirus measured virus remaining in the sample after treatment but did not consider virus activity in the collected polymer.

A comparison of aluminum hydroxide precipitation, membrane filtration and adsorption to an insoluble, cross-linked copolymer of isobutylene maleic anhydride (PE-60, Monsanto) was made and the results indicated that concentration with PE-60 was more rapid and efficient than the other two methods (Wallis et al., 1969). Relative recovery rates for the three methods were: Aluminum hydroxide, 56%; cellulose membrane, 64%; PE-60, 93%. Addition of prepared PE-60 to sewage, urine or feces at pH 5.0 - 6.0 allowed virus adsorption free from interference by organic matter. Efficient elution from the insoluble polyelectrolyte was accomplished by suspension in phosphate buffer containing 10% fetal calf serum, pH 8.0, or borate buffer at pH 9.0. In this same study, PE-60 was "sandwiched" between fiberglass filter pads to maintain the polyelectrolyte surface during filtration.

Eluates obtained were re-adsorbed to PE-52 and again eluted with physiological saline. Reduction of sample volume from 1 gallon to 3 ml was obtained and recovery efficiencies were maintained.

A later study by Wallis and Melnick (1970) found that thin layers of PE-60 prepared on 90 mm or 293 mm fiberglass filter pads could efficiently adsorb poliovirus from large volumes of artificially contaminated waters. Virus added to relatively small volumes (25 - 100 gallons) of water were recovered upon elution with borate buffer with an efficiency of up to 80%. A 300-gallon sample showed a depressed recovery of 40%.

England (1972) described a method of adsorbing viruses to the sulfate salt of protamine. Samples were adjusted to pH 7.5 after addition of bovine albumin to a final concentration of 0.25%. Protamine sulfate prepared as a 1.0% solution was added to a final concentration of 0.025% to 0.05% to the albumin supplemented sample. The suspension was allowed to mix at room temperature for 30 min at which time the precipitate was collected by passage through Tween-80 treated Millipore AP 20 filter discs. The precipitate was dissolved by addition of a small amount of 1 M NaCl to the filter and the sample was collected by vacuum into a receiving tube. The procedure was able to recover 80 - 100% of reovirus and adenovirus from artificially contaminated sewage. However, the recovery of enteroviruses varied.

The use of alkaline beef extract solutions as eluents precluded the re-adsorption of virus onto smaller filters for

reconcentration since adjustment to acidic pH caused considerable flocculation. Katzenelson and co-workers (1976) examined the fate of viruses in these precipitated protein solids. A 3.0% beef extract solution with added poliovirus was adjusted to a pH of 3.5 or 4.0 for precipitate formation and allowed to mix at room temperature for 30 min. The precipitate was collected by centrifugation and protein was resuspended with 0.15 M Na_2HPO_4, pH 9.0. Essentially 100% of the poliovirus added to the sample occurred in the pellet. Comparison of the organic flocculation method with glycine reconcentration showed the beef extract procedure to be best for the reconcentration of virus eluates. The tentative standard method using glycine reconcentration resulted in a mean recovery of 35% whereas organic flocculation recovered 74% of added virus.

Aqueous polymer two-phase systems have also been used successfully to concentrate viruses. The method involves dissolving two polymers in an aqueous solution under prescribed conditions of pH and ionic strength which will ultimately allow them to separate into two phases (Albertsson, 1974). The ionic composition influences the partitioning by producing an electrical potential between the two polymer phases and, hence, determines movement of charged virus particles into one of the two phases. Concentration is achieved by making the phase containing virus small in comparison to the original sample volume (Philipson et al., 1960).

The most common polymers used in the early application

of the two phase system were dextran sulfate and polyethylene glycol (PEG) with NaCl added to produce the electrical potential between the phases. Lund and Hedstrom (1966) used this system to recover poliovirus from artificially contaminated sewage with a resultant concentration factor of 100 and a 99% recovery efficiency. Results indicated that three times as many virus isolates were possible compared to direct plating methods.

Shuval et al. (1967) used a single-step method in which the lower dextran phase was collected and KCl was added to a final concentration of 1 M. The suspension was then centrifuged and the supernatant was assayed for virus content. Recovery ranged from 37% to 98% with concentration factors of 52 - 200. One trial of a two-step method was attempted in which NaCl rather than KCl was added to the collected dextran sulfate. The system was allowed to stand overnight and the resultant upper phase was assayed. Recoveries of 51% were reported with a 274-fold concentration of sample.

Further study by Shuval et al. (1969) of the two-step method resulted in concentration factors of 520 and recoveries up to 134%. In this method as well as in parallel studies of a one-step procedure, both the lower and interphases were collected. One-step recovery efficiencies were 87% with a final concentration of 173-fold.

Nupen (1970) made application of the aqueous two-phase polymer system in field trials which used a procedure similar to that developed by Shuval et al. (1969). All virus recoveries

were made prior to final treatment of sewage. Controlled experiments using one-step and two-step procedures resulted in 22% and 44% recoveries respectively.

Grindrod and Cliver (1969) determined that dextran sulfate interfered with the detectability of some viruses in cell culture. An alternative method (Grindrod and Cliver, 1970) caused little or no inhibition when dextran was substituted for the sulfated polymer. Recoveries with dextran ranged from 59% to greater than 100% compared to a 0.001% to 100% recovery range when dextran sulfate was used.

Application of polymer two-phase systems has been limited as a result of several disadvantages (Sobsey, 1976). The main disadvantage is the slow processing of samples due to the time required for phase development and the additional time needed for further concentration if a two-step procedure is used. Additionally, the system is not designed to recover virus from large volumes of water; its application being limited to the processing of several liters of sample. The sensitivity of many viruses to dextran sulfate requires the substitution of dextran if efficient recovery is to be made. Nevertheless, its ability as a second step concentration method of filter eluates has found useful application for viruses which are sensitive to pH extremes (Fields and Metcalf, 1975).

Ultrafiltration as a means of virus recovery from water samples involves the retention of virus by a membrane with a specified limiting porosity while allowing microsolutes and water to pass through freely. Gartner (1967) examined the

efficacy of soluble alginate filters as a device for ultrafiltration of virus from water. Filter paper treated with an electrolyte solution was used as a base for the formation of an alginate gel layer of uniform thickness which was then removed from the paper carrier for use. After filtration, virus was collected by dissolving the alginate filter in a 3.8% solution of sodium citrate which was then inoculated directly into tissue culture. Complete retention of virus input was determined and virus recoveries from the solubilized alginate gel varied from 25% to 100%.

Nupen (1970) evaluated a similar system using membrane filters (Sartorius) as the alginate filter support. Laboratory studies of this system resulted in a mean recovery of 40%. The soluble alginate filter system was not practical for use in the recovery of virus from turbid waters due to rapid membrane fouling.

Disc ultrafilters with a limiting porosity of 30,000 mw (Amicon PM 30) were used to examine 10-liter treatment plant effluent samples (Nupen and Standner, 1972). Virus was removed from the filters with a salt solution containing fetal calf serum and lactalbumin hydrolysate resulting in a recovery of 70%.

Ellender and Sweet (1972) examined the same filter using virus-inoculated distilled water. Average recovery was 50% while the sample was concentrated 45- to 72-fold.

Cellulose-acetate hollow fibers with a molecular weight cut-off of 30,000 were examined as an alternative method of

virus ultrafiltration (Belfort et al., 1974). Recoveries of poliovirus averaged less than 40% from 5-liter water samples due to concentration polarization and subsequent adsorption of virus to the membrane. Increased flow rates resulted in slightly higher average recoveries (47.3%). Further increases in recovery were attained by reversing the flow at the end of filtration by allowing the permeate to move from the outside of the hollow fibers into the lumen and collecting the small volume "backwash." Mean recovery after combination of initial concentrates with the respective backwash samples was 84%.

A subsequent study done with reusable asymmetric polysulfone hollow fibers (Amicon H10P10) resulted in average recoveries of 51.6% and 72.8% from tap water and distilled water respectively (Belfort et al., 1976).

Cliver (1967) examined hydroextraction using a polyethylene glycol concentration method. This method results in dehydration of a sample through movement of water across a dialysis membrane under the influence of PEG (strongly hydroscopic). The porosity of the semi-permeable membrane will restrict movement of the virus from the sample and also prevent diffusion of the hydroscopic substance into the virus sample. Cliver (1967) reduced 100-ml samples to about 2 ml in a 2 - 3 h period, but recovery efficiencies were low (10 - 30%). Shuval et al. (1967) reported recoveries from larger samples in the range of 20 to 50%. Virus losses in this procedure may be due partially to adsorption of virus to

dialysis membranes (Shuval et al., 1967). An additional drawback to this system is that it is only useful for work with small volumes and therefore application would be limited to highly contaminated samples (Sobsey, 1976).

MATERIALS AND METHODS

Cell Culture

Buffalo green monkey kidney cells (BGM) originally obtained from Dr. Richard Englebrecht (Dept. of Civil Eng., University of Illinois, Urbana, Ill.) and maintained in continuous cultivation in the Virus Laboratory, Department of Microbiology, University of New Hampshire, were used throughout the study (Dahling, 1974). Growth medium was minimum essential medium (MEM) containing Hank's balanced salts supplemented with Liebowitz medium (L-15) and 10% fetal calf serum (FCS). Maintenance medium was Melnick B supplemented with 2% FCS. All media contained 100 units penicillin G, 100 µg streptomycin sulfate and 100 µg fungizone (E.R. Squibb and Sons, Inc.) per ml. Cultures were grown in 1680 cm^2 roller bottles at 37°C and transfer was made using 0.1% versene - 0.125% trypsin, pH 7.2. Upon subculture to one-ounce (9 cm^2) prescription bottles (Brockway Glass) gentamicin sulfate (Schering Corp.) was added to a final concentration of 25 units/ml in lieu of penicillin and streptomycin.

Virus Stocks

Reovirus type 1 Lang (ATCC VR-230), echovirus type 7 Wallace (ATCC VR-37) and poliovirus type 2 MEF-1 (Flow Labs) were prepared in BGM cells (Dahling, 1974) in roller-bottle culture. Virus was inoculated onto confluent roller-bottle monolayers after removal of MEM-L-15 growth medium. Virus was allowed to adsorb for 1 h at 37°C after which 50 ml of

Melnick B were added and roller bottles were re-incubated. When advanced cytopathic effect was evident, virus was harvested by freezing (-60°C) and thawing (45°C) three times. Suspensions were then clarified of cell debris by low speed centrifugation. Supernatants were collected and extracted with an equal volume of Freon TF (E.I. DuPont de Nemours and Co.) (Bellamy, 1967; Smith, 1969). The aqueous phase was carefully removed from the Freon TF phase and aliquoted for storage at -60°C.

Coxsackievirus type B1 was a natural isolate, purified on BGM monolayers, identified by Lim Benyesh-Melnick serum pools and prepared as above.

Virus Assay

Standard plaque assays (Hsiung, 1957) were performed in bottles containing 9 cm^2 monolayers of BGM cells using an inoculum of 0.1 ml. Dilutions were made in phosphate buffered saline, pH 7.2 (enteroviruses), or 0.05 M glycine, pH 7.5 (reovirus). Adsorption of enteroviruses was for 1 h while reovirus adsorption ranged from 1.5 - 2.0 h. Cells were overlaid with 5 ml agar medium (Dahling, 1974) composed of the following per 100 ml: 40.0 ml 2x MEM; 1.0 ml L-glutamine (100x); 2.0 ml fetal calf serum; 1.0 ml non-essential amino acids (Microbiological Associates); 1.0 ml of a 1% $MgCl_2 \cdot 6\ H_2O$ solution; 0.6 ml neutral red (1:300; Gibco); 1.0 ml sterile liquid milk (Real Fresh Co.); 100 µg streptomycin sulfate and fungizone; 100 units penicillin G; 3.0 ml of a 7.5% $NaHCO_3$ solution; and 50 ml of a 1.5% Bacto-Difco agar solution. The nutrient portion was prepared from sterile components and

warmed to 45°C. The agar portion was sterilized by autoclaving and allowed to cool to 45°C in a waterbath. The nutrient and agar portions were then mixed, adjusted to pH 7.2 with 1N HCl, aliquoted to monolayers and allowed to solidify at room temperature after which overlaid monolayers were incubated at 37°C. Reovirus overlay differed in the addition of pancreatin (Oxoid) which was required for efficient plaque formation (Wallis, 1966; Dahling, 1974) and the use of a 1% Oxoid L-28 purified agar solution. Plaques were read at 3 - 5 days for enteroviruses and reovirus assays were allowed to proceed for 14 days before termination.

Sample Sterilization

Virus samples were treated with 100 units penicillin G, 100 µg streptomycin sulfate and 100 µg fungizone per ml and held at room temperature for 1 h. Anesthetic-grade diethyl ether was then added at a ratio of 1 ml per 10 ml of sample. Samples were mixed by vortex mixer and stored at 4°C for 18 h. Ether was removed by decanting samples into sterile glass petri dishes and allowing the ether to evaporate for 1 - 2 h at room temperature in a vented hood. After complete evaporation of residual ether, the remaining aqueous portion was collected and restored to its original volume by rinsing the respective petri dish with a volume of 0.05 M glycine, pH 7.5, equal to the difference between the original volume and the volume after evaporation. Samples were then stored at 4°C and assayed within 48 h.

pH Sensitivity of Reovirus 1

Reovirus type 1 Lang was tested for its stability at

pH 9.0, 10.0, 10.5, 11.0 and 11.5 in 0.05 M glycine at room temperature. Undilute virus stock was added to a volume of 0.05 M glycine at the appropriate pH and mixed continuously throughout the sampling period. At regular intervals 5.0 ml samples were withdrawn from assays at pH 9.0, 10.0 and 10.5 and adjusted to neutrality with a predetermined amount of 0.05 M glycine, pH 2.0. Samples from assays at pH 11.0 and 11.5 were adjusted to neutrality by a predetermined amount of 0.1 N HCl. As an additional precaution against inactivation beyond experimental design, all samples were immediately diluted 10^{-1} in 0.05 M glycine, pH 7.5. In all assays, controls at pH 7.0 were seeded in a similar fashion and representative samples were collected and treated with pH 7.5 glycine equal in volume to the amount of glycine or HCl added to assay samples.

Stability at the common adsorption pH of 3.5 (Sobsey et al., 1973) was determined in a manner similar to the method used for assays at alkaline pH. Samples were withdrawn at regular intervals and adjusted to neutrality with 0.05 M glycine, pH 9.0. Controls at pH 7.0 were collected and a volume of 0.05 M glycine, pH 7.5, was added which was equal in volume to the amount of glycine added to neutralize assay samples.

