DEVELOPMENT OF IMMUNOENZYMATIC METHODS FOR DETECTION
AND QUANTITATION OF POLIOVIRUS

by

Frederic K. Pfaender and Gary K. Whitmyre
Department of Environmental Sciences and Engineering
School of Public Health
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27514

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ABSTRACT

A major problem that must be faced in assessing the microbiological quality of a wide variety of water sources is the identification of human enteric viruses. Monitoring programs based on estimating bacteriological contamination of water are of questionable value in predicting virus concentrations. Routine monitoring of surface, drinking, and recreational waters for virus content is severely hampered by the absence of a reliable, specific, and simple procedure for quantification of the number of virus particles present. This research was directed toward development of a methodology useful for assessing entero-virus contamination. The method is based on an immunoenzymatic assay in which virus specific antibody, to which is bound other antibodies and enzymes, is reacted with the virus. The complex can subsequently be visualized by addition of enzyme reagents which produce a color change in the presence of virus particles. Two forms of this assay have been developed, one using a microscopic visualization of the colored particles and the other based on reactions in a test tube with total color produced being used to quantify virus. Both methods offer promise of being able to count virus particles in moderately contaminated waters. Further development offers the promise of greater sensitivity.
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1.0 Summary and Conclusions

Immunoenzymatic assays have been developed for detecting viruses using test tubes on filters. Both methods have a theoretical detection limit of about 100 plaque forming units (PFU). The test tube method is a modification of the ELISA technique in which virus particles are bound to polystyrene with antiviral antibody, and then assayed by addition of immunoenzymatic reagents to the tube. Development of a filter assay involved selection of a method for reducing nonspecific background staining, screening of filters for virus retention and suitability, and selection of optimal concentrations of immunoenzymatic reagents. For both the filter and tube assays, poliovirus type 1 was the model virus used for development of these assay systems due to its wide occurrence and persistence in the environment.

Although development of these methodologies was initiated by interest in the presence of virus in shellfish-growing waters, the techniques may be generally applicable to monitoring virus in water and wastewater samples, and may even have biomedical applications.

There are potential problems which will have to be addressed prior to application of this methodology to a routine monitoring situation. One involves modification of existing virus concentration techniques for the widely varying conditions that may occur in environmental samples, especially with regard to suspended particulate matter and particulate-bound virus. A second involves development of pre-assay techniques to render a concentrated virus sample suitable for assay. This could involve removal of particulate matter and other undesired material. A third is the elimination of interfering materials. For the immunoperoxidase technique, dissolved or suspended iron presents an interference problem for immunoenzymatic assays. This interference problem may be solvable by iron-removal methodologies now available. This interference may not exist for certain enzyme/substrate combinations.

Currently, these immunoenzymatic methods are limited to detecting virus in moderately to highly contaminated waters. Application to the low end of the virus concentration range (<100 PFU/100 gallons) will require further improvement in the sensitivity of the methods. The sensitivity can be maximized in several ways. Enzymes of higher specific activity and higher-titer antibodies improve the sensitivity. The nonspecific staining problem, which may be one of the greatest factors that limit sensitivity, can be reduced by using immunoenzymatic reagents of very high purity.

The ultimate applicability of these techniques to routine virus measurement and monitoring will depend on their reliability, ease of performance, cost, sensitivity, and availability of commercial preparations of appropriate antibodies.
2.0 Recommendations

Two major areas with regard to these quantitative immunoenzymatic methods require further research. First, additional effort should be put into improving the sensitivity, and thus the detection limit, of both the tube and filter assays. This goal can be partially achieved by developing new methods to reduce background. Second, follow-up research should be initiated to determine what modifications in these methods are necessary to make them suitable for assaying field samples. This could range from investigating what sample pretreatment measures are necessary, to making improvements in procedures and/or the equipment used in the assays to reduce the time required for the assay.

As new membrane filters are developed by manufacturers, they should be tested to see if they are more suitable for the immunoenzymatic filter assay than the filter that is currently proposed. New methods should be found for more efficiently immobilizing virus on filter surfaces or other solid matrices, and for minimizing the amount of virus lost through pretreatment.
3.0 Introduction

This project was initiated by the Water Resources Research Institute to identify and develop ways to quantify viruses that are not dependent on cell culture techniques. This was deemed important because cell culture assays are expensive, and not all types of viruses can be assayed by existing cell culture techniques. This prohibits routine use of cell culture in an extensive virus monitoring program. We have developed two immunoenzymatic methods for quantitation of poliovirus. Such methods should give a better indication of viral contamination than indirect methods like the coliform monitoring techniques, and can unequivocally demonstrate the presence and concentration of human enteric virus in contaminated water.

This report is organized into seven sections. Section 4.0 presents a review of relevant literature, and provides a background on enteric viruses, why they are of concern, and what methods currently exist for detecting virus in water. Section 5.0 presents the proposed immunoenzymatic procedure for detecting poliovirus. Section 6.0 summarizes the experimental protocols. Section 7.0 interprets the experimental results.
4.0 Literature Review

4.1 Introduction:

One of the built-in paradoxes of modern existence is that the per capita demand on our water resources is increasing, while at the same time less and less of this resource is available at the desired quality. Presently, each person in the United States uses from fifty to five-hundred liters of water each day, most of it being returned to the watershed from which it was drawn along with an added bonus - an anthropogenic residual of inorganic and organic chemical pollutants, and microorganisms including human enteric viruses (Shuval, 1976).

The enteric viruses which infect man are of increasing concern today, now that bacterial diseases have been well studied and, for the most part, are readily controlled with antibiotics. In contrast to the incidences of most bacterial diseases which have been declining over the last few decades, the incidence of human enteric viral diseases has increased (Committee on Environmental Quality & Management, 1970).

The pathways of virus to man are illustrated in Figure 4-1 (Gerba, et al., 1975). The water route is being increasingly appreciated as a significant mechanism through which man is infected by virus. This route is made possible for enteric viruses by their ability to survive in the natural environment, a point which will be discussed in further detail, and their ability to be transported to points far from their point of entry into the environment (Grinstein, et al., 1970; Lamb and Chin, 1964; Metcalf and Stiles, 1968; Shuval, et al., 1967).

4.2 Enteric Viruses - Background and Pathology:

4.21 Enteric Viruses - General Background. Fenner, et al., 1974, have defined enteric viruses as those which can survive the acidic and biliary secretions of the vertebrate digestive tract and initiate infection, either symptomatic or asymptomatic, in gut-associated tissue. These viruses are obligate parasites, relying on vertebrate animal cells for reproduction of their own kind. Berg, et al. (1971), have pointed out that enteric viruses ultimately find their way into aquatic environments, where they "are always present in small numbers that diminish with natural die-off and any treatment to which the water is subjected." The small residual of viable viruses is still significant, however, since even a single viable virus particle is capable of infecting a susceptible host.

The nomenclature of the enteric viruses by group and subgroup is shown in Table 4-1. Poliovirus is the most commonly reported enteric virus isolated from sewage and sewage-contaminated water. Adenoviruses are recoverable from fecal samples, but are mainly implicated in upper respiratory diseases (Sobsey, 1974). Some environmental virologists consider
FIGURE 4-1 - Pathways of Enteric Viruses to Man
**TABLE 4-1 - Human Enteric Virus Classification**

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>Number of Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteroviruses</td>
<td>Polioviruses</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Coxsackieviruses A</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Coxsackieviruses B</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Echoviruses</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>New Enteroviruses</td>
<td>4+</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>Reoviruses</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Rotaviruses (Duoviruses)</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>Hepatitis A Viruses *</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>Norwalk and Related Agents*</td>
<td></td>
<td>2(?)</td>
</tr>
</tbody>
</table>

* These viruses may also be classified as Parvoviruses

(Sobsey, 1977)
Hepatitis A, which appears to be a parvovirus on the basis of physical and chemical evidence (Shuval, 1972), the only virus for which a water route of transmission has been unequivocally demonstrated.

Nonbacterial gastroenteritis has traditionally been a wastebasket category for enteric diseases where an agent has not been isolated. There are two recently-recognized groups of viruses associated with outbreaks of nonbacterial gastroenteritis. These groups are the Norwalk and related agents, and the rotaviruses. These agents are quite fastidious and cannot be isolated by ordinary virological techniques. Furthermore, the quantitative assay methodology is not well developed for these agents (Sobsey, 1974). The Norwalk and related parvovirus-like agents cause an acute gastroenteritis with diarrhea and vomiting. This agent was responsible for the common source outbreak of acute infectious nonbacterial gastroenteritis that occurred in Norwalk, Ohio in 1968. The rotaviruses are the leading cause of winter gastroenteritis in infants where diarrhea and vomiting are symptoms, and a major cause of infant mortality in the Third World (Sobsey, 1972). Acute infectious nonbacterial gastroenteritis is second only to acute respiratory illness as the most frequent form of illness. This disease has a course from twenty-four to forty-eight hours with symptoms that can include, besides diarrhea and vomiting, low-grade fever, abdominal pain, headache, and malaise (Dolin, et al., 1973).

The Norwalk agent has been isolated from stool filtrates of infected individuals, and examined serologically and by immune electron microscopy (IEM). It has been characterized as a 27 nm particle that bears resemblance to a parvovirus on the basis of physical parameters (Kapikian, 1972). The Norwalk agent has been compared to two similar viruses - one isolated in Honolulu, Hawaii, and one isolated from Montgomery County, Maryland. All three agents produce clinically similar illnesses. The Norwalk and Montgomery County agents are similar antigenically, but the Hawaii agent is dissimilar from both of these viruses antigenically (Wyatt, et al., 1974).

The reovirus or rotavirus agent is involved in many cases of infantile gastroenteritis (Holmes, et al., 1975; Kapikian, et al., 1974; Petric, et al., 1975), and upon examination by IEM and complement fixation (CF) is seen to be antigenically related to viruses that cause diarrhea in infant mice, and to Nebraska Calf Diarrhea Virus (NCDV) (Kapikian, et al., 1974). Physically it is a particle with a sedimentation coefficient of 520-530 S, and a buoyant density in cesium chloride of 1.36 g/ml (Petric, et al., 1975). A breakthrough in the study of this group of agents was the accomplishment of successfully growing the virus in-vitro in human fetal intestinal organ cultures (Wyatt, et al., 1974).