Additional experiments at pH 4 - 7 were run in tap water to determine if virus losses were due to aggregation or lack of stability at acid pH. One milliliter of undilute reovirus was added to 700 ml of tap water adjusted to pH 7.0. Mixing was continuous throughout the assay. After a 10 min period,

three samples were taken: a 10 ml sample was added to 0.1 ml phosphate buffered saline (PBS), pH 7.5; a second sample of 5 ml was added to 5 ml 0.05 M glycine, pH 7.5; and a third sample was added to 5 ml of 0.05 M $MgCl_2$, pH 7.5. The pH of the batch suspension was then sequentially adjusted with 0.1 N HCl to pH 6.0, 5.0 and 4.0 with 10 min mixing periods at each pH interval and then sampled as above. Aliquots added to $MgCl_2$ at pH 4.0 and 5.0 were adjusted to neutrality with 0.1 N NaOH; all other samples needed no adjustment.

An alternative method was used to eliminate the effect of pH change in batch samples. Individual tap water samples to 350 ml at pH 4.0, 5.0, 6.0 and 7.0 were seeded with 0.5 ml of virus stock and allowed to mix for 10 min at room temperature. Virus samples were then removed and treated as above for collection from batch suspensions. All assays in tap water were designed so that samples could be tested directly with no intervening dilution steps which might contribute to aggregation.

Effect of Cationic Salts on Reovirus

Simple mixing experiments were performed to determine the effect of $MgCl_2$ and $AlCl_3$ on reovirus before each was considered for use as an enhancing salt in filtration-adsorption studies.

Undilute virus stock was added to 100 ml 0.05 M glycine, pH 7.0. The virus suspension was mixed for 5 min after which an input sample was taken. Magnesium chloride was added to a final concentration of 50 mM and the pH of the suspension

was adjusted to neutrality with 1 N NaOH. Samples of 5.0 ml were taken at 5, 10 and 15 min and diluted with an equal volume of 0.05 M glycine, pH 7.5. A final sample collected at 20 min was tested without dilution.

The protocol for testing the effect of $AlCl_3$ at 3 mM and 0.5 mM concentrations was identical to the protocol used for testing $MgCl_2$ effects.

Hemocyanin Collection and Preparation

Limulus polyphemus native to the estuaries of southern New Hampshire was used as a source of hemocyanin (Hcy). Whole hemolymph was collected from the hemocoel using an 18-gauge needle as previously described by Pistole (1976). Approximately 12 - 15 ml of whole hemolymph was collected from female crabs and 5 - 7 ml from the smaller males. The hemolymph was stored on wet ice for transport to the laboratory. Clotted cells and debris were removed by centrifugation at 16,300 x g for 15 min. Clarified hemolymph was stored at -20°C until it was concentrated. Concentration of hemolymph was performed by ultracentrifugation at 100,000 x g for 2 h (Fletcher et al., 1978; Nowak and Barondes, 1975). The deep-blue portion of the sample was aspirated from the bottom of the tube leaving behind the light blue interphase and the clear supernatant. The interphase and supernatant were decanted and the pelleted material was resuspended with 2 ml of distilled water overnight at 4°C and then pooled with the appropriate sample. Collection was standardized among lots so that a 10^{-2} dilution in 0.05 M glycine, pH 7.5, resulted in an A_{280} value of 1.3 (Beckman spectrophoto-

meter 600). The resultant hemocyanin-enriched fraction (HEF) was stored in serum bottles at -60°C until used.

HEF Purification

HEF was prepared for column chromatography by extensive dialysis at 4°C versus 0.05 M Tris-glycine, 0.01 M ethylene-diaminetetraacetic acid (EDTA), pH 8.9 (Sullivan et al., 1974). Attempts to separate contaminating serum proteins from HEF were made by using Sephadex G-100 and Sephadex A-50 columns (1.5 x 25.4 cm) equilibrated with the above buffer. The A-50 column was eluted with a linear gradient of 0 - 0.5 M NaCl in 0.05 M Tris-glycine, 0.01 M EDTA, pH 8.9, at a flow rate of 18 ml/h. The G-100 column was eluted with a continuous flow of equilibrating buffer at a flow rate of 26 ml/h. All fractions were collected at 25°C and read at A_{280} and A_{340}. Fractions exhibiting A_{340}:A_{280} (Copper:protein) ratios greater than or equal to 0.2 (Fager and Alben, 1972) were pooled and considered to contain only hemocyanin protein with associated copper. A-50 fractions pooled in this manner were compared with HEF for homogeneity by sodium-dodecyl-sulfate polyacrylamide slab-gel electrophoresis (Ornstein, 1964; Davis, 1964; Laemli, 1970). Protein determination was made by the Bio-Rad protein assay kit (Bio-Rad Co.) using a known concentration of bovine serum albumin to construct the standard curve. Column eluates were stored at -20°C until used in reconcentration procedures.

A crude method of purification was ultracentrifugation at 100,000 x g for 5 h (Nowak and Barondes, 1975; Fletcher et al., 1978). The clear supernatant was carefully removed

from the Hcy fraction and discarded. The deep-blue, concentrated Hcy fraction was collected and stored at 4°C. Pellets were resuspended overnight at 4°C with 2 ml of distilled water and pooled with the appropriate sample. Pooled samples were frozen at -20° until used in reconcentration procedures.

Virus Concentration

Reovirus 1 adsorption to different filter substrates was determined under various pH conditions. Epoxy-fiberglass Cox filters were examined at pH 4 through 9 at 1 $\log_{10}$ increments. Initial experiments were done using Cox filters with nominal porosities of 0.45 µm and 1.0 µm with high virus inputs in small sample volumes. After analysis of virus recoveries in Cox systems, Fulflo Commercial cartridge filters (Carborundum Co.) were examined at pH 6.0 with and without $MgCl_2$ added as enhancing salt.

Adsorption

Epoxy-fiberglass Cox filters (45 mm diameter) with porosities of 0.45 µm and 1.0 µm were examined for virus adsorption at pH 4.0 through 9.0 at room temperature. Reovirus was seeded into one liter of tap water adjusted to pH 7.0 and treated with 3.2 ml of a 1% sodium thiosulfate solution to neutralize residual chlorine. The suspension was allowed to mix for 5 min after which an input sample was taken and 100 ml aliquots were passed through 1.0 µm and 0.45 µm filters by positive pressure using 5 and 10 pounds per square inch (psi) N_2 respectively. Filtrate samples were taken by withdrawing 10.0 ml from the passaged

volume. The pH of the batch sample was then sequentially adjusted to pH 6.0, 5.0 and 4.0 using 0.1 N HCl. Aliquots were filtered at each pH interval and filtrates were sampled as above.

Virus passage through Cox filters was also examined at pH 8.0 and 9.0. The protocol adopted for filtration in the alkaline range was identical to that adopted for filtration in the acid range. Virus was seeded into one liter of tap water adjusted to pH 7.0 and treated with sodium thiosulfate. Batch samples were sequentially adjusted to pH 8.0 and 9.0 using 0.1 N NaOH and 100-ml aliquots were filtered at each pH interval and sampled as above.

Identical experiments were performed using Cox filters with virus suspended in one liter of 0.05 M glycine to determine if substantial aggregation was occurring.

Larger scale filtration-adsorption experiments were performed using 10-inch Fulflo Commercial cartridge filters of various composition (Table 1). Conditions for the examination of adsorption, determined by Cox filter studies, were established as tap water adjusted to pH 6.0 with and without added $MgCl_2$ at a final concentration of 0.05 M. Undilute virus was seeded directly into 5 liters of tap water and mixed throughout the assay. To avoid massive aggregation, subsequent experiments used a stock virus which was diluted initially into 50 ml of 0.05 M glycine, pH 7.5, as an input into the 5-liter tap water sample.

Filters exhibiting relatively poor adsorptive capacity were considered as clarifying filters in anticipation of the

Table 1.

Commercial Filter Composition

Designation	Porosity (μm)	Medium
019	10	Orlon
039	1	Orlon
K27	3	Fiberglass
W10A	1	Cellulose Acetate
E39	1	Viscose

Filters manufactured by Carborundum Co.,
Commercial Filter Division, Lebanon, Ind.

need to remove membrane-coating components (MCC) and suspended solids while still allowing virus to pass through the filter. Fulflo K-27 filters were examined at pH 7.1 in artificial seawater (Seven Seas) adjusted to a density of 1.22 - 1.26, tap water at pH 6.0 and tap water at pH 6.0 with 0.05 M $MgCl_2$. Virus was added to 50 ml of 0.05 M glycine, pH 7.5, and then seeded into 5 liters of the appropriate medium. Filters were pretreated by one of two methods: (i) Cyclic filtration of one liter of 3% beef extract (BE), pH 9.5, for 15 min using a peristaltic pump or (ii) soaking in a solution of 0.1% Tween-80 overnight. Filters were blown dry by N_2 positive pressure before use in filtration experiments. Filters treated with Tween-80 were rinsed with 20 liters of distilled deionized water before use. Controls were K-27 filters which were untreated but subjected to the same filtration conditions. Sample volumes were then passaged through pretreated filters and untreated controls using 10 psi N_2 positive pressure (ca. 5 liters/min) and filtrates were sampled for virus content.

Elution of Virus from Filters

Filters exhibiting a capacity to efficiently adsorb virus with or without the aid of an enhancing salt were subjected to various eluents in an attempt to remove adsorbed virus. Eluents primarily examined were 3% beef extract, pH 9.5, and 0.05 M glycine, pH 10.0. Studies examining the elution capability of HEF were performed using the following solutions: (i) 0.5% HEF, pH 9.5; (ii) 0.5% HEF, 0.15 M NaCl, pH 9.5; and (iii) 0.5% HEF, 1 mM Na_2HCO_3, pH 9.5.

Fulflo-cartridge filter holders were filled to capacity (ca. 950 ml) with the appropriate eluent and pumped through the filter using a peristaltic pump (Masterflex, Cole-Parmer). After 7 min the direction of the eluent flow was reversed and elution was continued for an additional 7 min. Eluents were then collected and adjusted to neutrality with 0.1 N HCl (HEF solutions) or 1.0 N HCl (beef extract and glycine) and tested for virus content.

Reconcentration Studies

Three methods of reconcentration were examined for the recovery of reovirus from filter eluates. Initial studies were performed under controlled conditions using mock eluates (3% beef extract solution) or water samples.

The method of Katzenelson et al., (1976) was examined using 500-ml volumes of 3% beef extract solution adjusted to pH 7.0. Monodispersed virus was added to the beef extract solution and allowed to mix for 10 min after which a 10-ml input sample was taken. The pH of the solution was then adjusted to pH 3.5 using 1.0 N HCl to precipitate beef extract protein. Mixing was continued for an additional 30 min. The solution was then centrifuged at 16,300 x g for 15 min at 4°C. Supernatant samples were collected and the remainder was discarded. Precipitates were resuspended in 0.15 M Na_2HPO_4, pH 9.5, neutralized and decontaminated before assay.

An aqueous two-phase polymer system described by Wesslen, Albertsson and Philipson (1959) was used to concentrate reovirus from 500-ml samples of beef extract. The

beef extract solution, adjusted to pH 7.2, was seeded with reovirus and mixed slowly throughout the procedure. The following reagents were added sequentially to the beef extract: NaCl to a final concentration of 0.15 M, 13.1 g of 20% (w/w) dextran 2000, 2.4 g methyl cellulose dissolved in 20 g hot 5 M NaCl. Approximately 10 ml of distilled water was used to rinse out the methyl cellulose slurry. The solution was mixed for 1 h at 4°C and then placed in a separatory funnel for 48 h at 4°C for phase development. The bottom and interphases were collected and mixed with an equal volume of 10% (w/w) polyethylene glycol 6000. After a 1-h mixing period at 4°C the solution was placed in a separatory funnel at 4°C for 24 h. After phase development, both the bottom and interphases were collected, ether treated and assayed. Upper phases at each step were also monitored for virus content.

Reconcentration of several representative enteroviruses and reovirus using hemocyanin enriched fraction was examined. Two different methods were evaluated. One system (HEF-method) involved the addition of HEF to a final concentration of 0.5% to tap water adjusted to pH 7.2. The pH of the suspension was adjusted with 0.1 N HCl to pH 6.0 - 6.2 for maximum floc formation. The solution was then mixed for 30 min at room temperature or 4°C followed by centrifugation at 16,300 x g for 15 min at 4°C. Supernatants were sampled for virus and the remainder was discarded. The precipitate was dissolved in 0.15 M Na_2HPO_4, pH 7.5, treated with ether and assayed.

In an alternative procedure, the hemocyanin-protamine sulfate method (HPS-method), samples were adjusted to pH 7.2 and HEF was added to a concentration of 0.5%. Protamine sulfate, prepared as a 1% solution, was then added to the sample for a final concentration of 0.01%. No further pH adjustment was necessary. Samples were then processed as in the HEF-method.

Similar reconcentration experiments with reovirus were performed using purified HEF to determine if hemocyanin was the active molecule or if other proteins in the hemolymph contributed to the observed activity. Purified hemocyanin prepared by Sephadex A-50 chromatography was used at a concentration equal to the amount of unpurified HEF added to obtain the 0.5% solution used in previous reconcentration experiments. Protein concentration of both A-50 eluates and HEF was determined by the Bio-Rad protein assay kit. Hemocyanin purified by Sephadex G-100 columns or ultracentrifugation at 100,000 x g for 5 h was added directly for maximum floc formation.

Experiments not using protamine sulfate for floc formation required Sephadex column eluates to be dialyzed against 5 mM NaCl, pH 9.5 to remove glycine-EDTA.

Model System

A model system was assembled from the methods described. Reovirus was added to 50 ml 0.05 M glycine, pH 7.5, and seeded into 15 liters of tap water adjusted to pH 6.0. The sample was then passed through a Tween-80 treated K-27 clarifying filter by positive pressure. Clarified sample

was received by a vessel containing a prepared solution of $MgCl_2$ to give a final concentration of 0.05 M and a pH of 6.0. With continuous mixing the sample was then passed through an E-39 filter previously rinsed with 20 liters of distilled water. E-39 filters were then cyclically eluted with 3% beef extract, pH 9.5. The beef extract was collected and adjusted to pH 4.0 for precipitate formation. Precipitate was collected by centrifugation at 16,300 x g (4^oC) and pellets were resuspended with 0.15 M Na_2HPO_4, pH 9.0, neutralized and decontaminated for assay.

RESULTS

pH Sensitivity of Reovirus

Adsorption and elution of viruses to filter surfaces is directly related to the net charge of the virus. Virus surface charge may be altered by pH adjustments and virus reactivity with various filters altered accordingly. Adsorption generally occurs at an acid pH whereas elution is accomplished in the alkaline range. Reovirus lability to acid and alkaline pH was examined to determine what pH values could be used for adsorption and elution without loss of virus.

Reovirus lability to alkaline conditions was examined at pH 9.0, 10.0, 10.5, 11.0 and 11.5. It was found that reovirus was extremely labile to 0.05 M glycine, pH 11.5 (Table 2). After one min at pH 11.5, only 46% of the input virus was recoverable and at the final sampling time of 10 min only 24% of the virus remained. These time periods are less than the time necessary for efficient elution in the field or in model experiments. Recovery of less than 1% of the input virus was made from test suspensions adjusted to pH 11.5 at the end of a 20 min period.

Virus losses were reduced at pH 11.0 and 10.5 but significant virus inactivation occurred within a 10 min period. Essentially all of the input virus was recoverable at pH 9.0 and 10.0 after one h; this was considered to be a reasonably safe and adequate time interval for efficient elution of virus.

Table 2

Reovirus Lability to Alkaline pH

Time (min)	pH 11.5	pH 11.5	pH 11.0	pH 10.5	pH 10.0	pH 9.0
0	3.9×10^6	2.8×10^5	1.7×10^6	6.3×10^4	5.0×10^6	2.2×10^6
1	1.8×10^6	-	3.2×10^6	1.5×10^4	3.3×10^6	3.1×10^6
4	7.5×10^5	-	5.3×10^6	1.9×10^4	4.1×10^6	3.1×10^6
6	1.3×10^6	-	3.4×10^5	2.2×10^4	4.1×10^6	1.8×10^6
10	9.5×10^5	-	2.4×10^5	2.0×10^4	5.1×10^6	1.8×10^6
20	-	5.0×10^1	2.7×10^5	2.5×10^4	-	2.2×10^6
30	-	0	1.3×10^5	2.3×10^4	-	-
40	-	0	1.5×10^5	-	5.0×10^6	-
60	-	0	1.8×10^4	-	-	2.4×10^6
120	-	0	-	-	-	-
180	-	0	-	-	5.3×10^6	-

- No Sample Taken

The acid lability of reovirus initially was examined at pH 3.5. This pH has been reported by many workers as an efficient pH for enterovirus adsorption and has not been found detrimental for enteroviruses over prolonged periods of time.

Experiments performed in 0.05 M glycine, pH 3.5, resulted in little or no loss of virus over a 3-h period (Table 3). However, subsequent adsorption studies, in which reovirus was seeded into tap water, resulted in poor recoveries from both controls and experimental samples. Virus lability was examined at pH 4.0 through 7.0 to determine whether reovirus was severely aggregated in tap water and did not disperse upon dilution for assay, or whether reovirus was actually labile within this range.

Batch tap water samples, in which one sample was prepared and the pH lowered sequentially, resulted in the greatest recoveries when the sample was diluted into 0.05 M glycine, pH 7.5, for assay (Table 4). Individual samples prepared at each pH and diluted into glycine also resulted in the highest recovery. Use of 0.05 M $MgCl_2$, pH 7.5, or phosphate buffered saline, pH 7.5, as diluent resulted in extremely low recoveries (Table 4).

From the above results, reovirus adsorption could occur with minimal aggregation and loss of infectivity at a moderate acid pH and elution could be safely performed in a pH range of 9 - 10. For accuracy of measurement in plaque assays, reovirus was diluted into glycine to minimize reduction of titer due to aggregative phenomena in the diluent.

Table 3

Reovirus Lability at pH 3.5

Time (min)	Trial 1	Trial 2
0	1.1×10^6	8.5×10^5
1	1.3×10^6	1.6×10^6
4	1.8×10^6	1.5×10^6
6	1.4×10^6	1.0×10^6
10	1.5×10^6	7.8×10^5
20	1.5×10^6	9.9×10^5
60	1.5×10^6	9.1×10^5
180	1.0×10^6	7.1×10^5

Table 4

Effect of pH and Diluent on Reovirus Aggregation in Tap Water

pH of virus Sample	Diluent	Percent Recovery	
		Batch Sample	Individual Sample
7	Phosphate	17.5	9.3
6	Buffered Saline,	0	8.3
5	pH 7.5	0.5	0.8
4		0	5.5
7	0.05 M Glycine,	52.0	56.7
6	pH 7.5	>100.0	53.3
5		47.8	50.0
4		50.0	67.8
7	0.05 M $MgCl_2$,	6.7	6.7
6	pH 7.5	3.3	1.1
5		2.2	4.4
4		4.4	0

Effect of Cationic Salts on Reovirus

When adsorption is performed at moderately acidic pH, the use of cationic salts enhances virus adsorption to filter surfaces. Both aluminum chloride and magnesium chloride have been used effectively for enhancement of adsorption but aluminum chloride has been shown to be much more efficient.

The effect of aluminum chloride on reovirus infectivity was determined at 3.0 and 0.5 mM concentrations. No reovirus was recovered from a 3.0 mM solution of $AlCl_3$ after 5 min when a low initial virus input was used (Table 5). Sensitivity of reovirus, using both high and low virus inputs, was then examined at the 0.5 mM $AlCl_3$ concentration commonly used for adsorption (Table 6). In both cases, less than 5% of reovirus was recoverable after a 5-min period.

Magnesium chloride at a concentration of 0.05 M was examined using high virus inputs (Table 7). After a 5-min period, essentially 100% of the virus was detectable and upon termination of the experiment the average recovery was 79%. Although $MgCl_2$ is a much less effective cation for adsorption, its use was made necessary because of the extreme lability of reovirus to low concentration of aluminum chloride.

Purification of Hemocyanin-Enriched Fraction

A hemocyanin-enriched fraction prepared for use in reconcentration and elution experiments by ultracentrifugation at 100,000 x g for 2 h was further purified to determine if the observed activity of the preparation was due to hemocyanin protein alone, or if other extraneous hemolymph

Table 5

Effect of 3.0 mM $AlCl_3$ on the Recovery of Reovirus from Tap Water

Time (min)	PFU/ml	Percent Recovery
0	250	-
5	0	0
10	0	0
15	0	0
20	0	0

Table 6

Effect of 0.5 mM $AlCl_3$ on the Recovery of Reovirus from Tap Water

	Trial 1		Trial 2	
Time (min)	PFU/ml	Percent Recovery	PFU/ml	Percent Recovery
0	1.98×10^4	-	8.93×10^2	-
5	3.60×10^2	1.8	4.00×10^1	4.4
10	5.00×10^2	2.5	2.60×10^1	2.9
15	6.90×10^2	3.5	0	0
20	7.80×10^2	3.9	0	0

Table 7

Effect of 50 mM $MgCl_2$ on the Recovery of Reovirus from Tap Water

	Trial 1		Trial 2		Trial 3	
Time (min)	PFU/ml	Percent Recovery	PFU/ml	Percent Recovery	PFU/ml	Percent Recovery
0	3.6×10^4	-	3.7×10^4	-	1.2×10^4	-
5	5.3×10^4	>100.0	4.0×10^4	>100.0	1.6×10^4	>100.0
10	4.7×10^4	>100.0	3.6×10^4	97.0	1.2×10^4	98.3
15	2.7×10^4	75.0	4.5×10^4	>100.0	1.1×10^4	93.2
20	2.8×10^4	77.7	2.3×10^4	86.0	8.8×10^4	74.5

components contributed to the activity. Three methods of purification were used: (i) Sephadex A-50 ion exchange, (ii) Sephadex G-100 chromatography and (iii) ultracentrifugation.

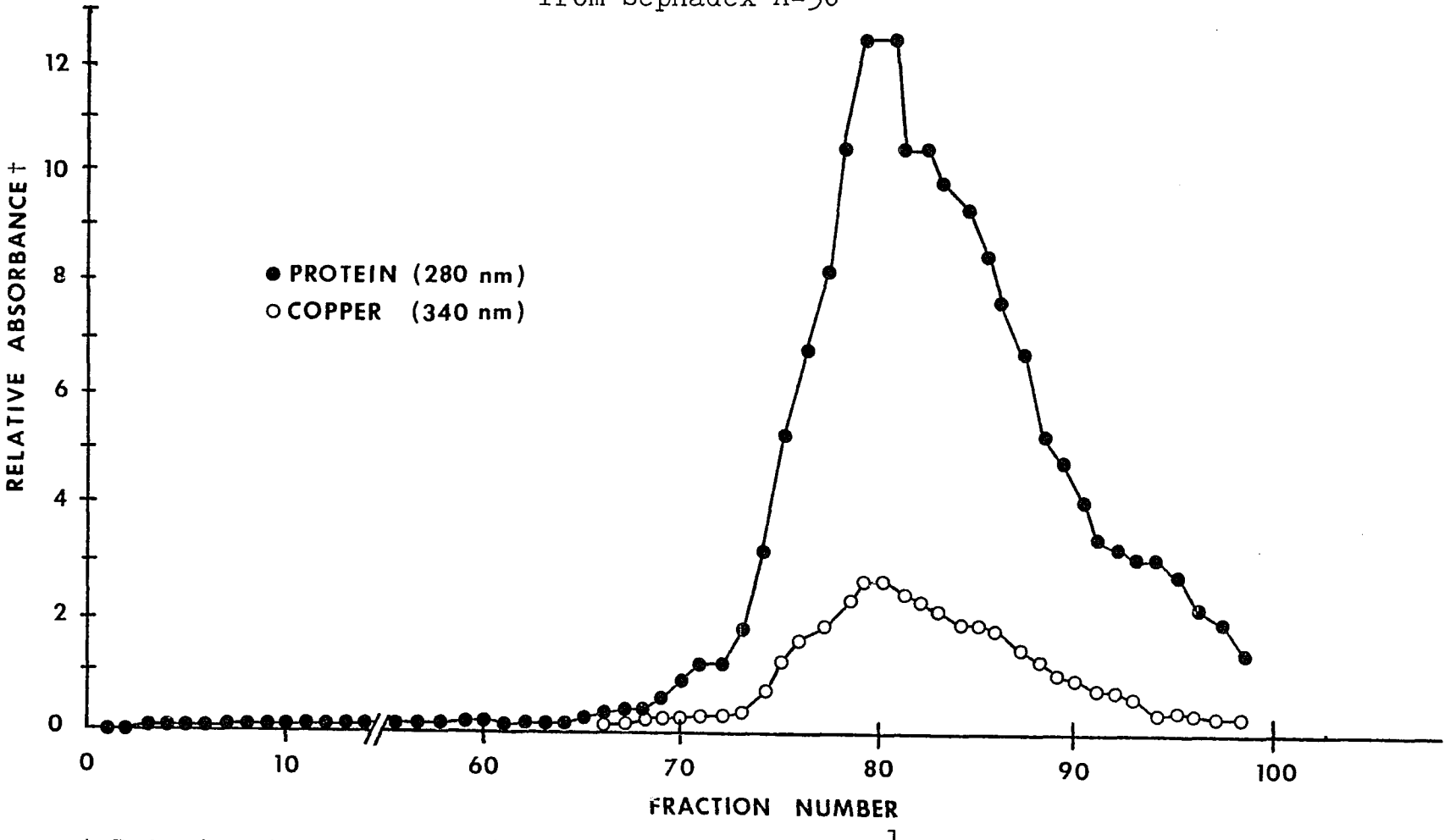
Eluates from A-50 columns were read at both A_{280} and A_{340} for protein and copper respectively. A ratio of A_{340}/A_{280} was calculated for each fraction to determine emergence of copper containing hemocyanin protein. Figure 1 shows that one or possibly two major protein fractions were resolved from HEF. Fractions 73 through 98 exhibited A_{340}/A_{280} ratios of 0.2 or greater and were considered as the purified preparation of hemocyanin-enriched fraction. Fractions 65 - 72, at the beginning of the peak indicate the presence of protein devoid of copper. This leading edge has been reported to represent apohemocyanin or hemocyanin protein without associated copper (Sullivan et al., 1976).

Construction of a standard curve was performed using the Bio-Rad protein assay kit with bovine serum albumin as the standard. Total HEF protein applied to the A-50 column was 864 mg in an 8-ml sample. Purified HEF obtained from collection of column fractions contained a total of 803.4 mg in a volume of 78 ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Sephadex A-50 eluates and unpurified HEF indicated no significant difference in banding patterns. Both samples exhibited a broad band of similarly migrating proteins, thus suggesting homogeneity (Figure 2).

Figure 1

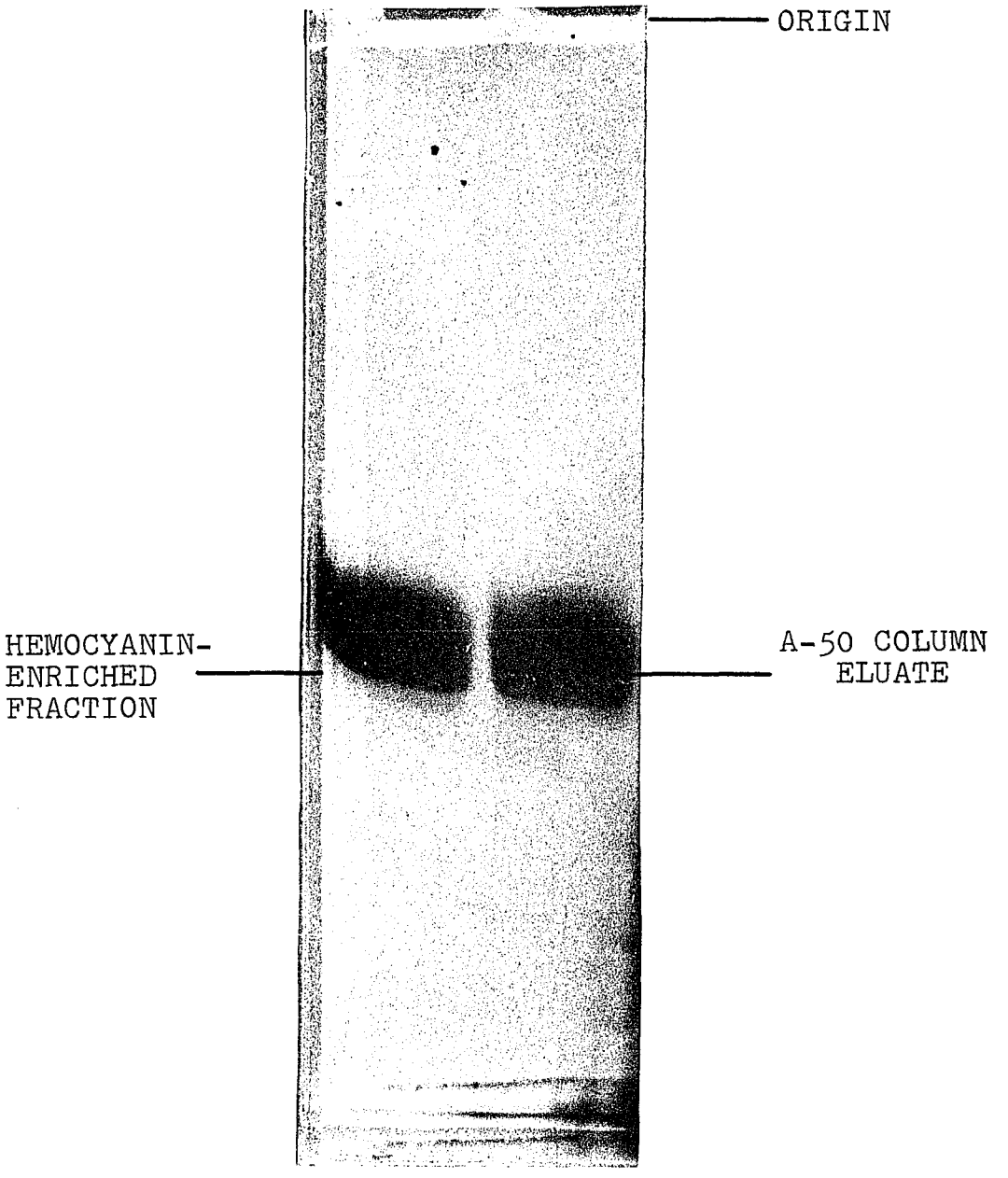
Elution Profile of Hemocyanin-Enriched Fraction
from Sephadex A-50



† Relative Absorbance - Samples were diluted 10^{-1} to obtain absorbance readings. Values presented are adjusted to full scale by multiplying by 10.