4.22 Water Borne Infections - Enteric Viruses. The fact that water is able to serve as a vector of enteric disease agents was recognized by John Snow about 1854. This was a long time prior to the sciences achieving any understanding of the causative pathogenic microorganisms involved (Shuval, 1976). Enteric viruses are thought to play a significant role in waterborne diseases because of their resistance to inactivation processes
and because of their common occurrence in man's environment.

A statistical breakdown of reported viral waterborne disease outbreaks in the United States between 1971 and 1975, inclusively, yields 17,752 cases (63 outbreaks) of gastroenteritis of unknown etiology and 369 cases (14 outbreaks) of Hepatitis A. Most outbreaks of waterborne disease occur in Spring or Summer, many involving semi-public water supplies, such as those in recreational areas, where treatment is lacking or unsophisticated. These outbreaks are usually associated with improper chlorination of drinking water (Sobsey, 1977). Clarke and Chang (1959) have reviewed the waterborne epidemics of infectious hepatitis in the United States and abroad, and discovered that the great majority of these outbreaks have resulted from sewage-contaminated water supplies, primarily those that are private or semi-public. The most notorious infectious hepatitis outbreak, however, occurred from an unanticipated gross sewage contamination of a river which served as the source for a public water supply. This occurred in 1955 in New Delhi, India, where an estimated 30,000 to 50,000 persons were affected (Visvanathan and Sindhu, 1957). Infectious hepatitis has also resulted from consumption of shellfish grown in sewage-contaminated waters (Bryan, 1972). Hundreds of cases of shellfish-transmitted hepatitis have been reported (Gerba, et al., 1975; Mason and McLean, 1962), and enteric viruses have been isolated from oysters taken from both the East and Gulf coasts of the United States (Gerba, et al., 1975). Another disease for which a water route has been implicated but not unequivocally demonstrated is poliomyelitis, the causative agent being poliovirus. There have been several outbreaks that have been associated with drinking water (Bancroft, et al., 1957; Little, 1964; Mosley, 1967).

The extent to which waterborne viruses threaten public health is controversial. Some persons propose that since one infectious unit is theoretically capable of producing infection in man, then the presence of one virus unit in a water supply poses a potential hazard (Gerba, et al., 1975). There are several arguments counter to this view. First of all, several or many virus particles are required to constitute a single infectious unit. This is illustrated in Table 4-2. For poliovirus this translates to up to 200 or 300 virus particles per infectious unit (Sharp, 1963). Secondly, ingestion of an infection with small amounts of virus does not necessarily lead to expression as disease. Thirdly, the risks represented by viruses in drinking water must be evaluated relative to other risks to man's health that are present in water, such as the myriad of carcinogens/mutagens present in chlorinated drinking water.

Most likely a person ingesting one or a few virions will be asymptomatic. These asymptomatic cases are unrecognized cases of infection and are thought to represent 99% or more of those infected. That means that only one per cent or less of those persons infected will show clinical symptoms (Jawetz, et al., 1974; Nenenson, 1970; Shuval, 1976; Sobsey, 1974). These asymptomatic cases are quite significant from a public health point of view, as these persons will excrete large quantities of virus in their
<table>
<thead>
<tr>
<th>Virus</th>
<th>Assay System</th>
<th>End Point Unit</th>
<th>Number of Infectivity Units</th>
<th>Number of Virus Particles per Infectious Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus 5</td>
<td>HeLa cells</td>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>10-103*</td>
<td>67</td>
</tr>
<tr>
<td>Cowpox virus</td>
<td>Rabbit skin</td>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>4-40</td>
<td>20</td>
</tr>
<tr>
<td>Feline pneumonitis virus</td>
<td>Egg</td>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Poliovirus (Mahoney)</td>
<td>Human amnion cells</td>
<td>PFU</td>
<td>22-56</td>
<td>36</td>
</tr>
<tr>
<td>Poliovirus (Mahoney)</td>
<td>Monkey kidney cells</td>
<td>PFU</td>
<td>35-253</td>
<td>34-250</td>
</tr>
<tr>
<td>Poliovirus (MEF-1)</td>
<td>Human amnion cells</td>
<td>PFU</td>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>Polyoma virus</td>
<td>Mouse embryo cells</td>
<td>PFU</td>
<td>33-55</td>
<td>33-56</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>L cells</td>
<td>PFU</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

(Sharp, 1963)
feces, which then ultimately find their way into the aquatic environment. These persons are also of tremendous importance epidemiologically as they can act as foci of infection. Thus from the few infected asymptomatic persons who obtained viruses from the water route, the viruses spread into the community by the fecal-oral route and by direct-contact transmission. Infection and the resulting disease following this pathway would appear to the epidemiologist as a classical picture of direct-contact transmission only, and not as the common-source transmission that it actually is (Berg, et al., 1971; Lenette, 1977).

Of course, there are many types of enteric viruses. Reinfections with the same type of virus are rare, and hence epidemic "peaks" of clinical and subclinical infections might be expected to occur when a sufficient number of susceptibles, usually children, is attained within the population.

4.23 Poliomyelitis. Poliomyelitis is an acute viral illness manifested by a wide variety of severities from asymptomatic infections to the paralytic disease (Nenenson, 1970). The disease is caused by a small enteric virus, poliovirus, of the enterovirus group. Poliovirus particles are 28 nm in size and relatively heat stable, especially in the presence of divalent cations like magnesium. The virus is inactivated by UV light, drying, and low residuals (0.1 ppm) of chlorine (Jawetz, et al., 1974).

There are 3 antigenic types, designated 1, 2, and 3 (Jawetz, et al., 1974). Additionally, there are genetic markers which distinguish the wild type virulent strains from the avirulent strains. For example, poliovirus type-1 strain Mahoney, a virulent strain, has the markers d+T+ and poliovirus type-1 strain Lsc, which is avirulent, has the markers d-T-.

The marker d+ designates the ability of viral plaques to form in cell monolayers in the presence of 0.07% sodium bicarbonate whereas d- designates the inability of viral plaques to form in the presence of 0.40% sodium bicarbonate. T+ indicates plaque-formation capability at 40°C and T- indicates that plaques will not form at temperatures greater than 37°C (Melnick and Melnick, 1959). Furthermore, heated poliovirus shows different surface antigens compared to native virus. Native virus has two major surface antigens, N and D, whereas heated virus has the antigens H and C. This presumably is due to denaturation of the virus coat proteins which expose new segments of the polypeptide chains (Beala and Mason, 1968).

Poliiovirus invades the alimentary tract, causing a viremia in some cases, followed by invasion of the central nervous system (CNS) and selective involvement of motor cells leading to, in the extreme case, paralysis (Nenenson, 1970). The incubation period for the disease is seven to fourteen days on the average, with a range of three to thirty-five days (Jawetz, et al., 1974). Paralysis is implicated in only about three to four percent of the clinically recognizable cases of poliomyelitis. The remainder involves a milder illness such as aseptic meningitis (Jawetz, et al., 1974; Paul, 1971). Transmission is thought to be primarily through direct contact with infected individuals.
The age distribution of the disease has been well studied. In isolated unvaccinated areas, polio attacks all age groups equally. In crowded primitive societies, where poor sanitation favors wide dissemination of the virus, polio continues to be a disease of infancy, with all children over four years of age possessing antibodies to the virus (Jawetz, et al., 1974). Unexplained, however, is the recent shift in the age incidence of poliomyelitis that started a few decades ago in western society. Poliomyelitis is now occurring more frequently in increasingly older age groups than it had in the past in the United States, England, Denmark, Sweden, and Australia (Keswick and Gerba, 1980). Currently there is concern that a false sense of security resulting from the sharp decline in poliomyelitis incidence in recent years is leading to a lapse in vaccination programs. This may be producing large numbers of susceptible youngsters that may eventually result in an epidemic. Figure 4-2 shows the seasonal pattern that the disease follows, with peaks in late Summer and early Fall.

As mentioned before the existence of a water route for poliomyelitis is supported by evidence. Poliovirus is present in sewage-contaminated water, and undoubtedly many persons ingest small quantities of virus daily. Interestingly, 13 of the 19 isolates of polioviruses that Wallis and Melnick (1967) found in municipal sewage had $d^+$ markers, which indicated that either the vaccine strains had partially reverted, or that wild-type strains were circulating in the population in the absence of clinical disease. To date eight reported polio outbreaks have occurred in which drinking water was thought to be the cause as summarized in Table 2-3. Six of the outbreaks occurred in Sweden during the 1930's and 1940's. In the U.S., the one confirmed water-borne outbreak occurred in 1952 in Huskerville, Nebraska in a cluster of row houses with a common contaminated water supply (Bancroft, et al., 1957). The Edmonton, Alberta outbreak of 1953 occurred in late Fall and accompanied the malfunctioning of the chlorination process for sewage effluent in a town twenty miles upstream from where Edmonton drew its water supply (Little, 1954).