Figure 2

Sodium-Dodecyl-Sulfate Polyacrylamide-Gel Electrophoresis of Hemocyanin-Enriched Fraction† and A-50 Eluate of Hemocyanin-Enriched Fraction



† Prepared by ultracentrifugation at 100,000 x g for 2 h.

Sephadex G-100 eluates with A_{340}/A_{280} ratios of 0.2 or greater were collected and considered as purified HEF. Figure 3 shows coincident peaks for copper and protein, with fractions 13 - 26 having A_{340}/A_{280} ratios of 0.2 or greater. Fractions 1 and 3 may indicate contaminating hemolymph protein but the possibility exists that these spikes in the profile may represent the hemocyanin protein devoid of copper as seen in the Sephadex A-50 profile.

Preparation of hemocyanin from whole hemolymph by ultracentrifugation for 5 h at 100,000 x g resulted in a distinct separation of the characteristic blue hemocyanin from the clear lymph component (Figure 4). This is unlike the HEF preparation from whole hemolymph in which a blue gradient is formed after centrifugation at 100,000 x g for 2 h.

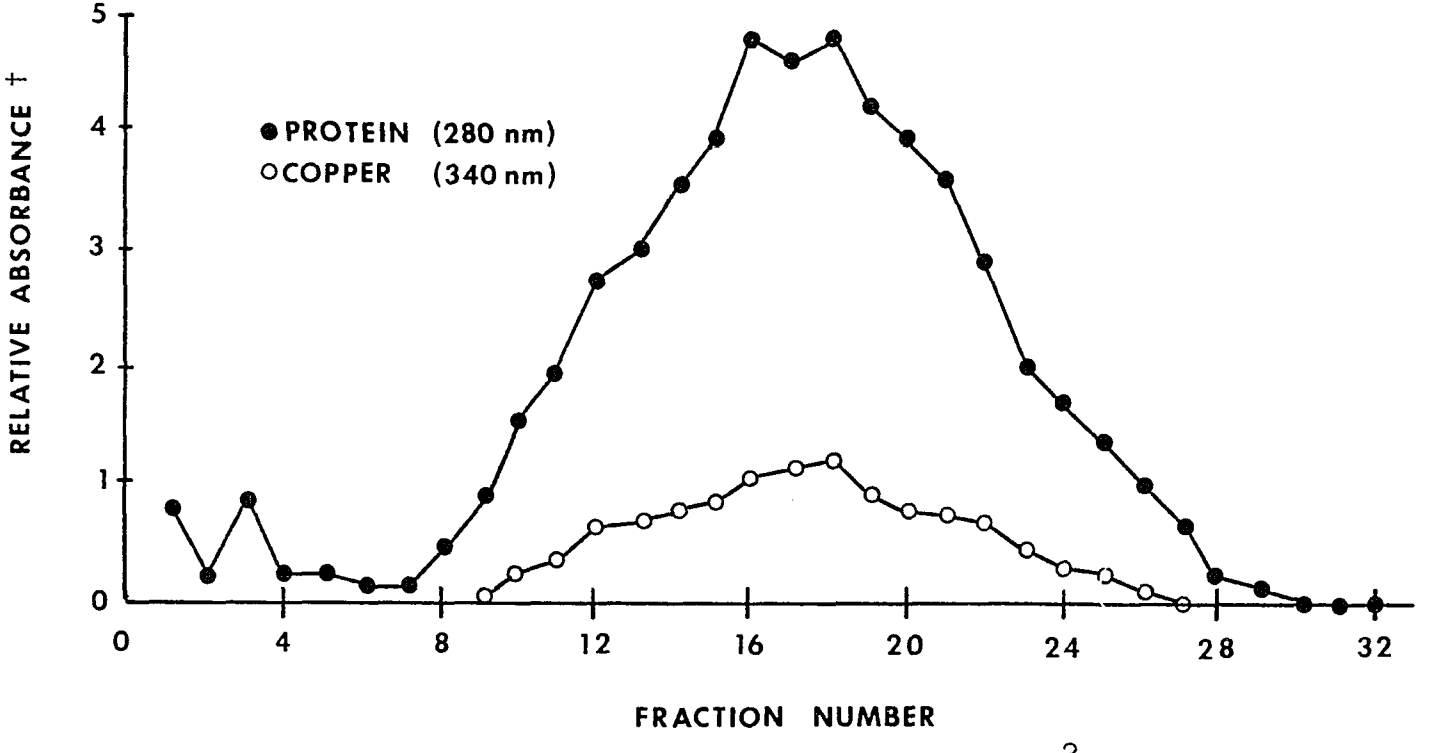
Adsorption and Clarification

Reovirus adsorption to 0.45 μm and 1.0 μm Cox filters was examined at pH 4 - 9 in both tap water (Table 8) and 0.05 M glycine (Table 9). In no instance was there any breakthrough of reovirus when the 0.45 μm filters were used. Additionally, very little breakthrough at any pH was exhibited when 1.0 μm filters were examined. Subsequent experiments in tap water resulted in poor recoveries of reovirus from input samples and massive aggregation was suspected.

Since adsorption studies using Cox filters yielded results that were anomalous, wound-fiber cartridge filters (Carborundum Co.) were examined under conditions established by pH and cation sensitivity studies (Table 10). The condi-

Figure 3

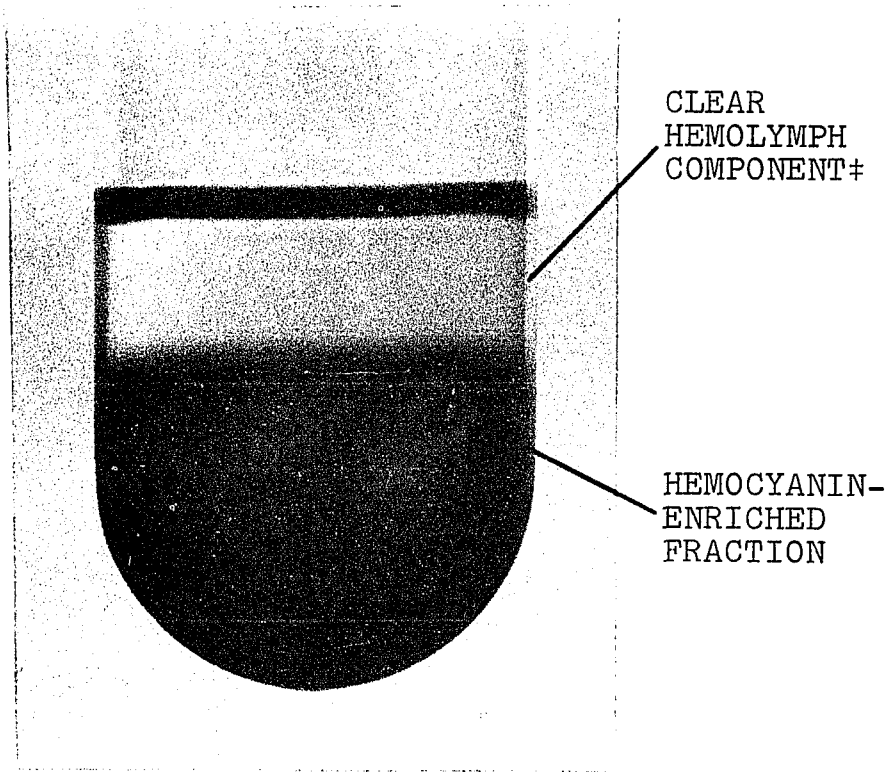
Elution Profile of Hemocyanin-Enriched Fraction
from Sephadex G-100



† Relative Absorbance - Samples were diluted 10^{-2} to obtain absorbance readings. Values presented are adjusted to scale by multiplying by 10.

Figure 4

Hemocyanin-Enriched Fraction
Prepared by Ultracentrifugation†



† 100,000 x g for 5 h

‡ Clear hemolymph component partially removed for demonstration of fractions

Table 8

Percent Adsorption of Reovirus in Tap Water to Cox Filters†

pH	Percent Adsorption 0.45 µm	1.0 µm
4	100.0	100.0
5	100.0	98.9
6	100.0	80.3
7	100.0	69.4
8	100.0	99.5‡
9	100.0	97.2‡

† Average of Four Replicate Experiments
‡ Average of Two Replicate Experiments

Table 9

Percent Adsorption of Reovirus
in 0.05 M Glycine to Cox Filters†

	Percent Adsorption	
pH	0.45 µm	1.0 µm
4	100.0	100.0
5	100.0	94.5
6	100.0	84.6
7	100.0	97.6
8	100.0	92.3
9	100.0	76.6

† Average of two replicate experiments

Table 10

Percent Reovirus Adsorption to Wound-Fiber Cartridge Filters
at pH 6.0
in the Presence or Absence of Magnesium Chloride

	Without Salt		With 0.05 M $MgCl_2$	
Filter†	Replicates	Average Percent Adsorption	Replicates	Average Percent Adsorption
E-39	2	49.1	2	99.8
W10A	5	80.2	6	99.3
K-27	2	46.2	2	98.3
O-19	2	62.5	2	96.6
O-39	3	65.1	3	93.2

† Commercial Filter Division, Carborundum Corp.

tions of moderate acidic pH aided in minimizing aggregative effects and inactivation of viruses. The use of $MgCl_2$ was dictated by the need of an enhancing salt at pH 6.0 for efficient adsorption and by the sensitivity of reovirus to the more efficient $AlCl_3$.

All filters were examined at pH 6.0 in the presence and absence of 0.05 M $MgCl_2$. Without the aid of $MgCl_2$ the W10A filters exhibited the best adsorptive capacity, whereas K-27 and E-39 filters were the poorest adsorbing filters. When $MgCl_2$ was added, all filters essentially behaved in a similar manner with the E-39 filters showing the best overall performance by a narrow margin.

When examining turbid waters, clarifying filters were used to eliminate suspended solids which might foul adsorbing filters and compete with virus for adsorptive sites. However, by eliminating the turbidity, solids-associated virus may also be retained by the clarifying filter.

Since the fiberglass K-27 filters demonstrated the poorest adsorptive capacity, they were considered for use as clarifying filters (Table 11). Without pretreatment of K-27 filters prior to the filtration of virus in tap water, it is evident that a substantial portion of the virus content would be lost to the filter (Table 11). Filtration of reovirus suspended in tap water through a Tween-80 treated K-27 filter at pH 6.0 resulted in no adsorption. However, once $MgCl_2$ was added to the sample the pretreatment was ineffective. Presumably, the magnesium chloride overcame the effect of the Tween-80 treatment.

Table 11

Effect of Pretreatment on Reovirus Adsorption to Fiberglass (K-27) Filters

Sample	Pretreatment	Percent Adsorption
Artificial Seawater, pH 7.1	none	46.9
Artificial Seawater, pH 7.1	none	50.0
Artificial Seawater, pH 7.1	3% beef extract pH 9.5	14.4
Artificial Seawater, pH 7.1	0.1% Tween-80	0
Tap water, pH 6.0	0.1% Tween-80	0
Tap water with 0.05 M $MgCl_2$, pH 6.0	0.1% Tween-80	81.3

Artificial seawater was examined as the suspending medium to determine if the constituent ions would also negate pretreatment. The ability of the K-27 filter to adsorb reovirus when using artificial seawater as the suspending medium was similar to its ability to adsorb reovirus suspended in tap water. Pretreatment with beef extract prior to filtration of the seawater sample reduced adsorption 3-fold and pretreatment with 0.1% Tween-80 eliminated adsorption completely. Therefore, seawater samples could safely be passed through Tween-80 treated K-27 filters for clarification prior to challenge of adsorption filters. However, if $MgCl_2$ was added to the sample to enhance adsorption, it had to be added after passage of the sample through a Tween-80 treated K-27 clarifying filter.

Elution

Choice of pH for elution is dictated primarily by the sensitivity of the virus to alkaline conditions. Under alkaline conditions, the virus surface charge is reversed and the filter and virus are electrostatically repulsed. The maximum allowable pH which reovirus could tolerate was determined to be pH 10.0. Therefore, the extreme condition of glycine at pH 11.5, commonly used for elution, was contraindicated. Glycine was examined at a maximum of pH 10.0, whereas beef extract and HEF solutions were examined at pH 9.5. Alkaline proteinaceous fluids are advantageous as eluents because of their ability to operate at a lower alkaline pH than glycine. In addition, proteins may competitively affect virus release from filter surfaces and

are directly compatible with a protein flocculation reconcentration method. Beef extract is also compatible with the aqueous two-phase polymer system. Hemocyanin enriched fraction was examined as an eluent since it was studied as an alternative reconcentration method.

Cyclic elution of reovirus from wound-fiber cartridge filters exhibiting efficient adsorption characteristics was examined with several different eluents (Table 12). Beef extract prepared as a 3% solution at pH 9.5 efficiently eluted reovirus from W10A and E-39 filters. However, elution from K-27 filters using alkaline beef extract solutions resulted in a somewhat lower average recovery of 72% with a range of 4.7 - 90.3%.

Elution from W10A and K-27 filters using 0.05 M glycine, pH 10.0, resulted in average recoveries of 51.7 and 0.27% respectively. The range of recovery in both cases varied considerably and further examination of glycine as an eluent was discontinued.

Various solutions of hemocyanin enriched fraction were considered as eluents. Solutions of 0.5% HEF adjusted to pH 9.5 with 1N NaOH failed to efficiently elute reovirus from any filter examined. These HEF solutions were unable to maintain their pH once passed through wound-fiber filters containing acidic residue from filtration. However, increasing the ionic strength of the HEF preparation by the addition of NaCl or buffering with sodium bicarbonate resulted in a considerable increase in virus elution efficiency from both W10A and K-27 filters.

Table 12

Elution of Reovirus from Wound-Fiber Cartridge Filters

	Percent Recovery Filter Material		
Eluent	W10A	K-27	E-39
3% beef extract, pH 9.5	43.0 - 100.0	4.7 - 90.3	40.5 - 100.0
0.05 M glycine, pH 10.0	24.0 - 79.8	0.007 - 0.27	-
0.5% HEF,‡ pH 9.5	2.1 - 93.0	0.41 - 0.67	0.40†
0.5% HEF,‡ +0.15 M NaCl, pH 9.5	59.7 - 100.0	>100.0†	10.9†
0.5% HEF,‡ +1 mM Na_2HCO_3, pH 9.5	41.1 - 54.1	>100.0†	0.04†

† Single Trial

‡ Hemocyanin Enriched Fraction

Reconcentration

Processing one-liter volumes of eluate in the laboratory is economically and logistically unreasonable. Second-step concentration methods are utilized to further reduce sample volume and maintain efficient processing. Methods examined for the reconcentration of reovirus from eluates include the aqueous two-phase polymer system, beef extract flocculation, HEF precipitation (HEF-method) and HEF co-precipitation with protamine sulfate (HPS-method).

Preliminary studies were performed to determine reconcentration method efficiencies. The aqueous two-phase polymer system of Wesslen, Albertsson and Philipsson involved the use of mock eluates of beef extract (Table 13). The lower dextran phase developed in the first step was more efficient in virus recovery than completion of the entire two-step procedure. However, the phase collected in the first step was considered to be too large for routine processing.

The organic flocculation method of Katzenelson was examined using 500-ml samples of 3% beef extract (Table 14). Recovery varied from 40 to 80% with an average recovery of 65.9%. Little or no virus was detected in supernatants. Unrecovered virus may have been the result of aggregation, binding to beef extract protein or inactivation by the conditions imposed in the procedure.

Reconcentration using HEF with or without protamine sulfate was examined using reovirus and several representative enteroviruses.

Table 13

Aqueous Two-Phase Polymer
Reconcentration of Reovirus

	Trial 1			Trial 2		
Sample	Volume (ml)	PFU	Percent Recovery	Volume (ml)	PFU	Percent Recovery
Input	500	5.4×10^6	-	500	3.0×10^5	-
Phase 1	75	5.7×10^6	>100	100	3.2×10^5	>100
Supernatant 1		4.6×10^5	9		1.2×10^4	4
Phase 2	14	3.3×10^6	63	23	9.5×10^4	32
Supernatant 2		6.7×10^5	13		5.1×10^4	17

Table 14

Beef Extract Organic Flocculation of Reovirus

Sample	Volume (ml)	Trial 1		Trial 2		Trial 3	
		PFU	Percent Recovery	PFU	Percent Recovery	PFU	Percent Recovery
Input	500	7.3×10^5	-	8.2×10^5	-	8.8×10^6	-
Supernatant	500	0	0	0	0	4.0×10^3	0.05
Precipitate	35	6.1×10^5	84	3.3×10^5	40	6.5×10^6	73.90

Occurrence of virus in supernatant or precipitate fractions as a function of mixing time was examined using poliovirus 2. Using hemocyanin enriched fraction, the majority of poliovirus occurred in the supernatant portion after 15 min of mixing (Table 15). With increased mixing time, successively greater amounts of the virus occurred in the supernatant and approximately 10% of the virus was recoverable from the precipitates at any time in the experiment.

Co-precipitation of HEF with protamine sulfate (HPS-method) using poliovirus resulted in an effect opposite to that observed when using HEF alone. Virus occurring in the precipitate increased with time to a maximum recovery of 85% at 45 min (Table 16). Further examination of the recovery efficiency of the HPS-method with poliovirus resulted in similar results; essentially 100% of the virus was recoverable in the precipitate and only 0.58 to 2.1% of the input virus was recoverable in the supernatant samples (Table 17).

The HPS-method also proved to be very efficient in concentrating coxsackievirus B1 (Table 18) and echovirus 7 (Table 19). Both viruses were poorly reconcentrated when the HEF-method was used with echovirus 7 (Table 20) exhibiting a somewhat better recovery efficiency than coxsackievirus B1 (Table 21).

Reovirus recovery with either the HEF- or HPS-method resulted in considerable variability. When temperature was monitored, it was found that both methods were unreliable in concentrating reovirus at 22°C. However, by performing the

Table 15

Effect of Mixing Time Upon the Recovery of Poliovirus in Supernatants and Precipitates Using 0.5% Hemocyanin-Enriched Fraction

Time (min)	Sample	PFU	Percent Recovery
0	Input	1.88×10^5	-
15	Supernatant	1.15×10^5	61.2
	Precipitate	2.13×10^4	11.3
30	Supernatant	1.46×10^5	77.7
	Precipitate	2.06×10^4	10.9
45	Supernatant	1.83×10^5	97.3
	Precipitate	1.81×10^4	9.6

Table 16

Effect of Mixing Time Upon the Recovery of Poliovirus in Supernatants and Precipitates Using 0.5% Hemocyanin-Enriched Fraction with Protamine Sulfate†

Time (min)	Sample	PFU	Percent Recovery
0	Input	1.88×10^5	-
15	Supernatant	1.16×10^4	6.2
	Precipitate	1.10×10^5	58.5
30	Supernatant	1.66×10^4	8.8
	Precipitate	1.46×10^5	77.7
45	Supernatant	6.60×10^4	3.5
	Precipitate	1.60×10^5	85.1

† Final concentration of protamine sulfate, 0.01%

Table 17

Recovery of Poliovirus II
Using 0.5% Hemocyanin-Enriched Fraction
with 0.01% Protamine Sulfate

Trial	Sample	PFU	Percent Recovery
1	Input	1.96×10^5	-
	Supernatant	4.30×10^3	2.10
	Precipitate	2.92×10^5	>100.00
2	Input	1.96×10^5	-
	Supernatant	1.66×10^3	0.85
	Precipitate	2.68×10^5	>100.00
3	Input	2.32×10^5	-
	Supernatant	1.00×10^3	0.58
	Precipitate	2.08×10^5	89.66
4	Input	1.42×10^5	-
	Supernatant	2.83×10^3	1.66
	Precipitate	2.26×10^5	>100.00

Table 18

Reconcentration of Coxsackievirus B-1
with 0.5% Hemocyanin-Enriched Fraction
and 0.01% Protamine Sulfate

	Trial 1		Trial 2	
Sample	PFU	Percent Recovery	PFU	Percent Recovery
Input	3.30×10^5	-	3.30×10^5	-
Supernatant	0		0	
Precipitate	4.28×10^5	>100	4.52×10^5	>100

Table 19

Reconcentration of Echovirus Type 7
with 0.5% Hemocyanin-Enriched Fraction
and 0.01% Protamine Sulfate

Sample	Trial 1		Trial 2	
	PFU	Percent Recovery	PFU	Percent Recovery
Input	6.03×10^6	-	6.04×10^6	-
Supernatant	9.60×10^5	15.9	8.44×10^5	13.9
Precipitate	4.88×10^5	80.9	4.48×10^6	74.4

Table 20

Reconcentration of Echovirus Type 7
with 0.5% Hemocyanin-Enriched Fraction

	Trial 1		Trial 2	
Sample	PFU	Percent Recovery	PFU	Percent Recovery
Input	6.00×10^5	-	5.70×10^5	-
Supernatant	6.70×10^5	>100.0	3.50×10^5	61.4
Precipitate	2.00×10^5	33.3	2.97×10^5	52.1

Table 21

Reconcentration of Coxsackievirus B-1
with 0.5% Hemocyanin-Enriched Fraction

	Trial 1		Trial 2	
Sample	PFU	Percent Recovery	PFU	Percent Recovery
Input	4.36×10^5	-	4.36×10^5	-
Supernatant	5.15×10^5	>100.0	5.20×10^5	>100.0
Precipitate	3.98×10^4	9.1	4.70×10^4	10.8

procedure at 4°C, the variability was minimized and the HEF-method resulted in reproducible recovery rates (Table 22).

Reconcentration of reovirus with HEF purified by column chromatography resulted in 100% recoveries with both HEF- and HPS-methods (Table 23). Purified HEF prepared by ultracentrifugation resulted in 100% recovery when co-precipitated with protamine sulfate, but a lower recovery resulted when only HEF was used.

Reconcentration procedures involving HEF were determined to be superior in recovery efficiency to organic flocculation and the aqueous two-phase polymer systems. The ability to recover enterovirus in the HPS-method and reovirus in the HEF-method suggested a differential reconcentration of virus from the same sample. Samples processed by the HEF-method would result, as the data presented suggest, in the efficient removal of reovirus by HEF whereas enteroviruses would generally remain in the supernatant portion. After readjustment of HEF to a final concentration of 0.5%, reprocessing of the same sample by the HPS-method could conceivably concentrate the enteroviruses remaining in the sample.

Solutions of 0.5% HEF were determined to be poor eluents for reovirus. Increased ionic strength or buffering allowed HEF to elute reovirus with reasonable efficiencies from K-27 and W10A filters. However, attempts to reconcentrate reovirus from these modified HEF eluents resulted in poor virus recoveries and a considerable protein floc after attempted resuspension. Thus the differential ability of HEF could not be examined in model filtration-elution experiments.

Table 22

Effect of Temperature on the
Reconcentration of
Reovirus Using Hemocyanin-Enriched Fraction

Method	Temperature	Replicate	Percent Recovery
HEF†	22°C	1	8.0
		2	68.2
		3	28.5
	9°C	1	>100.0
		2	>100.0
		3	>100.0
		4	64.1
		5	81.5
HPS‡	22°C	1	>100.0
		2	7.4
		3	3.6
	9°C	1	>100.0
		2	49.2
		3	>100.0
		4	30.1
		5	12.5

† 0.5% Hemocyanin-Enriched Fraction

‡ 0.5% Hemocyanin Enriched-Fraction and 0.01% Protamine Sulfate

Table 23

Reovirus Reconcentration with Purified Hemocyanin-Enriched Fraction†

Purification Method	Sample	0.5% Hemocyanin-Enriched Fraction PFU	0.5% Hemocyanin-Enriched Fraction Percent Recovery	0.5% Hemocyanin-Enriched Fraction and 0.01% Protamine Sulfate PFU	0.5% Hemocyanin-Enriched Fraction and 0.01% Protamine Sulfate Percent Recovery
A-50 Column	Input	6.9×10^4	-	5.6×10^3	-
	Supernatant	0	0	0	0
	Precipitate	8.1×10^4	>100	3.1×10^4	>100
G-100 Column	Input	2.6×10^5	-	2.6×10^5	-
	Supernatant	0	0	0	0
	Precipitate	2.9×10^5	>100	3.8×10^5	>100
Ultracentrifugation	Input	3.9×10^5	-	2.4×10^5	-
	Supernatant	1.0×10^4	2.5	0	0
	Precipitate	2.4×10^5	61	5.3×10^5	>100

† $4^{\circ}C$, Tap water

Model System

A model system for the recovery of reovirus from aqueous environments was devised based upon the results previously described (Table 24).

Adsorption was performed at pH 6.0 in the presence of 0.05 M $MgCl_2$. Viscose E-39 filters gave superior results under these conditions, and hence, were used as the adsorbing filter. However, both W10A and K-27 filters could have served equally as well. Since all filters adsorbed reovirus relatively efficiently in the presence of $MgCl_2$, clarification of influent samples was performed before the addition of magnesium chloride to the filter stream.

Fiberglass K-27 filters were used as clarifying filters since they were the least efficient adsorber in the absence of $MgCl_2$. Tween-80 treatment of K-27 filters preceeded their use and eliminated all detectable adsorption activity.

Cyclic elution was performed using 3% beef extract, pH 9.5. The use of beef extract was dictated by its efficiency in eluting reovirus from E-39, K-27 and W10A filters and by the inability of HEF eluents to reconcentrate reovirus.

In two trials of the model system, recovery efficiencies of 40 and >100 percent were attained using elution times of 20 and 30 min respectively.

Table 24

Model System for Concentration
of Reovirus from Aqueous Systems

	Trial 1 †				Trial 2‡			
Sample	PFU/ml	Volume (ml)	Total PFU	Percent Recovery	PFU/ml	Volume (ml)	Total PFU	Percent Recovery
Input	2.28×10^2	15000	3.42×10^6	-	1.28×10^2	15000	1.87×10^6	-
Eluate	1.38×10^3	1000	1.38×10^6	40.3	1.75×10^3	1000	1.75×10^6	93.5
Reconcentrate		37	1.37×10^6	40.0	7.23×10^4	35	2.53×10^6	>100

† Elution for 20 min
‡ Elution for 30 min

DISCUSSION

Although animal viruses have been readily demonstrated in finished and unfinished waters, no single established method exists for the recovery of all of these viruses from aqueous environments. Ideally, a standard method should be (i) insensitive to the type of water processed, (ii) able to concentrate most viruses known to occur in surface waters, (iii) relatively easy and economical to perform and (iv) able to detect aggregates (Wallis et al., 1979). If an indicator system is to be used as a viable alternative, the indicator microorganism should always be present in the original source of pollution and it should be readily detectable (Katzenelson and Kedmi, 1979).

Virus Indicator Systems

Enteroviruses have been the choice as the virologic indicator of fecal wastes since they are constantly replicated in the mammalian gut, excreted in high numbers, and can withstand adverse environmental conditions for relatively long periods of time.

The need to detect animal viruses directly has been emphasized by the inability of indicator systems to accurately correlate the indicator organism to the virus content of a given sample. The fecal coliform standard has been shown to be deficient since coliforms are much more sensitive to chlorine treatment than the enteroviruses (Berg et al., 1978). Samples free of fecal coliforms may not insure the absence of animal viruses.

Like the enteroviruses, various bacteriophages have been shown to be insensitive to chlorine treatment (Kott, 1974) and, thus, could serve as simple alternative indicators. However, the use of phage as an indicator of viral pollution has resulted in conflicting data regarding the significance of their occurrence in a sample (Kott, 1976; Vaughn and Metcalf, 1975).

Adsorption of Virus to Filters

Emphasis has been directed toward the recovery of enteroviruses rather than enteric viruses in general. Enteroviruses are generally adsorbed to filter substrates at a pH below either isoelectric point (pI). Under these conditions the virus would have a net positive charge (Mandel, 1971) and would be electrostatically attracted to the negatively charged filter surface (Kessick and Wagner, 1978).