4.3 Enteric Viruses - Removal/Survival in Treatment Processes:

4.31 Removal/Survival in Wastewater Treatment Processes. Wastewater treatment processes, besides reducing the biological oxygen demand (BOD) of the wastewater, also significantly reduces the number of infectious virus particles present in the wastewater. Concern about enteric viruses would end at the treatment plant if conventional sewage treatment yielded 100% inactivation of these viruses. However, this is not the case (Berg, 1973; Malina, 1976; Schwartzbrod, et al., 1973; Sproul, 1974). The effluent of a typical properly-operated and maintained treatment plant contains about fifty plaque forming units (PFU) of enteric viruses per liter (Berg, 1971), although this varies. The proportion of virus removed in a treatment plant depends on the virus, the types of processes used, and the efficiencies at which they are operated. With primary treatment, which is essentially just a settling process, virus removal varies between 0 and 30% with 3 hours of
FIGURE 4-2 - Seasonal Occurrence of Poliomyelitis

Source: Morbidity and Mortality (1971)
### TABLE 4-3 - Published Reports of Poliomyelitis Attributed to Contaminated Drinking Water

<table>
<thead>
<tr>
<th>No.</th>
<th>Year of occurrence</th>
<th>References</th>
<th>Country</th>
<th>Place or type of population</th>
<th>No. cases of type attributed to supply</th>
<th>Attack rate per 100,000</th>
<th>Character of episode</th>
<th>Duration of water-borne phase</th>
<th>Type of supply held responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1913(7)-39</td>
<td>Spack, 1941</td>
<td>Sweden</td>
<td>Rural district</td>
<td>10b</td>
<td>—</td>
<td>Sporadic cases</td>
<td>Years</td>
<td>Private well, pond</td>
</tr>
<tr>
<td>2</td>
<td>1944</td>
<td>Kline, 1947</td>
<td>Sweden</td>
<td>Town</td>
<td>63</td>
<td>0.5</td>
<td>Epidemic</td>
<td>5 Months</td>
<td>Municipal system, Filtered surface water</td>
</tr>
<tr>
<td>3</td>
<td>1944</td>
<td>Kline, 1947</td>
<td>Sweden</td>
<td>Town</td>
<td>53</td>
<td>0.2</td>
<td>Epidemic</td>
<td>3 months</td>
<td>Municipal system, Untreated deep well water</td>
</tr>
<tr>
<td>4</td>
<td>1948</td>
<td>Faahraeus et al., 1950</td>
<td>Sweden</td>
<td>Stockholm suburb</td>
<td>9</td>
<td>—</td>
<td>Sporadic cases</td>
<td>3 Months</td>
<td>Municipal system, Proximate contamination</td>
</tr>
<tr>
<td>5</td>
<td>1948</td>
<td>Faahraeus et al., 1950</td>
<td>Sweden</td>
<td>Town</td>
<td>63*</td>
<td>0.2</td>
<td>Epidemic</td>
<td>7 Months</td>
<td>Municipal system, Untreated deep well water</td>
</tr>
<tr>
<td>6</td>
<td>1949</td>
<td>Huns et al., 1952</td>
<td>Sweden</td>
<td>Malmö</td>
<td>138</td>
<td>0.1</td>
<td>Epidemic</td>
<td>6 Months</td>
<td>Municipal System, Filtered surface water</td>
</tr>
<tr>
<td>7</td>
<td>1952</td>
<td>Baneroff et al., 1957</td>
<td>U.S.A.</td>
<td>&quot;Husker vi, la&quot;</td>
<td>45</td>
<td>6.7</td>
<td>Epidemic</td>
<td>5 Weeks</td>
<td>Municipal system, Proximate contamination</td>
</tr>
<tr>
<td>8</td>
<td>1953</td>
<td>Little, 1954</td>
<td>Canada</td>
<td>Edmonton</td>
<td>75b</td>
<td>&lt;0.1b</td>
<td>Epidemic</td>
<td>2 Weeks</td>
<td>Municipal system, Chlorinated surface water</td>
</tr>
</tbody>
</table>

* Unadjusted rate among persons presumably consuming contaminated supply.

b Estimated from author's data.

Includes cases attributed to other modes of transmission.

Source: Berg, 1967
settling time. In secondary, or biological treatment, virus removal can be as high as 97% (Safferman and Morris, 1976). This is followed by a disinfection process, usually chlorination, which reduces the levels of virus even further. The concentrations of bacteriophage and human viruses have occasionally been used as an indication of the effectiveness of water and wastewater treatment processes (Clarke, et al., 1961; Malina, et al., 1975; Safferman and Morris, 1976; Shelton and Drewry, 1973).

Virus removal has been well studied for secondary and tertiary sewage treatment. In laboratory studies, trickling filters have reduced virus levels by up to 2 "logs", or 99% (Shuval, 1970), and activated sludge reduced virus levels with a widely varying efficiency. For activated sludge Coxsackievirus type A9 was reduced 96 to 99%, but poliovirus type 1 was reduced as little as 38% after six to eight hours of exposure to the process (Clarke, et al., 1961). Tertiary treatment, or advanced chemical treatment of wastewater leads to greater reductions in virus concentrations than primary or secondar treatment. Chemical coagulation-flocculation can be particularly effective in removing virus, although again the variability in removal efficiencies is great. In laboratory studies, aluminum sulfate (alum) coagulation has yielded virus reductions as high as 99.8 to 99.9% (Wolf, et al., 1974). Lime treatment, besides removing virus by adsorption to or entrapment in the floc, has the added advantage of inactivating the virus by the high pH attained in this process, sometimes up to 4 "log" or 99.99% removal (Berg, et al., 1968; Sattar, et al., 1976).

Further reduction in viable virus can be achieved by disinfection. Chlorine is now the most widely used disinfectant for this purpose, although ozone, iodine, and bromine are receiving serious consideration as alternative disinfectants. Disinfection efficiency depends on a number of variables, including the type of virus present, its physical state, the type of disinfectant used, the quality and nature of the waste, pH, temperature, and length of treatment (Gerba, et al., 1975; Sharp, et al., 1976). If halogens are used it is necessary to add amounts beyond the demand of the endogenous organic and inorganic material present before any significant reduction in virus occurs (Freund, 1976). For secondary effluent, one must raise the dose to as high as 40 mg/l for ten minutes to obtain a 3-log removal. However, this high a dose of chlorine does not come without its own problems. The chlorinated organic compounds formed show toxicity toward various forms of aquatic life as well as providing a source of contamination of the environment with potentially carcinogenic compounds (Gerba, et al., 1975).

The average enteric virus density in domestic raw sewage is estimated at 7000 infectious units per liter, with a high range limit of 463,500 infectious particles per liter (Gerba, et al., 1975). The amount present varies widely as a function of hygiene in the community, the socioeconomic level, and the time of year. A seasonal peak for virus excretion in the United States occurs in late Summer and early Fall (Gerba, et al., 1975; Clarke and Kobler, 1964). The levels of virus in raw sewage undoubtedly reflect the endemic/epidemic status of enteric viral diseases in the community,
fluctuating from week to week and season to season (England, 1972). Even apparently healthy individuals excrete enteric viruses, especially children under the age of 15 (Ramao-Alvarez and Sabin, 1956). On the average about 10% of the children in this age group are shedding viruses at any given time (Gelfand, et al., 1957; Honig, et al., 1956; Sabin, 1955). In infected individuals, enteric viruses are excreted in concentrations as high as 100,000 TCID₅₀ per gram of feces, but the average concentration is a magnitude lower, or about 10,000 TCID₅₀ per gram. Sabin (1955), (assuming that 10% of the population under age 15 are asymptomatic excreters, and that the symptomatic individuals will be few) calculated an average value of 311 TCID₅₀ per gram feces for the total population. Between 16% and 100% of this virus is associated with suspended solids in the input stream and throughout the treatment process (Wellings, et al., 1976). The types of virus that have been isolated from sewage are noted in Table 4-4.

4.32 Removal/Survival During Water Treatment Processes. Water treatment processes were originally designed to reduce turbidity and improve the bacteriological quality of water. Conveniently it also removes enteric viruses from the water quite effectively (Guy, et al., 1977). This removal, however, is not complete (Guy, et al., 1977; Mangravite, et al., 1975; York and Drewry, 1974). From this arises the public health concern about viruses in drinking water.

Rivers which receive raw or treated sewage may contain one to ten PFU per 100 milliliters or higher, depending on flow conditions (Shuval, 1976). Physical-chemical treatment, as for wastewater, is by far the most effective treatment process for removing viruses from water. With this process alone a removal of 3 or 4 logs (99.9% - 99.99%) is possible (Gerba, et al., 1975; Farrah, et al., 1978). For water this involves alum (aluminum sulfate) addition followed by lime (calcium hydroxide). It is important to note that all viruses are not equally removed by the resulting floc (Farrah, et al., 1978). Disinfection is the final barrier between the viruses present in the raw water and the glass of water consumed by man. For chlorine a 5 mg/l free chlorine residual over a 30 minute exposure period leads to about a 3 log reduction in titre, although this varies from virus to virus, and also as a function of environmental conditions such as organic content, pH, and temperature. Another disinfectant, used less frequently, is ozone. It, unlike the halogens, has a threshold effect. There is little inactivation until one has about 0.2-1 milligram of ozone per liter. A problem with this disinfectant is that it lacks a residual (Sobsey, 1974). Bromine is receiving increasing attention as a disinfectant and shows promising disinfection qualities toward viruses (Sharp, et al., 1975).

There is little information available on the occurrence of viruses in drinking water. Two French studies in Paris in the 1960's showed enteric viruses in 18% and 8%, respectively, of the finished water samples examined.

*TCID₅₀ is the dose of virus that will lead to infection of 50% of replicate cell cultures.
TABLE 4-4 - Types of Viruses Isolated From Sewage

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Types Isolated</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1964</td>
<td>Chicago, Ill.</td>
<td>Polioviruses 1,2,3</td>
<td>Same types isolated from upper Illinois R. below sewage outfall.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coxsackieviruses A &amp; B</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Echovirus 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reovirus 1</td>
<td>Corresponded with poliomyelitis outbreak - 44 cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1967</td>
<td>Southern California</td>
<td>Adenoviruses/Echo-viruses/Reoviruses/Polioviruses/Coxsackieviruses B</td>
<td>13 of 19 poliovirus isolates had d+ markers</td>
</tr>
<tr>
<td>1967</td>
<td>Houston, Texas</td>
<td>Polioviruses 2,3/Echoviruses(9)/Coxsackieviruses (2)</td>
<td></td>
</tr>
<tr>
<td>1972</td>
<td></td>
<td>Polioviruses 1,3/Echoviruses 3,6,7/Coxsackieviruses B3, B5</td>
<td>Wide range of virulence. Majority of isolates with intermediate virulence characteristics.</td>
</tr>
</tbody>
</table>

The average virus concentration in the first of the two studies was determined to be 1 infectious unit per 300 liters. More recently, enteric viruses have been isolated from drinking water of communities in South Africa and New England (Gerba, et al., 1975). Admittedly, enteric viruses have been detected only rarely in drinking water that has been properly treated (Shuval, 1976), but it is not certain whether this is due to their absence or to the lack of sufficient effort in monitoring drinking water for the presence of virus. To date, there is no routine program for virus monitoring in drinking water except in Paris, where the gauze pad method, one of relatively low efficiency, is employed (Shuval, 1976).