Mandel (1971) reported that poliovirus 1 (Brunhilde) exhibited two isoelectric points occurring at pH 4.5 (state B) and 7.0 (state A). The virus population, as a whole, was electrophoretically homogeneous and each particle could exhibit either pI depending upon the pH of the suspending medium. Above pH 7.0 and below pH 4.0, the viruses showed pIs of 7.0 and 4.5 respectively. At intermediate pH values, the population was in equilibrium between the two states. Floyd and Sharp (1978) reported a pI of 8.2 for poliovirus 1 (Mahoney), which is somewhat different than the A-state, at pH 7.0, reported by Mandel (1971). Generally, the pI value of 4.5 is considered as the accepted value, and, therefore, poliovirus is described as negatively charged at a moderate

acid pH. However, if the equilibrium condition of poliovirus is operating at a pH range of 4 - 7, a certain number of viruses would be negatively charged at a given pH and the remainder would be positively charged. Proteins with more than one isoelectric pH will have a small net charge over a broad range of pH due to the balance of ionizable groups present (Mix, 1973). Mandel (1971) demonstrated the small variability of poliovirus 1 (Brunhilde) mobility rates as a function of pH in an electrophoretic field. Between pH 4.5 and 5.5 the virus exhibited a small net negative charge and between pH 5.5 and 7.0 the virus was net positive. This effect was directly linked to the two pIs of this poliovirus strain. Considering this effect, addition of cationic salts would facilitate the adsorption of any net negatively charged viruses to the filter surface and also cross-complex any residual negative charge available on those viruses having a net positive charge. This would result in the observed overall enhanced adsorption. However, most literature (Wallis et al., 1979; Mix, 1973) ignores the equilibrium condition of poliovirus in the pH range of 4 - 7 and simply states that the virus is net negatively charged above pH 4.5. Mandel (1971) showed that the virus was stabilized in the B-state (pI 4.5) when the virus was exposed to conditions that resulted in the loss of infectivity. Therefore, the equilibrium state may account for poliovirus activity at the filter surface more accurately. Most other viruses have one pI and the situation is greatly simplified.

Reovirus adsorbed to various wound-fiber filters at pH 6.0 in the absence of cationic salts with an efficiency range of 46 - 80%. These conditions may allow for the adsorption of virus particles if a significant residual positive charge was still available.

Reovirus-3-Dearing has a reported pI of 3.9 (Floyd and Sharp, 1978). Assuming that the three major types of reovirus have pI values which are similar ($\pm$ 1 pH unit), Reovirus-1-Lang should have a net negative charge at pH 6.0 and, according to electrostatic theory, should not adsorb to the filter surface efficiently. The degree of adsorption found under these conditions (pH 6.0) may be due to simple entrapment of virus in the wound-fiber depth filter. However, since these experiments were performed in tap water, a sufficient amount of cationic species (especially Ca^{+2}) may be present to cause some specific adsorption.

When reovirus adsorption was attempted with the same filter materials in the presence of 0.05 M $MgCl_2$ at pH 6.0, the efficiency of adsorption in all cases was nearly 100%. As with the enteroviruses, cations enhanced adsorption of virus to the filter surface. Since both the reovirus and filter would be net negatively charged, cationic salt bridging may be responsible for virus adsorption. The divalent cations would react with both virus and filter surfaces with the net effect being an increase in the virus pI (a less negatively charged particle at pH 6.0) and a reduction of negative charges on the filter surface (Kessick and Wagner, 1978). Adsorption of the cation to both surfaces would not necessarily

mean the elimination of all available negative charge and, thus, the possibility of cross-complexing between the two surfaces simultaneously is likely.

Magnesium chloride was used as the enhancing salt since experimental results indicated that reovirus was sensitive to low concentrations of aluminum chloride. Aluminum chloride would be the preferred enhancing salt since it could be used at concentration one magnitude lower than $MgCl_2$ without a loss in adsorption efficiency. Referring to the Hofmeister series in which adsorption increases with decreases in the hydrated radii of the ions (Mix, 1973), a trivalent cation should exhibit a greater affinity for a negatively charged surface.

The inability to recover reovirus from dilute solutions of $AlCl_3$ may be related to a tenacious binding of the Al^{+3} ions to the surface of the virion. This may ultimately interfere with the sites necessary for virus adsorption to monolayers during assay. However, at neutrality, aluminum hydroxide flocs could be present and virus may have been entrapped in the floc or electrostatically attracted to various aluminum hydroxide complexes (Weber, 1972). Floyd and Sharp (1978) showed that a pH 5.0 reovirus is either trapped or aggregated in the polymeric form of the hydroxide. These complexes are soluble at alkaline pH but none of the samples were treated in this manner since, traditionally, the flocs are dissolved in pH 11.5 glycine solutions (Berg, 1971; Wallis et al., 1979) and reovirus was shown to be sensitive to alkaline conditions greater than pH 10.0. If reovirus was

severely aggregated in these aluminum flocs, it would be an advantage during filtration to have these complexes adsorb to the filter surface. The drawbacks of the concept are: that the flocs will foul the filters more rapidly and a high alkaline pH would be necessary to solubilize the complexes after filtration. Reducing the alkalinity of the eluate would only decrease the solubility of the aluminum hydroxide flocs and, consequently, reduce reovirus recovery by driving the reaction closer to the original condition which optimized floc formation (Weber, 1972). Therefore, $AlCl_3$ was not considered as an enhancing salt.

Magnesium chloride is less efficient as an adsorption enhancing salt, but reovirus was readily recoverable from 50 mM $MgCl_2$ solutions. Magnesium complexes are not affected by alkaline conditions in the same manner as aluminum hydroxide flocs. Solubilization of Mg^{+2} cation bridges between virus and filter would not occur at alkaline pH (Kessick and Wagner, 1978). Experiments involving the use of 0.05 M glycine, pH 10.0, as an eluent for reovirus adsorbed in the presence of Mg^{+2} ion indicate that low or variable recovery occurred. Hence, the use of glycine as an eluent was discontinued.

Sample Clarification

Fiberglass K-27 filters were used for clarification since they exhibited the poorest adsorptive capacity at pH 6.0 in the absence of $MgCl_2$. Kessick and Wagner (1978) found that epoxy-fiberglass materials exhibited a significant negative electrophoretic mobility in a pH range of 4 - 6 with the

greatest net negative charge occurring at pH 5.0. It was suggested that this charge was due to the -SiOH groups available for ionization. Therefore, reovirus would not be efficiently adsorbed to fiberglass filters at pH 6.0 in the absence of cationic salts.

Treatment of the K-27 filters with Tween-80 completely eliminated any residual adsorptive capacity as long as a substantial concentration of divalent cations did not come into contact with the filter during virus concentration.

Elution and Reconcentration

Alkaline proteinaceous fluids have been employed successfully as an alternative method of elution in instances where glycine is ineffective. A major advantage of protein eluents is that they operate at a lower pH, thus, reovirus could be eluted without inactivation. Protein solutions at alkaline pH could possibly compete with virus for adsorption sites, bind residual cations, and allow charge reversal because of their pH.

Beef extract was shown to be the superior eluent for the recovery of reovirus adsorbed to filters in the presence of magnesium chloride. The small difference in the time required to increase reovirus recoveries during elution from filters in the model system suggests that forces other than electrostatic charge reversal due to pH are responsible for virus release. Shear forces at the filter surface created by eluent flow probably aid in the release of trapped virus and may be responsible for reduction of virus aggregation.

The inability of 0.5% HEF solutions to operate in a manner similar to beef extract was due to the drastic change in pH which occurred once the eluent came into contact with the acidic residue retained by the filter. The solution, as prepared, did not have the buffering capacity of the beef extract solution. Altering the ionic strength of the HEF solutions increased the elution of reovirus from K-27 and W10A filters but a change from alkaline to acidic pH still occurred. Virus elution could be attributed to the adsorption of Na^{+} ions to the remaining negative charge at the filter and virus surface. The final result would be an increase in the degree of repulsion between virus and filter due to the increase in the net positive charge on both surfaces. Floyd and Sharp (1978) showed that at high cation concentrations, aggregation is prevented since all the viruses in a suspension are strongly positively charged. Unless the concentration is increased to the point where the anionic component affects a charge reversal at the surface, the virus will remain dispersed. The same mechanism may be operating at the filter surface with the NaCl supplemented HEF. The additive effect of residual Mg^{+2} and Na^{+} ions in the eluent could cause a net positive charge at both surfaces with the resultant release of virus due to repulsion. The virus could be viewed as being salted off the membrane in a manner analogous to removal of proteins from ion exchange columns.

Sodium bicarbonate-HEF solutions afforded buffering capacity plus an increase of available cations. Shifts in

the pH of this eluent did not occur and elution ability was equal to the NaCl supplemented HEF eluents.

Hemocyanin enriched fraction prepared as a 0.1% solution was capable of eluting virus if the pH change was not drastic. Application of this method to an adsorption-elution method for bacteriophage MS2 using Cox epoxy-fiberglass filters resulted in elution efficiencies of 100% (Blaine, 1979). However, the fluid retention capacity of the flat Cox filters is much less than the wound-fiber depth filters. Very small amounts of acidic residue would be present at the end of filtration and the maintenance of the alkaline pH of the HEF solution could be assured. In this same procedure, use of the HEF-method for reconcentration of MS2 after adjustment of the HEF content of the eluent to 0.5%, also resulted in efficient recovery.

Examination of the reconcentration methods involving hemocyanin proteins revealed that a possible differential reconcentration ability existed. The HPS-method concentrated both enteroviruses and reovirus, whereas the HEF-method was able to reject enteroviruses while concentrating reovirus. The ability of hemocyanin proteins to flocculate from a solution and to adsorb virus is dependent upon the nature of the protein itself and the conditions under which flocculation is attempted.

Hemocyanins are high molecular weight globular proteins used for oxygen transport in various invertebrate genera. The hemocyanin of Limulus polyphemus has subunits of molecular weights varying from 65,000 - 70,000 Daltons (Sullivan et al.,

1976). Amino acid analyses of the five major zones resolved by Sephadex G-100 fractionation showed a high degree of similarity among the subunits. Due to the large number of aspartic and glutamic acid residues, the aggregate has an acidic characteristic with an isoelectric pH of 5.5. Maximum flocculation of the protein would occur at its isoelectric point since the proteins become increasingly dissimilar as they approach this pH and overcome the repulsive effect that allows them to remain soluble above or below the pI. This particular pH would be relatively harmless to most viruses of interest occurring in water systems. In the HEF-method, maximum floc formation occurred at pH 6.0. This slight shift in pI could be due to the presence of divalent cations (especially Ca^{+2}), which occur in tap water, binding to the protein and altering the overall charge. Kessick and Wagner (1978) suggested that cation binding to virus surfaces would cause shifts in the pI to a higher value and specific binding of cations to filter surfaces could also cause a net increase of positive charge. A similar phenomenon may be responsible for the apparent shift in the pI of hemocyanin.

At pH 6.0, aspartic and glutamic acids would contribute to the negative charge of the protein, since the protein is at (or near) its isoelectric pH, other ionizable species will contribute to the net zero charge that should occur at the isoelectric point. Lysine, histidine and arginine residues, which are also predominant in hemocyanin proteins, will contribute the necessary positive charge at the pI.

In the HPS-method the addition of protamine sulfate, rather than pH adjustment, causes floc formation. Protamines are low molecular weight proteins (6,000 - 17,000 mw) which contain a large number of arginine residues. The predominance of arginine is responsible for the high pI value found in this class of proteins. Protamine sulfate has a pI of 12.0 and will be positively charged in most aqueous preparations (Cookson, 1973).

Due to the attraction of the oppositely charged protein species, addition of protamine sulfate to a 0.5% HEF solution at a slightly alkaline pH (ca. pH 8.0) will cause the formation of a floc and a shift in the pH to 7.0.

Viruses are generally negatively charged near neutrality and, hence, would be attracted to the positive charges on the protamine sulfate and hemocyanin. A similar mechanism was suggested by Cookson (1973) for the protamine sulfate concentration method examined by England (1972) in which bovine serum albumin was used to enhance flocculation.

The aggregation of colloidal particles occurs in two steps: (i) particle transport to cause interparticle contact and (ii) particle destabilization to allow attachment once contact occurs (Weber, 1972). The effectiveness of transport is dependent upon temperature, viscosity, agitation and the concentration of reactants in the solution. Brownian motion is responsible for some of the effects of aggregation but would not be an efficient mechanism for virus removal in the absence of additional colloids or metal precipitates to drive the reaction. The use of HEF as an

ampholytic polyelectrolyte and the subsequent pH adjustment or addition of protamine sulfate allows for sufficient interparticle contact and aggregate growth during mixing. The mixing simply allows for additional contact between particles. When agitation contributes to the aggregation of particles, the process is termed orthokinetic flocculation as opposed to the random motion due to Brownian movement (perikinetic flocculation) (Weber, 1972). With increased contact, particle growth (aggregation) will increase and the effective size of any virus is increased if the contact is productive. Increases in aggregate size will result in more effective virus removal upon centrifugation.

Particle destabilization can be attained by several mechanisms: (i) Double-layer compression; (ii) adsorption and charge neutralization; (iii) enmeshment in a precipitate and (iv) adsorption with interparticle bridging (Weber, 1972). The latter two mechanisms could best explain the activity of HEF proteins in aqueous suspensions. Enmeshment is dependent upon the degree of oversaturation and the charge of the precipitate. The rate of precipitation is increased if the precipitate is positively charged and anions are present in the solution. Additionally, colloids can serve as the nuclei for precipitate formation. In the case of concentration of reovirus with HEF, two colloids are actually involved. In the HEF-method, hemocyanin protein would contain both positive and negative charges at or near its pI. The virus would be negatively charged and would behave as the anionic component. Since the HEF will be

attracted to itself at the isoelectric point, it serves as the nucleus for aggregate formation and eventually involves virus in the complex.

Adsorption and interparticle bridging is dependent upon a polymeric component with a sufficient number of ionizable groups to allow for reaction and complexing. Initially some adsorption of virus will occur to HEF protein and the remainder of the protein extends into the solution and is available for other viruses or virus-protein aggregates. Particle-polymer-particle complexes are formed and additional bridging will occur between adjacent complexes. In the HPS-method, complexing could be enhanced by the presence of protamine sulfate.

The inability of the HEF-method to concentrate poliovirus could be due to the extended mixing time. It has been shown that interparticle bridges could be reversed with sufficient agitation even after aggregation has occurred (LaMer and Healy, 1963). Alternatively, since the amount of poliovirus found in the precipitate remains constant, complexing may not have occurred to any degree. The increase in virus in the supernatant may be due simply to the disruption of virus aggregates by shear forces.

Enterovirus concentration by the HPS-method was attributed to the strongly positive protamine sulfate which would adsorb virus and also cause extensive cross-complexing.

Reovirus was inconsistently concentrated by both methods at 22°C. Upon reduction of the temperature to 9°C, reovirus concentration using the HEF-method became efficient and

reproducible but results with the HPS-method were still variable. Reovirus may have been poorly released from the heavy floc collected in the HPS-method since a considerable precipitate remained after resuspension was attempted. Temperature reduction would reduce the solubility of the HEF protein and account for the more efficient reovirus removal upon centrifugation.

Virus size, as the criterion for the differential concentration ability of HEF for enterovirus and reovirus, is not consistent with results obtained using bacteriophage MS2. This bacteriophage is approximately the same size as poliovirus yet it is concentrated by the HEF-method. Other undescribed surface phenomena may be responsible for the ability of HEF to concentrate some viruses while rejecting others. Reovirus and poliovirus were shown to bind divalent cations differently (Floyd and Sharp, 1978) and the specific sites for the uptake of these cations were operative at a wide range of pH. The conditions which allow the aggregation of the virus could not be extrapolated to other viruses under the same experimental conditions. Adsorption may be dependent upon a similar mechanism.

Although reovirus was eluted from depth filters using HEF supplemented with NaCl or prepared in buffer, reconcentration was unsuccessful. Unlike model HEF-reconcentration, considerable floc remained after resolubilizing these precipitates and virus was not recovered from precipitates or supernatants. Since virus was recovered upon elution but not after reconcentration by either HEF- or HPS-methods using

supplemented HEF solutions, reovirus may still be entrapped in these residual precipitates and could not be released by the procedures used. England (1972) used 1 M NaCl to dissolve precipitates, whereas the method presented here resuspended precipitates in 0.15 M Na_2HPO_4, at pH 7.5. Virus-salt-protein complexes were probably not fractionated under the ionic conditions used.

Conclusions

From the data presented, the critical factor in the recovery of reovirus would not appear to be the conditions necessary for adsorption but, rather, the choice of the proper eluent-filter combination. Berg (1971) and co-workers found that elution of reovirus from cellulose-nitrate filters required sonication and was enhanced by the addition of pancreatin. Decreasing porosity had a marked effect on the recovery rate upon elution, with the smaller porosities yielding better recoveries. The rigorous elution method indicates the difficulty with which reovirus is eluted from filters.

A possible relationship of filter porosity and reovirus adsorption was seen when flat epoxy-fiberglass Cox filters were used. No breakthrough occurred at any pH examined when 0.45 μm filters were used as adsorption filters for reovirus suspended in tap water or glycine. Use of 1.0 μm Cox filters resulted in some correlation of adsorption and pH. In the range of pH 4 - 7, adsorption decreases with increases in tap water pH. A similar trend was indicated at pH 4 - 6 when virus adsorption to 1.0 μm Cox filters was examined

using glycine as the suspending medium. Floyd and Sharp (1977) showed that severe virus aggregation occurred at low pH except at pH 6.0 where little aggregation was indicated. The results reported here are consistent with Floyd and Sharp's results since least adsorption of reovirus to filters occurred at pH 6.0 and 7.0. At these pH values, the least aggregation would be expected and increased breakthrough should occur. Results showing adsorption at an alkaline pH may be due to experimentally induced aggregation. In the alkaline range, reovirus was seeded into pH 7.0 tap water or glycine. The pH was then sequentially adjusted to pH 8.0 and 9.0. Aggregates induced during seeding may not have been properly dispersed at these low alkaline values. Addition of a sufficient amount of glycine, pH 11.0, to acidic tap water samples for a final pH of 7.0 was shown to reverse reovirus aggregation (Floyd and Sharp, 1977). Experiments performed in glycine showed a decrease in reovirus adsorption with pH increases from 7.0 to 9.0. Under these conditions, the buffer may have been responsible for some degree of aggregate dispersal since this reversal did not occur in tap water samples. Therefore, the anomalous results were due to the retention and/or adsorption of viral aggregates to Cox filter surfaces and did not accurately reflect the behavior of individual virions. To prevent aggregation of virus upon addition to water samples, further adsorption studies with wound-fiber filters were performed in larger volumes and reovirus was initially diluted into 0.05 M glycine, pH 7.5.

Moderations of the conditions imposed by current adsorption-elution technology could allow the reproducible recovery of reovirus from water samples. Alternative methods of reconcentration should be sought to selectively isolate various viruses, especially in cases where the predominance of one virus in a sample will cause the masking of the presence of another potentially pathogenic virus.

Poliovirus has generally been used as the indicator of the virologic quality of water. Studies have determined that poliovirus could become increasingly insensitive to chlorine treatment in the laboratory (Bates et al., 1977) and a similar adaptive mechanism may be occurring in the environment. This suggests that virus monitoring could be the critical factor in the estimation of water quality even after acceptable chlorine treatment. This may be the case especially where virus is protected by various organic solids (Hejkal, 1979) and made unsusceptible to chlorination. Katzenelson (1979) showed, however, that despite the routine administration of "live" polio-vaccine to the infant population, poliovirus was not detectable in 50% of sewage samples, effluents from oxidation ponds and spray irrigation lines.

Reovirus has been recovered from chlorinated primary effluents in excess of any enteroviruses isolated under the same conditions (Sattar and Westwood, 1978). Therefore, reovirus may occur in various surface waters long after enteroviruses have ceased to be detectable. In addition, reovirus survived anaerobic digestion of sludge whereas poliovirus did not (Ward and Ashley, 1977). The virucidal

agent in this digested sludge was determined to be ammonia in its uncharged state (i.e. above pH 8.0). This insensitivity of reovirus to the routine processing of wastewaters and sludges suggests that reovirus may be a superior indicator of the degree of viral disinfection.

With the increased interest in the occurrence of rotavirus as a pathogen transmitted by the water route and the inability to detect natural rotavirus, it has been suggested that reovirus might serve as a model for the ability to recover rotavirus (Farrah et al., 1978). The similarities between the rota- and reoviruses extend beyond morphological characteristics and include the possible occurrence of carbohydrate moieties on their surface, inability to be recovered with aluminum hydroxide flocs and difficulty in routine assay. Therefore, evidence suggests that not only could reovirus serve as a model for the recovery of rotaviruses, but could also serve as an indicator of the virologic quality of various waters and sewage sludges intended for land disposal.

LITERATURE CITED

Aiken, E.W., W.H. Benton and W.F. Hill, Jr. 1971. Enteric viruses in ground and surface waters: a review of their occurrence and survival. Pages 59-74 in V. Snoeyink (ed.), Viruses and water quality: occurrence and control. University of Illinois, Urbana.

Albertsson, P.A. 1974. Concentration of viruses by two phase separation. Pages 16-18 in J.F. Malina and B.P. Sagik (eds.), Virus survival in water and wastewater systems. Water Resources Symposium No. 7. University of Texas, Austin.

Alder, J.L. and R. Zickl. 1969. Winter vomiting disease. J. Infect. Dis. 119:668-673.

Bates, R.C., P.T.B. Shaffer and S.M. Sutherland. 1977. Development of poliovirus having increased resistance to chlorine inactivation. Appl. Env. Microbiol. 34: 849-853.

Belfort, G., Y. Rotem and E. Katzenelson. 1974. Virus concentration using hollow fiber membranes. Water Res. 9:79-85.

Belfort, G., Y. Rotem and E. Katzenelson. 1976. Virus concentration using hollow fiber membranes - II. Water Res. 10:279-284.

Beard, J.W. 1967. Host-virus interaction in the initiation of infection. Pages 167-192 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Berg, G., D.R. Dahling and D. Berman. 1971. Recovery of small quantities of viruses from clean waters on cellulose nitrate filters. Appl. Microbiol. 22:608-614.

Berg, G., D.R. Dahling, G.A. Brown and D. Berman. 1978. Validity of fecal coliforms, total coliforms, and fecal streptococci as indicators of viruses in chlorinated primary sewage effluents. Appl. Env. Microbiol. 36: 880-884.

Blaine, K. 1979. A model system for the concentration of coliphage MS2 from polluted surface waters. M.S. Thesis. University of New Hampshire, Durham. 52 pp.

Bolivar, R., R.H. Conklin, J.J. Vollet, L.K. Pickering, H.L. DuPont, D.L. Walters and S. Kohl. 1978. Rotavirus in travelers' diarrhea: study of an adult student population in Mexico. J. Infect. Dis. 137:324-327.

Cliver, D.O. 1965. Factors in the membrane filtration of enteroviruses. Appl. Microbiol. 13:417-425.

Cliver, D.O. 1967. Detection of enteric viruses by concentration with polyethylene glycol. Pages 109-120 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Cliver, D.O. 1968. Virus interactions with membrane filters. Biotechnol. Bioeng. 10:877-889.

Cookson, J.T. 1973. The chemistry of virus concentration by chemical methods. Pages 160-173 in E.D. Murray and A.W. Bourquin (eds.), Developments in industrial microbiology, Vol. 15. American Institute of Biological Sciences, Arlington, VA.

Dahling, D.R., G. Berg and D. Berman. 1974. BGM, a continuous cell line more sensitive than primary rhesus and African green monkey kidney cells for the recovery of virus from water. Hlth. Lab Sci. 11:275-282.

Davis, B.J. 1964. Disc electrophoresis - II. Method and application to human serum proteins. Ann. NY Acad. Sci. 121:404-427.

Dienstag, J.L., I.D. Gust, C.R. Lucas, D. Wong and R.H. Purcell. 1976. Mussel-associated viral hepatitis type A: serological confirmation. Lancet. 1:561-564.

Dismukes, W.E., A.L. Bisno, S. Katz and R.F. Johnson. 1969. An outbreak of gastroenteritis and infectious hepatitis attributed to raw clams. Am. J. Epidemiol. 89:555-561.

Dolin, R., N.R. Blacklow, H. DuPont, S. Formal, R.F. Buscho, J.A. Kasel, R.P. Chames, R. Hornick and R.M. Chanock. 1971. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. J. Infect. Dis. 123:307-312.

Drouhet, V. 1960. Lésions cellulaires provoquées par les réovirus (virus ECHO 10). Anticorps flourescents et études cytochimique. Ann. Inst. Past. 98:618-621.

Elias, M.M. 1977. Distribution and titers of rotavirus antibodies in different age groups. Br. J. Med. 24:2-9.

Ellender, R.D. and B.H. Sweet. 1972. Newer membrane concentration processes and their application to the detection of viral pollution of waters. Water Res. 6:741-746.

England, B. 1972. Concentration of reovirus and adenovirus from sewage effluents by protamine sulfate (salmine). Appl. Microbiol. 24:510-512.

England, B., R.E. Leach, B. Adame and R. Shiosaki. 1967. Virologic assessment of sewage treatment at Santee, California. Pages 401-417 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Englebrecht, R.S. and E.O. Greening. 1978. Chlorine-resistant indicators. Pages 243-296 in G. Berg (ed.), Indicators of viruses in water and food. Ann Arbor Science Publishers Inc., Ann Arbor, MI.

Fager, L.Y. and J.O. Alben. 1972. Structure of the carbon monoxide binding site of hemocyanins studied by fourier infrared spectroscopy. Biochem. 11:4786-4792.

Farrah, S.R., C.P. Gerba, C. Wallis and J.L. Melnick. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Appl. Env. Microbiol. 31:221-226.

Farrah, S.R., C.P. Gerba, S.M. Goyal, C. Wallis and J.L. Melnick. 1977a. Regeneration of pleated filters used to concentrate enteroviruses from large volumes of tap water. Appl. Env. Microbiol. 33:308-311.

Farrah, S.R., S.M. Goyal, C.P. Gerba, R.H. Conklin and E.M. Smith. 1978. Comparison between adsorption of poliovirus and rotavirus by aluminum hydroxide and activated sludge flocs. Appl. Env. Microbiol. 35:360-363.

Farrah, S.R., S.M. Goyal, C.P. Gerba, C. Wallis and J.L. Melnick. 1977b. Concentration of enteroviruses from estuarine water. Appl. Env. Microbiol. 33:1192-1196.

Fletcher, M.A., T.M. Lo and K.E. Caldwell. 1978. Interaction of limulin with membrane glycoproteins. Pages 644-655 in Progress in clinical and biological research, Vol. 29, Biomedical applications of the horseshoe crab (Limulidae). Alan R. Liss, Inc., New York.

Flewett, T.H., A.S. Bryden, H. Davies, G.N. Woode, J.C. Bridger and J.M. Derrick. 1974. Relationship between viruses from acute gastroenteritis of children and newborn calves. Lancet. ii:61-63.

Floyd, R. and D.G. Sharp. 1977. Aggregation of poliovirus and reovirus by dilution in water. Appl. Env. Microbiol. 33:159-167.

Floyd, R. and D.G. Sharp. 1978. Viral aggregation: effects of salts on the aggregation of poliovirus and reovirus at low pH. Appl. Env. Microbiol. 35:1084-1094.

Gartner, H. 1967. Retention and recovery of polioviruses on a soluble ultrafilter. Pages 121-127 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Gelb, L.D. and A. Martin Lerner. 1965. Reovirus hemagglutination and inhibition by N-acetylglucosamine. Science. 147:404-405.

Gomatos, P.J. and I. Tamm. 1963. The secondary structure of reovirus RNA. Proc. Natl. Acad. Sci., USA. 49:707-714.

Gomatos, P.J., I. Tamm, S. Dales and R.M. Franklin. 1962. Reovirus type 3: physical characteristics and interaction with L cells. Vir. 17:441-454.

Gravelle, C.R. and T.D.Y. Chin. 1961. Enterovirus isolations from sewage; a comparison of three methods. J. Infect. Dis. 109:205-209.

Grindrod, J. and D.O. Cliver. 1969. Limitations of the polymer two phase system for detection of viruses. Archiv. Ges. Virusforsch. 28:337-347.

Grindrod, J. and D.O. Cliver. 1970. A polymer two phase adapted to virus detection. Archiv. Ges. Virusforsch. 31:365-372.

Hartley, J.W., W.P. Rowe and R.J. Huebner. 1961. Recovery of reoviruses from wild and laboratory mice. Proc. Soc. Exp. Biol. (NY). 108:390-395.

Hejkal, T.W., F.M. Wellings, P.A. LaRock and A.L. Lewis. 1979. Survival of poliovirus within organic solids during chlorination. Appl. Env. Microbiol. 38:114-118.

Hill, W.F., Jr., E.W. Akin, W.H. Benton, C.J. Mayhew and T.G. Metcalf. 1974. Recovery of poliovirus from turbid estuarine water on microporous filters by the use of celite. Appl. Microbiol. 27:506-512.

Hill, W.F., Jr., W. Jakubowski, E.W. Akin and N.A. Clarke. 1976. Detection of virus in water: sensitivity of the tentative standard method for drinking water. Appl. Env. Microbiol. 31:254-261.

Hilton, M.C. and G. Stotzky. 1973. Use of coliphage as indicators of water pollution. Can. J. Microbiol. 19: 747-751.

Hsiung, G.D. and J.L. Melnick. 1957. Morphological characteristics of plaques produced on monkey kidney monolayer cultures by enteric viruses (Poliovirus, Coxsackievirus, Echovirus groups). J. Immunol. 78:128-136.

Hull, R.N., J.R. Minner and C.C. Mascoli. 1958. New viral agents recovered from tissue cultures of monkey kidney cells. III. Recovery of additional agents both from cultures of monkey tissues and directly from tissues and excreta. Am. J. Hyg. 68:31-44.

Hull, R.N., J.R. Minner and J.W. Smith. 1956. New viral agents recovered from tissue cultures of monkey kidney cells. I. Origin and properties of cytopathic agents SV_1, SV_2, SV_4, SV_5, SV_6, SV_{11}, SV_{12}, and SV_{15}. Am. J. Hyg. 63:204-215.

Jakubowski, W., W.F. Hill, Jr., and N.A. Clarke. 1975. Comparative study of four microporous filters for concentrating viruses from drinking water. Appl. Microbiol. 30:58-65.

Johnson, J.H., J.E. Fields and W.A. Darlington. 1967. Removing viruses from water by polyelectrolytes. Nature. 213:665-667.