4.33 Occurrence and Survival in Wastewater Treatment Sludge. Sludge arises from wastewater treatment settling processes such as primary treatment, and from biological treatment processes like activated sludge. The sludge generated in this process is sometimes concentrated by such processes as anaerobic sludge digestion, which involves elevated temperatures, centrifugation, and evaporation. Viruses are associated with sludge by adsorption and entrapment. The fate of this sludge-associated virus needs to be known since sludge, whether viewed as a waste product to be disposed of, or as a resource to be utilized, will end up in the environment.

Raw sludge solids are quite protective of viruses (Ward, et al., 1976). It has been demonstrated that virus removed by adsorption to or entrapment within alum and lime flocs during water treatment is not inactivated (Wolf, et al., 1974). The same is true for sludges obtained in municipal wastewater treatment processes (Gilcreas and Kelly, 1955; Stevenson, et al., 1956; Ward, 1977). Virus has been demonstrated in fully-digested sludge (60 days old, 34°C) and dried sludge. The types of viruses that have been isolated from treated sludge included polioviruses, reoviruses, and echoviruses (Wellings, et al., 1976).

Raw sludge is quite protective for poliovirus in particular (Ward, 1977). Even without applied heat, poliovirus is more rapidly inactivated in anaerobically digested sludge than in raw sludge (Ward, et al., 1976; Ward, 1978). In this case ammonia formed in the digestion process in the virucidal agent, the presence of which is reflected by the 2 orders-of-magnitude higher pH in digested sludge compared to raw sludge (Ward, et al., 1976; Ward and Ashley, 1977; Ward, 1978). It is important to make the qualification that ammonia is not a general virucidal agent. Reovirus is indifferent to the presence of ammonia in the concentrations experienced in digested sludge (Ward, 1978). When sludge is reduced in volume by de-watering through evaporation, some virus inactivation occurs, although it is not complete. Poliovirus, coxsackievirus and reovirus are known to be partially inactivated by the de-watering process (Ward and Ashley, 1977).

4.4 The Occurrence and Survival of Enteric Viruses in the Environment

4.41 Occurrence and Survival in the Aquatic Environment
4.41 Occurrence and Survival in Water. It has been general practice to discharge sewage, treated or untreated into the nearest available body of water. Often, due to insufficient concentration of contact time with disinfectant, much of the virus present in sewage is discharged in a viable state (Lenette, 1977). Laboratory and field studies have proven that enteric viruses, depending on virus type and environmental conditions, can survive in the environment for times ranging from days to months (Akin, et al., 1971; Shuval, 1976).

Poliovirus has been isolated from contaminated well water (Kling, 1940) and contaminated surface water (Paul and Flisk, 1941). Herrmann, et al., (1974) have demonstrated that in lake water (in-situ), 10% of the original virus introduced into the water were still infectious after 8 days. Coxsackie A9 and poliovirus type 1 were inactivated more rapidly in untreated lake water than in sterile lake water indicating a biological factor might be involved in the inactivation. In an in-situ study which utilized virus that was labelled via C14-leucine, Herrmann and coworkers (1974) showed that the virus coat proteins were degraded, presumably by the microorganisms indigenous to the lake water.

In contaminated reaches of the Illinois River Lamb and Chin (1964) isolated viruses including polioviruses 1, 2, and 3, coxsackieviruses A and B, echovirus type 7, and reovirus type 1. The presence of these viruses in this study corresponded with a poliomyelitis outbreak in the Chicago metropolitan area involving the type 1 and type 3 virus.

Human pathogenic viruses may also find their way into coastal waters vis direct discharge of wastes, or from sewage contaminated rivers that flow through coastal estuaries. The survival times for viruses in seawater vary. DeFlora, et al., (1975) studied water, sand, and sediments in coastal water near a sewage outfall. Many of the viruses isolated were vaccine strains of poliovirus. In in-situ studies they noted a 10% survival of poliovirus after 6 days in the seawater phase. This compares to a 10% survival of poliovirus after 1 to 2 days found by Shuval (1976) for Mediterranean seawater. This three-fold difference in apparent survival may reflect differences in seawater temperature.

Virus inactivation rates have been seen to be dependent on many factors, but especially on temperature. O'Brien and Newman (1977) looked at the inactivation of polioviruses types 1 and 3, and coxsackieviruses A-13 and B-1 in-situ in the Rio Grande River using membrane dialysis chambers. Inactivation rates were exponential, being primarily a function of temperature. Of the viruses tested, poliovirus type 1 and coxsackievirus type B-1 were the most stable. Poliovirus 1 was reduced one log (90%) in 25 hours at 23-27°C, but at 4-8°C it required 46 hours, almost twice as long, to achieve the same reduction. Virus inactivation was considerably lower in autoclaved river water, indicating a heat-labile, possibly microbial inactivation factor.
4.412 Occurrence and Survival in Sediments. In aquatic environments, the majority of the virus present is bound to particulate matter. When these particulates settle out of the water phase, they become part of the sediments, an association that is a reversible one. Turbulence can cause resuspension of these particulates. This, along with certain changes in the physical/chemical environment, can cause elution of virus particles from the particulates.

In the in-vitro studies by DeFlora and co-workers (1975), 10% of the original poliovirus in the sediments was still viable after 30 days. Viruses were transferred from the sediments to the water phase by "simulated wave action" (simple mechanical shaking). Gerba and co-workers (1977) found that poliovirus adsorbs readily to natural marine sediments, to the extent that they observed a 10 to 100-fold concentration of viruses in the sediments relative to the overlying water. Metcalf, et al. (1974), were able to isolate enteroviruses from bottom mud samples of the Houston Ship Canal. Mud samples taken from some oyster-growing beds have proved positive for coliphages and human enteric viruses (Vaugh and Metcalf, 1975). In-vitro studies show that viruses are provided enhanced survival in saline water when adsorbed to clay particles (Bitton and Mitchell, 1974; Gerba and Schaiberger, 1975). This has implications both from public health and environmental sampling viewpoints.

It is likely that sediments could act as a reservoir for viruses (DeFlora, et al., 1975; Gerba and Schaiberger, 1975), being released into the water phase when conditions favoring desorption exist. Viruses can be released by any one of a number of processes, both natural and man-made, including wave-action, dredging, and increases in the organic content of the overlying water (Gerba and Schaiberger, 1975). Indeed, this virus "time-bomb" in the sediments may endanger recreational and shellfishing uses of the water long after contamination has occurred. A similar type of phenomena may occur in freshwater sediments.

4.413 Occurrence and Survival in Shellfish. Laboratory studies indicate that oysters, clams, and mussels can concentrate enteric viruses to levels 10 to 60 times higher than their surrounding water (Crovari, 1958; Hedstrom and Lycke, 1964; Mitchell, et al., 1966). The survival of enteroviruses that are taken in by shellfish is significantly prolonged. Coxsackievirus B-3 and echovirus 6 persist at detectable levels for up to four months in the eastern oyster Crossostrea virginica (Metcalf and Stiles, 1967; Metcalf and Stiles, 1968). The high incidence of shellfish-induced hepatitis epidemics may be the result of the virus surviving for longer periods in shellfish than they would in water. The fact that most shellfish are filter feeders accentuates the problem, for they filter the virus particles out of the water into the protected shellfish. These viruses do not grow within the shellfish cells, they merely survive for long periods.

Significantly, human enteric viruses have been isolated from oysters harvested from seawater which was within the 70 coliform bacteria per 100 milliliter standard approved for and considered "safe" for shellfish growing waters (Gerba, et al., 1975; Metcalf and Stiles, 1968).
Fugate, et al., (1975) additionally have isolated echovirus type 4 and polioviruses types 1 and 3 from oyster-growing waters that met the bacterial standard. The types of enteroviruses that have been isolated from shellfish exposed to sewage-contaminated water includes echoviruses 5, 6, 8, and 12; polioviruses 1 and 3; and coxsackievirus A-18 (Bendenelli and Rucchi, 1969). Additionally, Australia antigen, which is the antigenic marker for Hepatitis type B virus, was found in clams contaminated by drainage of untreated hospital sewage (Mahoney, et al., 1974). In this study virus was found to pass from infected clams to previously uninfected clams.

4.42 Occurrence and Survival in the Terrestrial Environment. Viruses contaminate the terrestrial environment primarily through land disposal of domestic wastewater effluents or sludges, and through landfill disposal of solid wastes containing viruses. The virus hazards of disposal of sewage-related materials on land should be apparent. That of municipal solid waste disposal may be less obvious. The materials comprising solid waste are quite heterogeneous, and one would expect viral pathogens to be present in such items as disposable diapers and pet excreta (Peterson, 1971). A potential problem may be the possibility that viruses are leaching from landfill areas and entering groundwater. The ability of virus particles to do this depends on interaction of the virus with other components in the waste, with its physical/chemical environment, and with other soil factors which affect its ability to migrate through the soil (Lenette, 1977). The average density of viruses in municipal solid waste is estimated at 32 virus infectious units per 100 grams. Viruses may survive and appear in landfill leachate for up to 90-140 days (Gerba, et al., 1975; Lenette, 1977). Virus "leakage" from the landfill area is especially likely if the underlying soil does not have a high clay content (Gerba, et al., 1975).

With increasing state and federal restrictions and requirements on the discharge of sewage into bodies of water, more and more municipalities are giving serious consideration to disposal of domestic sewage on land. A recent EPA report indicates that more than 1000 communities in the U.S. are currently utilizing land sites for disposal of sewage sludge and effluents (Lenette, 1977). Deep-well injection is also being considered as a method for disposing of sewage sludges, in particular. This may pose a threat to groundwater quality. Some comfort is derived from the fact that many soils adsorb viruses strongly, but this is offset by the relatively slow inactivation rate of soil-bound viruses, and of the factors which favor virus desorption from soil particles, such as soil soaking by heavy rains, (Gerba, et al., 1975).