Jordan, L.E. and H.E. Mayor. 1962. The fine structure of reovirus, a new member of the icosahedral series. Vir. 17:597-599.

Joyce, G. and H.H. Weiser. 1967. Survival of enteroviruses and bacteriophage in farm pond waters. J. Am. Wat. Wks. Assoc. 59:491-501.

Kapikian, A.Z., H.W. Kim, R.G. Wyatt, W.L. Cline, J.O. Arrobio, C.D. Brandt, W.J. Rodriguez, D.A. Sack, R.M. Chanock and R.H. Parrot. 1976. Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. New England J. Med. 294:965-972.

Kapikian, A.Z., R.G. Wyatt, R. Dolin, T.S. Thornhill, A.R. Kalica and R.M. Chanock. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J. Virol. 10:1075-1081.

Katzenelson, E., B. Fattal and T. Hostovesky. 1976. Organic flocculation; an efficient second-step concentration method for the detection of viruses in tap water. Appl. Env. Microbiol. 32:638-639.

Katzenelson, E. and S. Kedmi. 1979. Unsuitability of polioviruses as indicators of virological quality of water. Appl. Env. Microbiol. 37:343-344.

Kessick, M.A. and R.A. Wagner. 1978. Electrophoretic mobilities of virus adsorbing filter materials. Water Res. 12:263-268.

Kott, Y. 1966. Estimation of low numbers of Escherichia coli bacteriophage by use of the most probable number method. Appl. Microbiol. 14:141-144.

Kott, Y., N. Roze, S. Sperber and N. Betzlr. 1974. Bacteriophage as viral pollution indicators. Water Res. 8:165-171.

Krystal, G., J. Perrault and A.F. Graham. 1976. Evidence for a glycoprotein in reovirus. Vir. 72:308-321.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

LaMer, V.K. and T.W. Healy. 1963. Adsorption-flocculation reactions of macromolecules at the solid-liquid interface. Rev. Pure App. Chem. 13:112-132.

Liu, O.C., D.A. Brashear, H.R. Seraichekas, J.A. Barnick and T.G. Metcalf. 1971. Virus in water. I. A preliminary study on a flow-through gauze sampler for recovering virus from waters. Appl. Microbiol. 21:405-410.

Loh, P.C., H.R. Hohl and M. Soergel. 1965. Fine structure of reovirus type 2. J. Bacteriol. 89:1140-1144.

Lou, T.Y. and H.A. Wenner. 1963. Natural and experimental infection of dogs with reovirus, type 1: pathogenicity of the strain for other animals. Am. J. Hyg. 77:293-304.

Luftig, R.B., S.S. Kilham, A.J. Hay, H.J. Zweerink and W.K. Joklik. 1972. An ultrastructural study of virion and cores of reovirus type 3. Vir. 48:170-181.

Lund, E. and C.E. Hedstrom. 1966. The use of an aqueous polymer phase system for enterovirus isolation from sewage. Am. J. Epidemiol. 84:287-294.

Mandel, B. 1971. Characterization of type 1 poliovirus by electrophoretic analysis. Vir. 44:554-568.

Mason, J.O. and W.R. McClean. 1962. Infectious hepatitis traced to the consumption of raw oysters: an epidemiologic study. Am. J. Hyg. 75:90-111.

Mayor, H.D., R.M. Jamison, L.E. Jordan and M. van Mitchell. 1965. Reoviruses II. Structure and composition of the virion. J. Bacteriol. 89:1548-1555.

McNulty, M.S., G.M. Allan, G.R. Pearson, J.B. McFerran, W.L. Curran and R.M. McCracken. 1976. Reovirus-like agent (rotavirus) from lambs. Inf. Imm. 14:1332-1338.

Melnick, J.L. 1947. Poliomyelitis virus in urban sewage in epidemic and non-epidemic times. Am. J. Hyg. 45:240-253.

Melnick, J.L. 1976. Viruses in water. Pages 3-11 in G. Berg et al. (eds.), Viruses in water. American Public Health Assoc., Inc., Washington, D.C.

Metcalf, T.G. 1961. Use of membrane filters to facilitate the recovery of virus from aqueous suspensions. Appl. Microbiol. 9:376-379.

Metcalf, T.G., C. Wallis and J.L. Melnick. 1974. Environmental factors influencing isolation of enteroviruses from polluted surface waters. Appl. Microbiol. 27:920-926.

Mix, T.W. 1973. The physical chemistry of membrane interaction. Pages 136-142 in E.D. Murray and A.W. Bourquin (eds.), Developments in industrial microbiology, Vol. 15. American Institute of Biological Sciences, Arlington, VA.

Mosley, J.W. 1959. Water-borne infectious hepatitis. New England J. Med. 261:703-708, 748-753.

Mosley, J.W. 1965. Transmission of viral disease by drinking water. Pages 5-23 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Murphy, A.M., M.B. Albrey and E.B. Crewe. 1977. Rotavirus infections in neonates. Lancet. 2:1149-1150.

Newman, J.F., E.F. Brown, J.C. Bridger and G.N. Woode. 1975. Characterization of a rotavirus. Nature (London). 258: 631-633.

Nowak, T.P. and S.H. Barondes. 1975. Purification with formalinized horse erythrocytes as the affinity adsorbent. Biochem. Biophys. ACTA. 393:115-123.

Nupen, E.M. 1970. Virus studies in the Windhoek waste-water reclamation plant (south-west Africa). Water Res. 4: 661-672.

Nupen, E.M. and G.J. Stander. 1972. The virus problem in the Windhoek wastewater reclamation plant. Pages 133-142 in S.H. Jenkins (ed.), Advances in water pollution research. Proceedings of the 6th International Conference. Pergamon Press, New York.

Ornstein, L. 1964. Disc electrophoresis - I. Background and theory. Ann. NY Acad. Sci. 121:321-349.

Philipson, L., P.A. Albertsson and G. Frick. 1960. The purification and concentration of viruses by aqueous polymer phase system. Vir. 11:553-571.

Pistole, T.G. and R.M. Furman. 1976. Serum bactericidal activity in the horseshoe crab, Limulus polyphemus. Inf. Imm. 14:888-893.

Plotkin, S.A. and M. Katz. 1965. Minimal infective doses of viruses for man by the oral route. Pages 151-166 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Portnoy, B.L., P.A. Mackowiak, C.T. Caraway, J.A. Walker, T.W. McKinley and C.A. Klein, Jr. 1975. Oyster-associated hepatitis: failure of shellfish certification to prevent outbreaks. JAMA. 233:1065-1068.

Rao, V.C., U. Chandorkar, N.U. Rao, P. Kumaran and S.B. Lakhe. 1972. A simple method for concentrating and detecting viruses in wastewater. Water Res. 6:1565-1576.

Rao, N.U. and N.A. Labzoffsky. 1969. A simple method for the detection of low concentrations of viruses in large volumes of water by the membrane filter technique. Can. J. Microbiol. 15:399-403.

Rao, V.C., R. Sullivan, R.B. Read and N.A. Clarke. 1968. A simple method for concentrating and detecting viruses in water. J. Am. Wat. Wks. Assoc. 60:1288-1294.

Rhim, J.S., K.O. Smith and J.L. Melnick. 1961. Complete and coreless forms of reovirus (ECHO 10); ratio of number of virus particles to infective units in the one step growth cycle. Vir. 15:428-435.

Rodger, S.M., R.D. Schnagl and I.H. Holmes. 1975. Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. J. Virol. 16:1229-1235.

Rodger, S.M., R.D. Schnagl and I.H. Holmes. 1977. Further biochemical characterization, including the detection of surface glycoproteins of human, calf and simian rotaviruses. J. Virol. 24:91-98.

Rosen, L. 1962. Reoviruses in animals other than man. Ann. NY Acad. Sci. 101:461-465.

Rosen, L. 1968. Reoviruses. Pages 73-107 in S. Gard et al. (eds.), Virology monographs, Vol. 1. Springer-Verlag, New York.

Rosen, L. and F.R. Abinanti. 1960. Natural and experimental infection of cattle with human types of reoviruses. Am. J. Hyg. 71:250-257.

Rosen, L., F.R. Abinanti and J.F. Hovis. 1963b. Further observations on the natural infection of cattle with reoviruses. Am. J. Hyg. 77:38-48.

Rosen, L., H.E. Evans and A. Spickarp. 1963a. Reovirus infections in human volunteers. Am. J. Hyg. 77:29-37.

Rosen, L., J.F. Hovis, F.M. Mastrota, J.A. Bell and R.J. Huebner. 1960. Observations on a newly recognized virus (Abney) of the reovirus family. Am. J. Hyg. 71:258-265.

Ruddy, S.J., R.F. Johnson, J.W. Mosley, J.B. Atwater, M.A. Rosetti and J.C. Hart. 1969. An epidemic of clam-associated hepatitis. JAMA. 208:649-655.

Sabin, A.B. 1956. The significance of viruses recovered from the intestinal tracts of healthy infants and children. Ann. NY Acad. Sci. 66:226-230.

Sabin, A.B. 1959. Reoviruses, a new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. Science. 130:1387-1389.

Sattar, S.A. and J.C.N. Westwood. 1978. Viral pollution of surface waters due to chlorinated primary effluents. Appl. Env. Microbiol. 36:427-431.

Scarpine, P.V., G. Berg, S.L. Chang, D. Dahling and M. Lucas. 1972. A comparative study of the inactivation of viruses in water by chlorine. Water Res. 6:959-965.

Schangl, R.D. and I.H. Holmes. 1976. Characteristics of the genome of human infantile enteritis virus (rotavirus). J. Virol. 19:267-270.

Shuval, H.I., S. Cymbalista, B. Fattal and N. Goldblum. 1967. Concentration of enteric viruses in water by hydroextraction and two phase separation. Pages 45-55 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Shuval, H.I., B. Fattal, S. Cymbalista and N. Goldblum. 1969. The phase separation method for the concentration and detection of viruses in water. Water Res. 3:225-240.

Sobsey, M.D. 1976. Methods for detecting enteric viruses in water and wastewater. Pages 89-127 in G. Berg et al. (eds.), Viruses in water. American Public Health Assoc., Inc., Washington, D.C.

Sobsey, M.D., C. Wallis, M. Henderson and J.L. Melnick. 1973. Concentration of enteroviruses from large volumes of water. Appl. Microbiol. 26:529-534.

Spendlove, R.S., M.E. McClain and E.H. Lenette. 1970. Enhancement of reovirus infectivity by extracellular removal or alteration of the virus capsid by proteolytic enzymes. J. Gen. Virol. 8:83-94.

Spendlove, R.S. and F.L. Schaffer. 1965. Enzymatic enhancement of infectivity of reovirus. J. Bacteriol. 89:597-602.

Sproul, O.J. 1976. Removal of viruses by treatment processes. Pages 167-179 in G. Berg et al. (eds.), Viruses in water. American Public Health Assoc., Inc., Washington, D.C.

Sullivan, B., J. Bonaventura, C. Bonaventura and G. Godette. 1976. Hemocyanin of the horseshoe crab, Limulus polyphemus. J. Biol. Chem. 251:7644-7648.

Tierney, J.T., R. Sullivan and E.P. Larkin. 1977. Persistence of poliovirus 1 in soil and on vegetables grown in soil previously flooded with inoculated sewage sludge or effluent. Appl. Env. Microbiol. 33:109-113.

Todd, D. and M.S. McNulty. 1976. Characterization of pig rotavirus RNA. J. Gen. Virol. 33:147-150.

Vasquez, C. and P.Tournier. 1962. The morphology of reovirus. Vir. 17:503-510.

Vasquez, C. and P. Tournier. 1964. New interpretation of the reovirus structure. Vir. 24:128-130.

Vaughn, J.M. and T.G. Metcalf. 1975. Coliphages as indicators of enteric viruses in shellfish and shellfish raising estuarine waters. Water Res. 9:613-616.

Wallis, C., S. Grinstein, J.L. Melnick and J.E. Fields. 1969. Concentration of viruses from sewage and excreta on insoluble polyelectrolytes. Appl. Microbiol. 18:1007-1014.

Wallis, C., M. Henderson and J.L. Melnick. 1972. Enterovirus concentration on cellulose membranes. Appl. Microbiol. 23:476-480.

Wallis, C. and J.L. Melnick. 1962. Cationic stabilization - a new property of enteroviruses. Vir. 16:504-506.

Wallis, C. and J.L. Melnick. 1967a. Concentration of enteroviruses on membrane filters. J. Virol. 1:472-477.

Wallis, C. and J.L. Melnick. 1967b. Concentration of viruses from sewage by adsorption on Millipore membranes. Bull. World Hlth. Org. 36:219-225.

Wallis, C. and J.L. Melnick. 1967c. Concentration of viruses on aluminum phosphate and aluminum hydroxide precipitates. Pages 129-139 in G. Berg (ed.), Transmission of viruses by the water route. John Wiley and Sons, New York.

Wallis, C. and J.L. Melnick. 1972. A portable virus concentrator for use in the field. Water Res. 6:1249-1256.

Wallis, C., J.L. Melnick and J.E. Fields. 1970. Detection of viruses in large volumes of natural waters by concentration on insoluble polyelectrolytes. Water Res. 4: 787-796.

Wallis, C., J.L. Melnick and C.P. Gerba. 1979. Concentration of viruses from water by membrane chromatography. Ann. Rev. Microbiol. 33:413-437.

Wallis, C., J.L. Melnick and F. Rapp. 1966. Effects of pancreatin on the growth of reovirus. J. Bacteriol. 92: 155-160.

Wallis, C., K.O. Smith and J.L. Melnick. 1964. Reovirus activation by heating and inactivation by cooling in $MgCl_2$ solutions. Vir. 22:608-619.

Ward, R.L. and C.S. Ashley. 1977. Identification of the virucidal agent in wastewater sludge. Appl. Env. Microbiol. 33:860-864.

Weber, W.J., Jr. 1972. Coagulation and flocculation. Chapter 2. Pages 61-109 in W.J. Weber, Jr., Physiochemical processes for water quality control. John Wiley and Sons, New York.

Westwood, J.C.N. and S.A. Sattar. 1976. The minimal infective dose. Pages 61-69 in G. Berg et al. (eds.), Viruses in water. American Public Health Assoc., Inc., Washington, D.C.

Wesslen, T., P.A. Albertsson and L. Philipson. 1959. Concentration of animal viruses using two-phase systems of aqueous polymer solutions. Archiv. Ges. Virusforsch. 9:510-520.

Woode, G.N., J.C. Bridger, G.A. Hall, J.M. Jones and G. Jackson. 1976b. The isolation of reovirus-like agents (rotaviruses) from acute gastroenteritis of piglets. J. Med. Microbiol. 9:203-209.

Woode, G.N., J.C. Bridger, J.M. Jones, T.H. Flewett, A.S. Bryden, H.A. Davies and G.B.B. White. 1976a. Morphological and antigenic relationships between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals. Inf. Imm. 14:804-810.