Laboratory studies using soil columns indicate that virus may move considerable distances through soil, particularly wet or soggy sandy soil. In general the residence times for active virus in the soil are long, varying as a function of several factors, especially temperature (Lenette, 1977; DuBoise, et al., 1976). At 4°C, 10% or more of the infectious virus could be recovered after 84 days. At 20°C, only 0.001% of the infectious virus input was recoverable after the same length of time. Due to the amphoteric nature of the viral capsid proteins, adsorption to and release from soil
particles occurs with fluctuations in the ionic strength and pH (DuBoise, et al., 1976).

4.43 Factors Affecting Virus Survival in the Environment. There are two competing and opposing processes in the environment which are of concern to virus survival. One set of processes are those which favor survival of the infectious unit, this includes factors such as aggregation, and autochthonous and allochthonous organic matter (Berger, et al., 1970; Clarke, et al., 1956; Joyce and Weiser, 1967). The second set of processes are those which tend to favor inactivation of the infectious virus particles, and includes factors such as microbial enzymes, heavy metals, etc. (Lycke, et al., 1965; Mitchell, 1971; Mitchell and Jannasch, 1969).

Viruses can be transported great distances in natural waters (Grinstein, et al., 1970; Lamb and Chin, 1964; Metcalf and Stiles, 1968; Shuval, et al., 1971; Theios, et al., 1967). This may be mainly a function of the minimum water velocity threshold that prevents virus-laden particles from settling by gravity or to coagulate and settle, this threshold depending mainly on particle size (Schaub and Sagik, 1975). The particulate matter to which the majority of virus in nature are attached consists of inorganic material (clays, silts) and organic matter (living and dead microbial cells, cell debris, detritus). It has been well demonstrated that enteric viruses adsorb strongly to this particulate matter under a wide range of temperature, pH, and metal ion concentrations in both fresh water and marine aquatic environments (Carlson, et al., 1968; DeFlora, et al., 1975; Gerba, et al., 1975; Hamblet, 1969; Schaub and Sagik, 1975; Wellings, et al., 1976). Such solids-associated virus is still infectious in tissue culture and whole animals (Gerba, et al., 1975; Schaub and Sagik, 1975) and plays an important role in aiding survival of viruses in the environment (DeFlora, et al., 1975; Wellings, et al., 1976).

Another factor which enhances virus survival in the environment is virus particle aggregation. Poliovirus is released from infected cells in a highly-aggregated state which maintains itself in water of low ionic strength. Young and Sharp (1977) noted that such aggregates are stable even after a one-step 7000-fold dilution into distilled water. Aggregated poliovirus also is not dispersed by a one-step dilution into lake water or tap water, but is dispersed if the diluent is clarified secondary sewage effluent (Young and Sharp, 1977). Gerba and Schieberger (1975) found that extensive aggregation of previously monodispersed virus occurs when it is added to artificial seawater, and, to a lesser extent, when it is added to natural seawater. The virus aggregates formed become as large as groups of 4 and adsorb to particulate matter as small as 0.1 μm in size. The presence of soluble organic matter dispersed these virus aggregates. However, since this study was done using a bacteriophage (T-2) it is uncertain what this means for human enteric viruses.

In the real world some of the aggregates of virus may actually be complexes of aggregated virus with suspended particulate matter (Young and Sharp, 1977). Fluctuations in pH, salinity, metal ion concentrations, and
dissolved organic matter (DOM), such as commonly occurs in estuaries, could lead to changes in the aggregation state of the virus and, thus, changes in the number of infectious foci, since one viral aggregate is known to produce a single plaque upon assaying (Galasso and Sharp, 1962; Gerba and Schaiberger, 1975).

Under laboratory conditions poliovirus and reovirus aggregate to clumps of up to 100 or more virions upon 10-fold dilution into distilled water. Interestingly, this aggregation did not occur for either virus when the dilution was 100-fold or greater. Presumably this is due to the kinetic limitations of one particle finding another, since this is a diffusion-limited process (the collision frequency is a function of the square of the particle concentration, so a 10-fold dilution of the particles leads to a 100-fold drop in the collision frequency). Poliovirus aggregates that formed in distilled water were reversible by dilution into phosphate-buffered saline (PBS) or 0.14 M sodium chloride, but reovirus aggregates did not disperse under similar conditions. Poliovirus aggregates to some extent at pH 3.5, and 6, but this aggregation was reversible by adjusting the pH to 7. Poliovirus aggregation at less-than-neutral pH values could be prevented by addition of salt (0.1 M at pH 6 and 2.5 M at pH 3) or divalent magnesium cation (0.25 M at pH 3). Aggregation does not occur at alkaline pH values equivalent to that experienced by water undergoing alum and lime treatment (Floyd and Sharp, 1977).

Virus inactivation in the environment is a rate-dependent process, being dependent on temperature, the presence of organic matter, salinity, agitation, and microbial activity (Akins, et al., 1971; DiGirolamo, et al., 1977; Gerba and Schaiberger, 1975; Hamblet, 1969; Matossian and Gababedian, 1967). Temperature plays a very important part in virus inactivation. For poliovirus type 1, echovirus type 6, and coxsackievirus type B-5 little inactivation occurred in estuarine water at 15°C over a 9 to 10 week period. At 25°C, however, a 4-log loss of infectivity was seen in the same period of time (Lo, et al., 1976). High temperatures attack both the protein coat and the nucleic acid core of the virus, leading to denaturation and loss of capsid protein followed by liberation of nucleic acids, resulting in empty capsids. The loss of biological activity in heated virus is thought to be primarily a function of the loss of native conformation of the capsid protein (Breindi, 1971).

Another significant force favoring virus inactivation is secretion of proteolytic enzymes into the environment by microbial, primarily bacterial, cells. Poliovirus type 1 was relatively unaffected by a wide variety of proteolytic enzymes, whereas coxsackievirus A-9 was quite sensitive (Cliver and Herrmann, 1972). Other factors interact with the microbial one. For example, poliovirus was sensitive to inactivation by bacterial growth, but the rate was pH-dependent. Studies utilizing coxsackievirus A-9 doubly labelled with C14-leucine and P32 indicate that the main effect of bacterial action is on the protein coat of the virus (Cliver and Herrmann, 1972).
4.5 The Need for Virus Standards for Water

There has been some controversy over what constitutes a safe level of virus in drinking water. One could propose a "no-threshold" position and demand their complete removal from drinking water. This, however, is probably an unreasonable, uneconomic, and unattainable goal. This is particularly true when viewed relative to other risks in society. Viruses constitute a risk of limited and familiar dimensions relative to such things as the myriad of anthropogenic carcinogens in man's environment, for example.

To date the coliform bacterial standard based on the MPN method has been the primary standard for determination of the microbiological safety of drinking water, recreational water, shellfish-raising areas, and shellfish (APHA, 1976; Finstein, 1972; Nenehson, 1970; Wilt, 1975). The wisdom of checking the bacterial content of drinking water first became apparent around 1900 when it was realized that there was some connection between removal of bacteria from water and reductions in bacterial disease mortality rates. The evolution of the standard since that time has paralleled the improvements in engineering technology's ability to remove bacteria from water. In the early 1900's engineering experience with slow-sand filtration of drinking water, along with some epidemiological input, led to adoption of a drinking water standard of 69 coliforms per 100 milliliters. The development of disinfection technology allowed the USPHS to formulate a more stringent standard of 2 coliforms per 100 milliliters in 1910. Research since that time into areas such as minimal infectious dose and the ratio of pathogens to coliforms has resulted in further adjustment of the standard (Shuval, 1976).

The coliform standard currently in use has several drawbacks. It is unable to differentiate between bacteria of fecal origin and those arising from natural sources such as vegetation and soil (Wilt, 1974). Some of the coliform bacteria as isolated from the water are "injured" and may not grow, depending on the method used, leading to undercounting the coliforms. Membrane filter methods, especially, add additional stress to these already stressed organisms. Also, coliform tests are subject to interferences by large populations of non-coliform bacteria, or when turbidity is considerable (Sobsey, 1977).

To circumvent some of the problems of the present standard (total coliforms), some have suggested basing a standard on the more specific fecal coliform and E. coli organisms (Wilt, 1974). Alternatively, the ratio of fecal coliforms to fecal streptococci has been proposed as a possible standard for fecal contamination (Geldreich, 1962; McFeters, et al., 1974). Coliforms of human origin are inactivated more rapidly in well water than human enterococci. The reverse pattern is seen for fecal organisms from wildlife. Similar results have been obtained in an ice-covered Alaskan river (Gordon, 1972), wastewater treatment lagoons (Klock, 1971), and in other natural waters (Geldreich, et al., 1968; Gyllenberg, et al., 1960).
The usefulness of bacterial standards, however, for determining the viral quality of water is limited. It is generally agreed that the absence of coliforms in water does not guarantee its safety from a virological standpoint. Although viruses are present in sewage in considerably smaller numbers that coliforms (1000 PFU per 100 milliliters compared to $10^7$ to $10^8$ per 100 milliliters for bacteria) the bacteria die-off much faster than the enteric viruses (Sobsey, 1974). This leads to a situation, which has been reported several times, of being able to isolate viable virus from drinking water, shellfish-raising water, and shellfish that met their respective bacterial standard (Bendenelli and Rucchi, 1969; Fugate, et al., 1975; Metcalf and Stiles, 1968).

The levels of bacteriophages and polioviruses have been used to determine the effectiveness of different water and wastewater treatment processes and could be used as a measure of the viral quality of water (Clarke, et al., 1961; Malina, et al., 1975; Safferman and Morris, 1976; Shelton and Drewry, 1973). The current trend in water quality indicates that municipalities will eventually be required to monitor viruses in their drinking water and wastewater effluents on a routine basis. Virus standards of 1 PFU per 100 gallons for drinking water and 1 PFU per 10 gallons for recreational water have been proposed (Hill, et al., 1976; Lenette, 1977; Shuval, 1976).

4.6 Current Methods for Concentration and Detection of Enteric Viruses From Environmental Samples

Current methods for detection of viruses in water involve a concentration step followed by quantitation, usually by the plaque-formation technique, although other methods such as immunofluorescence and complement fixation can be utilized.

The concentration method of choice is quite often membrane filtration. Viruses adsorb to such filters reversibly, adsorption being primarily due to electrostatic attraction of the filter for the virus. Cliver (1968) surveyed 28 membrane filters with four different enteroviruses (poliovirus type 1 - strain CHAT, coxsackievirus A-9, coxsackievirus B-2, echovirus 6) and one reovirus (type 1) and noted that adsorption of virus to membrane filters is governed by three factors - the chemical composition of the filter itself, the ratio of particle diameter to pore size, and the presence of substances, such as proteinaceous materials, which interfere with virus adsorption. Added to this list should also be substances which enhance virus adsorption, e.g. divalent magnesium cation. Cellulose acetate filters adsorb virtually no virus when the pore size exceeds the virus particle diameter by a factor of 3 or more. Cellulose nitrate filters, on the other hand, in the absence of interfering substances, adsorb enteroviruses significantly at a pore size as much as 285 times the virus particle diameter (Cliver, 1968). Other filters such as epoxy-fiberglass depth-cartridge units have been applied successfully to virus concentration. Adsorption of virus occurs at low pH, and elution of virus once concentration is complete is typically achieved using protein solutions or high pH buffers.
Several groups of researchers have addressed the problem of virus concentration from sewage, one of the most notable groups being Wallis and Melnick and their coworkers at Baylor University. One of their early methods (Wallis and Melnick, 1967) was adsorption of virus on cellulose ester filters after prefiltration, treatment with anionic exchange resins to remove organic compounds that interfere with virus adsorption, and addition of magnesium chloride to enhance adsorption. Elution was achieved by hand-homogenization of the filters in serum. Later they modified the technique, employing fiberglass and epoxy-fiberglass filters, followed by activated carbon and anionic exchange resin treatment to remove interfering organics, and then reconcentration on a 47 mm 0.25 to 3.0 μ pore size filter (Farrah, et al., 1976). A similar procedure, using acid adsorption and elution at high pH, was capable of detecting even solids-associated viruses in sewage effluent (Gerba, et al., 1978).

Beatrice England has used protamine sulfate treatment to obtain a 50 to 400-fold concentration of virus from sewage (England, 1972; England, 1971). This method was somewhat selective for reovirus and adenovirus over entero-viruses. Other workers have used a combination of protamine sulfate, alum, resin, and filter techniques (Moore, et al., 1970). Methods have also been developed for detection of viruses in sludges from anaerobic digestors (Eisenhardt, et al., 1977; Glass, et al., 1978; Lund, 1970; Lund and Ronne, 1973; Moore, 1975).

Farrah and coworkers (1977), have developed a technique for concentrating viruses from estuarine water using pleated cartridge filters. Methods for eluting viruses from marine sediments using proteinaceous, alkaline, or chelating solutions have been successful (Gerba, et al., 1977). Virus detection methods for coliphages and human enteric viruses in sediments and water from shellfish-raising areas are available (Vaughn and Metcalf, 1975). In addition, virus detection methods for shellfish tissue are well developed (Benedenelli and Rucchi, 1969; Carrick and Sobsey, 1977; DiGirolamo, et al., 1970; Fugate, et al., 1975; Hamblet, 1969; Konowalchuk and Speirs, 1973; Kostenbader and Cliver, 1972; Mitchell, et al., 1966; Sobsey, et al., 1975).

Wallis, Melnick and Metcalf have also developed a method for isolating enteroviruses from contaminated surface water that is similar to their technique for drinking water with the addition of three clarifying filters (Metcalf, et al., 1974). Several other groups of workers have approached this methodology problem for both human and nonhuman enteric viruses (Rao and Labzoffsky, 1969; Tschider, et al., 1975).

There has been considerable activity in the area of developing a standard method for detection of viruses in drinking water. All the methods to date utilize a microporous adsorbing filter which adsorbs viruses at low pH and desorbs them with either protein eluants or glycine buffer pH 9 or 11.5 (Akubowski, et al., 1975; Farrah, et al., 1976; Hill, et al., 1976; Sobsey, et al., 1973). Systems that have been tested include the MF nitrocellulose membrane filters, AA Cox M-780 epoxy-fiberglass, the K-27 yarn-wound fiber-
glass cartridge filter plus the AA Cox M-780, and the Balston epoxyfiber-glass tube-type filter. Acceptable virus recovery is possible with the Balston and MF 293 mm nitrocellulose filters down to 12 PFU poliovirus type 1 (Lsc) input levels (Akubowski, et al., 1975). Flows of 10 gallons per minute or higher are attainable with these filters, with recovery efficiencies averaging about 50%, and representing up to a 100,000-fold concentration of virus in a matter of hours (Farrah, et al., 1976). With all of the above filter methods, the presence of particulates, organic matter, and salts affect virus recovery efficiencies (Hill, et al., 1971).

The new tentative standard method for detecting viruses in drinking water (APHA, 1976) is already being put into use by the U.S. Environmental Protection Agency (EPA) and the Center for Disease Control (CDC) to survey water supplies and to investigate outbreaks of potential waterborne disease (Sobsey, 1977).

Only two methods are currently being utilized widely for quantitating viruses in clinical samples and environmental concentrate. They are determination of tissue culture infective dose (TCID) and plaque formation in mammalian cell cultures overlaid with semi-solid agar. The TCID method is based on formation of cytopathological effects (CPE) and has a reproducibility of 0.3 log. The plaque-counting method, while going more reliable counts, is not universally applicable since not all viruses form plaques, and plaque assays have not been well-developed for some viruses that could potentially form plaques. Probably most significant in terms of precluding application to widespread routine environmental monitoring of viruses is the need for tissue culture facilities with both techniques.

4.7 Immunoenzymatic Methods for Detection of Viral and Nonviral Antigens

4.71 Qualitative Methods. Several methods currently exist for identifying both viral and nonviral antigens with the use of specific antibody that is coupled to enzymes or other useful markers. In the case of viruses, a single virion is capable of binding many molecules of antibody. Steric hindrance by coupling of that antibody to a marker as large as an enzyme may result in some decrease in the maximum binding possible. Interestingly one or only a small number of molecules of antibody are sufficient to neutralize viral infectivity (Mandel, 1971).

Coupling of the enzyme to the antibody of interest can be achieved in several ways, but currently the most popular method is the use of glutaraldehyde to form a permanent covalent link between the two molecules. This technique has seen application to other areas, such as forming cross-linked insoluble preparations of antigen or antibody which can then be used as specific immunoadsorbents for concentrating or purifying a given antigen or antibody (Avrameas and Ternynck, 1961a). For the preparation of enzymes covalently linked to proteins, glutaraldehyde was the most effective of several alternative coupling agents (Avrameas and Ternynck, 1969b). For coupling enzymes to antibodies, glutaraldehyde was far more effective than other coupling reagents for producing conjugates which retained both signi-
ficant enzymatic and immunological activities (Avrameas, 1970).

Several different enzymes have been identified as being useful for immunoenzymatic techniques that employ enzyme coupled to antibody. These enzymes include horseradish peroxidase, acid phosphatase, alkaline phosphatase, glucose oxidase, tyrosinase, and lactic dehydrogenase (Avrameas, 1970; Nakane and Pierce, 1967). The conjugates are both enzymatically and immunologically active after coupling with bifunctional reagents. Since any one molecule of enzyme can react with many molecules of substrate, the use of a substrate that produces a highly colored and insoluble product adds considerable "amplification" to the detection sensitivity that would be available by antibody alone (Nakane and Pierce, 1967). Exceedingly small amounts of antigen can be detected by enzyme-antibody conjugates when incubation with substrate is prolonged until a significant quantity of enzyme reaction product has accumulated.

The most satisfactory and reproducible results are obtained with enzymes of high specific activity (Avrameas, 1970). Use of highly purified antibodies is also necessary to reduce nonspecific background staining, a problem which will be addressed later in further detail. In general, the more impure the enzyme preparation used and the higher the content of nonspecific proteins in the antibody preparation, the more significant is the nonspecific staining. By comparison, peroxidase is a better marker enzyme than alkaline or acid phosphatase because it produces cleaner conjugate preparations that yield lower nonspecific background staining.

Immunoenzymatic methods have been used in several studies of the effects of virus on the host cell. In particular, the structural antigens of simian virus 40 (SV40), adenovirus 12, and rat K virus, as well as the nuclear antigens induced by these viruses, have been detected by antibody linked to horse-radish peroxidase, alkaline phosphatase, and glucose oxidase (Avrameas, 1970; Miller, et al., 1974). Several of these antigens have been detected in tissue simultaneously by coupling each specific antibody used to a different enzyme and by using substrates that produced differently colored reaction products. Immunoperoxidase techniques (the use of specific antibody coupled to peroxidase) has been used for identifying myxoviruses, paramyxoviruses, poliovirus, herpes simplex and SV40 in cultural cells (Benjamin, 1974; Benjamin and Ray, 1974; Herrman and Morse, 1973; Miller, et al., 1974). In the cases of herpes simplex, myxoviruses and paramyxoviruses this technique was used for typing (Benjamin, 1974; Benjamin and Ray, 1974).

Peroxidase-antibody conjugates have also been used for identification and study of nonviral antigens. These include spirochetes, Staphylococcal enterotoxin A, and alpha - antitrypsin (Palmer, et al., 1974; Saunders and Bartlett, 1977; Steinberger, et al., 1970).

4.72 Quantitative Methods. Only one quantitative immunoenzymatic technique has reached the stage of development in which it is useful as a
research tool. This is the enzyme-linked immunoadsorbent assay, or "ELISA" (Engvall and Perlmann, 1972). This is a sensitive and simple method for measurement of either antigens or antibodies. When the test is set up for detecting antibodies, polystyrene tubes or microplates are coated with antigen and then incubated with antiserum. This is then followed by treatment with an enzyme-labeled preparation of anti-immunoglobulin. The enzyme remaining in the tubes after washing is then reacted with a colorimetric reagent. The optical density or absorbance of the reaction products, measured spectrophotometrically, is a measure of the amount of antigen originally adsorbed to the tubes. This method is analogous to the indirect double-layer qualitative methods described above except that the antigen being detected is adsorbed to polystyrene instead of bound to tissue. This method has been utilized for quantitating substances as diverse as aflatoxin B (Lawellin, et al., 1977) and plant viruses (Clark and Adams, 1977; Voller, et al., 1976).

The sensitivity of this technique is limited, however, especially for virus quantitation, for several reasons. First, only a limited amount of the antigen or antibody will bind to the polystyrene. In addition, some of the adsorbed antigen or antibody is released from the tubes during the incubations and lost during the subsequent washings. Also the sensitivity of this technique is limited by the detection limits of the spectrophotometric or colorimetric assay.

ELISA is more sensitive than use of immunodiffusion or immunological precipitation techniques (Clark and Adams, 1977), and about equivalent in sensitivity to fluorescent or radioimmunoassay techniques (Burgett, et al., 1977; Green, et al., 1974; Katzuelson, 1976). For viruses, ELISA sensitivity is at the nanogram level, with 10-100 ng/ml plant viruses being detectable. The usefulness of this technique for detection of enteric virus in water and wastewater is uncertain as we are working at virus concentrations orders of magnitude lower.
5.0 A Proposed Immunoenzymatic Method for the Detection of Poliovirus in Water

The objective of this project was the development of an immunoenzymatic technique that would allow routine and rapid quantitation of the viral contaminants of water. Additionally, these immunoenzymatic methods would circumvent the need for mammalian cell culture facilities that increase the cost and complexity of virus enumeration.

The immunoenzymatic technique that will be investigated depends on the isolation of poliovirus on membrane filters followed by the binding of enzyme to the virus particles through the mediation of specific antibodies. When a dye is added that can participate in the enzymatic reaction, a highly-colored insoluble product is formed which precipitates around the virus particle. If the reaction is allowed to proceed for a sufficient amount of time, the reacted dye particles produced are visible under a conventional light microscope and permit visualization and enumeration of the virus particles.

The development of a simple, sensitive and specific technique for enumerating enteroviruses in natural waters is now possible because the recently-developed filtration techniques mentioned in Section 4.6 permit the concentration of enteroviruses from large quantities of water. Also, immunoenzymatic methods have been developed to the point where they are sufficiently sensitive and specific that they could be applied to such a determination. As evident from Section 4.7, immunoenzymatic methods have been utilized primarily for visualization of various antigens, including viruses, in tissue culture, but the methods lend themselves to other uses.

In development of this methodology for detection of human enteroviruses, it was decided to identify an indicator virus toward which the emphasis of the project could be directed. In this case the most truely representative virus is poliovirus. Poliovirus has many characteristics in common with other human enteroviruses in terms of survival in sewage treatment processes, resistance to disinfection and viability in natural waters (Metcalf, 1971; Wilner, 1965). The other factor which makes polioviruses an excellent choice as an indicator virus is that it is being excreted by a significant portion of the U.S. population as a result of administration of live attenuated poliovirus in the form of the Sabin polio vaccine. It should, therefore, occur in water in large numbers relative to other enteroviruses and provide a convenient marker for their presence in water.

Immunoenzymatic methods developed for poliovirus are quite specific by nature of the specificity inherent in the antibodies used in the procedure. This specificity arises from the three-dimensional structure of the particular antibody molecule. The relevant antibodies are of the immunoglobulin G or IgG type; which have molecular weights of about 150,000 Daltons. IgG molecules consist of three distinct parts - a large region which has
essentially constant amino acid composition for all types of IgG antibodies synthesized in that animal, and 2 smaller lobes which each contain a sequence of amino acids which varies depending on the antigen that triggered synthesis of the antibody in the animal. It is in these two variable regions of the molecule that binding with specific antigen takes place. Thus, anti-poliovirus antibody will react only with poliovirus, and this reduces the likelihood of obtaining counts that are too low or too high. This is in contrast to coliform detection methods where counts vary according to the viability status of the bacteria sampled or plated (Ray and Speck, 1973). Also, some coliform detection methods themselves, especially filter assays, apply stress to the organisms in the form of drying and hydrostatic pressure which may result in cell death or injury and yield low counts. Measurement of viruses via immunoenzymatic means possesses potentially greater sensitivity and accuracy than either the plaquing technique used for viruses (Berg, et al., 1963) or the MPN counts of coliforms (Finstein, 1972). The immunoenzymatic methods can detect human pathogens without being subject to interferences from the presence of viruses of non-human origin. This is in contrast to coliform assays which are only indirect indicators of pathogens and are subject to false positives from bacteria of non-fecal origin.

The immunoenzymatic procedure can be used with a test-tube assay or filter assay. The flexibility of this technique should also be noted. If, at some later time, assay for another virus is desirable (like coxsackievirus, or even hepatitis virus, when techniques for the cultivation are developed) a different specific antibody could be used to prepare the enzyme-antibody conjugate and it could then be used with the same procedure. Essentially, the enzyme-antibody conjugate is used like a reagent and to look for different viruses it would only require changing reagents.

All immunoenzymatic techniques employ either specific antibody covalently linked to an enzyme, or a series of antibodies and enzyme. A colorless compound which can serve as substrate for the enzyme is added and converted by the enzyme to a highly colored reduced form. Thus, spectrophotometric detection of solubilized reduced product or microscopic detection of clumps of insoluble reduced product is possible.

Figure 5-1 diagramatically presents the 3 basic types of immunoenzymatic techniques for detecting viruses. These are referred to as the direct, indirect, and mixed enzyme-antibody methods. The direct method employs a conjugate of antiviral-antibody and enzyme that is produced by reaction with a coupling agent such as glutaraldehyde. The indirect method first allows binding of antiviral antibody to the virus, followed by a conjugate of enzyme and an antibody to the antivirus antibody. The mixed enzyme-antibody method does not utilize a covalently-coupled conjugate, but relies on adsorption to the virus particle of a series of antibodies followed by adsorption of enzyme. The emphasis in this project is on the mixed enzyme-antibody approach since it provides more "amplification" than the other two methods as a result of ultimately binding a greater number of enzyme molecules per virion. Thus, this technique will likely have the greatest sensitivity of the three methods.
**DIRECT METHOD**

![Diagram of Direct Method](image)

**INDIRECT METHOD**

![Diagram of Indirect Method](image)

**MIXED ENZYMEL-ACTIVITY**

![Diagram of Mixed Enzyme-Antibody](image)

**FIGURE 5-1**

**IMMUNOENZYMATIC METHODS**

**KEY**

- Virus Particle
- Antibody Molecule
- Enzyme Molecule
6.0 Materials and Methods

6.1 Cell Culture

Vero or Green Monkey Kidney (BGMK) cells were propagated by trypsinization of existing cell cultures, dilution into fresh growth media and distribution to cell culture bottles. Small volumes (5-20 ml) of 1 X trypsin or phosphate-buffered saline (PBS) were used as a rinse prior to trypsinization to remove interfering materials. Twenty ml 1 X trypsin was used for trypsinizing large glass roller bottles at 37°C for 20 minutes. The detached cells were then pelleted by low speed centrifugation in 50 ml centrifuge tubes containing 1-2 ml fetal calf serum (FCS) and resuspended in growth media. The usual passage ratios were 1:3 or 1:4 for producing roller bottles and 1:100 for producing 1 oz. bottles, for plaque assay, from roller bottles. These cell cultures were grown to confluent monolayers, whereupon they were utilized for passaging or virus production in the case of roller bottles, or for virus assays in the case of 1 oz. bottles. The growth media consisted of 100 ml of autoclavable Minimal Essential Media (MEM, Grand Island Biological Co.) supplemented with 1.5 ml 7.5% sodium bicarbonate, 10 ml FCS, glutamine, and antibiotics.

6.2 Poliovirus Production

Roller bottles containing confluent monolayers of BGMK or Vero cells were infected in high multiplicity with 1-2 ml of stock poliovirus 1-Lsc (virus titre = 10^7 - 10^8 plaque-forming units/ml) after rinsing the monolayers with PBS or tris-buffered saline (TBS). After a few minutes of incubation to allow virus attachment, 80 ml of maintenance media with or without serum, was added to the bottle. Maintenance media was the same as growth media except that it contained twice as much bicarbonate and either no serum or only 2% serum (2 ml/100 ml MEM). The infected bottles were incubated at 37°C and harvested after 16-24 hours by freezing and thawing 3 times. A crude virus stock was obtained by pelleting (2000 rpm X 20 min., Sorvall RC2-B) cell debris from the virus-containing supernatant produced by the 3 freeze-thaw cycles.

6.3 Purification of Antibody by Immunoadsorption

Immunoadsorption was examined as a possible method for providing highly purified antibody, free of nonspecific antibodies and unwanted serum proteins, for use in development of the immunoenzymatic assay. The method used was similar to the batch-wise procedure of Avrameas and Ternyck (1969; 1971). To 10 ml of freeze-thaw lysate from infected roller bottles of Vero cells was added 300 mg lysozyme and 400 mg bovine serum albumin (Calbiochem) along with 2 ml of 1 M phosphate buffer, pH 7. To this mixture 2 ml of 2-5% glutaraldehyde (Applied Sciences) in PBS was added dropwise to the stirred solution.
After incubation at room temperature for fifteen minutes, a gel formed. This gel was suspended in cold PBS and homogenized, in portions, in a hand homogenizer. The suspension was vortexed and centrifuged for five minutes at 1000 rpm at room temperature in a clinical centrifuge to remove unreacted virus and protein. This was repeated two or three times. The gel particles were suspended and resuspended several times in 0.1 M glycine buffer, pH 1.4. This mixture was then suspended and resuspended into 0.1 M phosphate buffer, pH 7.0. Five milliliters of whole antipoliovirus antiserum was incubated with the rinsed immunoadsorbent gel particles at 4°C for 24 hours, or at 25°C for thirty minutes. This material was then washed free of unbound antibody with 0.1 M phosphate buffer, pH 7.0. Bound antibody was eluted with 0.1 M glycine buffer pH 1.4 for fifteen minutes at 4°C. The eluate was collected by low-speed centrifugation, neutralized, and an aliquot was taken from VNT determination by the procedure in Section 6.8.

6.4 Preparation of Monodisperse Virus

Monodisperse poliovirus type 1 - Lsc was prepared by the method of Sharp et al. (1976; 1977). This procedure consisted of Freon extraction of infected cell lysate, followed by centrifugation in sucrose gradients (in 0.05 M phosphate, pH 7.2) at 25,000 rpm in a Beckman SW27 rotor (4°C, 2 hours). The virus was collected from the top of the centrifuge tubes, fractionated, and assayed for virus by particle counts and infectivity (plaque assay).

6.5 Virus Plaque Assay

Virus samples are serially diluted in PBS with serum or TBS with serum (2%). Aliquots of 0.1 ml volume are applied to the monolayer side of 1-ounce bottles of Vero or BGMK cells. The bottles are tilted to evenly spread the virus suspensions, and then incubated at 37°C for one hour, tilting again every 15 minutes to assure even distribution of the virus. The overlay media for this assay is prepared as follows: 100 ml of 2.8% agar (Difco), previously autoclaved, is heated in a steril bottle until melting occurs. The melted agar is combined with various nutritional and preservative components which have been prewarmed to 45°C. These include 100 ml 2X Minimal Essential Medium (MEM), 10.5 ml 2.8% sodium bicarbonate, 1.25 ml 4 M MgCl₂, 1 ml 200X antibiotics, and 1X neutral red. This mixture is maintained in a 45°C water bath while dispensing 5 ml quantities with a Hamilton Syringe to the 1-ounce bottles of Vero or BGMK cells that have received virus aliquots. The overlay media is applied to the inside face of the bottles that does not have the cell monolayer attached. The bottles are turned and the overlay is allowed to harden on top of the monolayers for 20 minutes at 25°C. The bottles are then inverted and incubated at 37°C. After 3 days an initial plaque count is done, and this is verified by final plaque counts at days 4 and 5. An average value is taken for each set of triplicate bottles.
6.6 Physical Assay of Purified Virus

The number of virus particles in a virus preparation were quantitated by the use of the kinetic attachment method of sharp (1974). Virus particles were allowed to attach to a collodion grid at room temperature, after which the grids were shadowed and counted under the electron microscope.

6.7 Production of High-Titre Antiserum to Poliovirus

6.71 Production and Purification of Poliovirus Antigen. Twenty roller bottles of Vero cells were infected with poliovirus at high multiplicity as in section 6.2 to produce a large volume of crude virus stock. This virus stock was then purified by a series of filtrations and treatments. It was first passed through a 0.45 µm Cox filter pretreated with 100 ml sterile 1% Tween-80. It was then passed through a sterile PM-30 Amicon membrane filter at 40 psi until a volume of 20 ml remained. The retentate and PBS washings of the Amicon filter were then Freon-extracted with an equal volume of cold Freon 113 and blended in a small pre-cooled Waring blender for one minute according to the method of Sharp and Floyd (1975; 1976). The phases were separated by low speed centrifugation (500 rpm x 10 min.) in a Sorvall GLC-1 centrifuge. The aqueous phase was combined with the aqueous phase from a similar re-extraction of the Freon phase and passed through a sterile 0.45 µm Cox filter (with a 1.9 µm AP-200 prefiltler) at pH 3.5 in the presence of 0.0005M aluminum chloride. The adsorbed, concentrated virus was then eluted twice with 7 ml of pH 11.5 0.05M Glycine-NaOH and neutralized to pH 7-8.

These eluates were then combined for inactivation of the virus, which was accomplished by the method of Salk (1955). Virus was inactivated by incubation with 0.05% formaldehyde in distilled/deionized water at 37°C for 24 hours in the presence of 2 X antibiotics. Unlike the Salk method, no sodium bisulfite was used to neutralize excess formaldehyde. The pH of the inactivated virus preparation was 7.6. Plaque assays revealed no remaining infectivity in this preparation.

6.72 Antibody Production. The formaldehyde-inactivated virus produced according to Section 6.71 was mixed at a ratio of 1:1 with complete Freund's adjuvant (Cappel Laboratories), emulsified, and injected into a young, female sheep at days 0, 21, 28, and 35. At each injection time, injections were made at 5 sites, 2 ml per site. The sites used included 3 locations on the neck and one on each flank.

The sheep was bled on each day of immunization just prior to immunization, blood being taken using a 17 guage needle donor kit (Cutter Labs, Inc.) and a 500 ml Travenol sterile vacuum bottle (Travenol Laboratories). The whole blood was transferred immediately to plastic centrifuge bottles and allowed to clot at room temperature for 1-2 hours. The clot was then ringed with a spatula and the bottles were refrigerated for 4 hours. These bottles were spun for 20 min. in a refrigerated Sorvall RC2-B at 6000-8000 rpm (GSA rotor, 20 min.). The serum was removed from the bottles by careful
pipetting and stored at -20°C.

Virus Neutralization Titre's (VNT's) were performed on all serum samples according to the procedure in section 6.8. Day 41 antiserum was tested for anti-Vero cell activity by performing VNT's in both Vero and Hep-2 cells. Hep-2 cells used for the VNT were grown on medium 199 with glutamine, antibiotics and fetal calf serum (5%) added. The overlay for the Hep-2 VNT consisted of 15 ml 10X medium 199, antibiotics, 7.5 ml heat-inactivated fetal calf serum, 4.9 ml, 7.5% sodium bicarbonate, 0.24 ml 4M magnesium chloride, 1.0 ml 0.3% neutral red, and 120 ml 1.25% agar.

6.8 Virus Neutralization Titer Determinations. Virus neutralization titer (VNT) is defined here as the dilution of antiserum, which when mixed 1:1 with a live poliovirus suspension in TBSS (TBS) containing 2 ml Δ FCS* per 100 ml) yields on 80% reduction in the plaque count. 0.4-0.5 ml of two virus dilutions (3-4 X 10^3 PFU/ml, 3-4 X 10^2 PFU/ml) were each mixed with an equal volume of serial dilutions of antiserum in PBS. Antibiotics were added and the mixtures were incubated for 1.5 hours in a 37°C water bath. Both positive (virus only) and negative controls (no virus) are included. The incubated virus-antibody mixtures were then plaqued directly in 1 oz. bottles according to the plaque assay procedure.

6.9 Gel Diffusion. Gel diffusion was carried out with all antibody preparations by the method of Ouchterlony (1962) to determine the purity and specificity of the preparations. Diffusion plates used were 7-well IDP-II cells (Cordis Laboratories) possessing a 5 mm maximum diffusion distance (Lot #71395). They consist of 1% agarose in saline-phosphate buffer, pH 7.2, containing 0.1% sodium azide as a preservative. The 50 μl wells were loaded with 40 μl of PBS, antigen, or antibody using a Hamilton syringe. The syringe was rinsed with PBS several times between loadings. After incubation for 3 days the templates were removed and the gels were rinsed in PBS for 15 hours, with a charge of PBS after the first 40 minutes. The gels were stained with nigrosin for 3 hours, followed by destaining with several charges of 2% acetic acid.

6.10 Reduction of Nonspecific Background Staining. Various pretreatments were applied to several different filter types to determine their relative effectiveness in reducing nonspecific background staining. Filters including 0.01 μm Sartorius, 0.025 μm Millipore, and 0.05 μm Sartorius filters were pretreated by incubation at room temperature, or filtration with, 0.01% Siliclad, 1% Tween-80, 10% Tween-80, 0.7% albumin, or a combination of 10% Tween-80 and 0.7% albumin. The pretreated filters were then exposed to the entire immunoenzymatic filter assay procedure described in Section 6.14, but without the addition of virus. The filters were then incubated with diaminobenzidine reagent, also described in Section 6.14,

* ΔFCS = heat inactivated fetal calf-serum.
for several hours. Staining on the upper and lower sides of the filter were estimated on a scale of 0 (no staining) to (maximum staining) by visual comparison.

6.11 Virus Retention on Filters. A variety of filters were screened for their ability to retain Poliovirus Lsc-1. Virus stock was diluted to a titer of approximately \(1 \times 10^4\) PFU/ml in TBSS. One-half to five ml quantities of this dilution were filtered through the test filters at low pressures (< 40 psi N2). Both filtrates and original dilutions of virus were titered by the plaque assay in triplicate. Filters tested included 0.01 \(\mu\)m cellulose nitrate (Sartorius), 0.03 \(\mu\)m polycarbonate (Nucleopore), XM 100 A and XM 300 (Amicon), 0.01-0.02 \(\mu\)m cellulose acetate (Sartorius), 0.025 \(\mu\)M MVS (Millipore), 0.05 \(\mu\)M (Millipore), 0.05 \(\mu\)M (Sartorius), 0.08 \(\mu\)M (Sartorius) and 0.9 \(\mu\)M epoxyfígiberglass (Versapor). The more promising filters were also tested for virus retention under conditions where the filters were pretreated with Tween-80 and serum albumin. As mentioned in section 6.10 this treatment was necessary in the immunoenzymatic assay to reduce nonspecific background staining.

6.12 Determination of Properties of Alginate Filters. To determine the compatibility of alginate filters with reagents in the immunoenzymatic procedures, 13 mm alginate filters (0.05 \(\mu\)m, Sartorius) were subjected to phosphate buffered saline concentrations of 0.007 to 0.14 M, and to poliovirus antiserum concentrations ranging from 0.1 to 5 percent. Solutions were placed on the filter in a 13 mm ultrafiltration cell and drawn through the filter under low pressure. The filtrates were collected in pre-rinsed 13 X 100 mm capped glass tubes to be tested for turbidity and eluted carbohydrate. Turbidity was determined using a Hach 2100 A Turbidimeter, standardized to 0.61 NTU with a chlorobenzene standard and to 10 NTU with a Hach standard. Eluted alginate in the form of suspended carbohydrate was determined using the orcinol-sulfuric acid method (Bruckner, 1953). Reacted samples were read at 520 nm on a Gilford 300-N spectrophotometer equipped with an automatic sampler. Negative controls for both turbidity and carbohydrate determinations were unfiltered dilutions of phosphate buffered saline or serum.

6.13 Immunoenzymatic Tube Assay. An immunoenzymatic assay system based on the principles of the ELISA technique (Clark and Adams, 1977; Engvall and Perlmann, 1972; Lawellin, et al., 1977; Voîler, et al., 1976) was tested. The procedure is outlined in Figure 6-1. Polystyrene tubes for the assay were coated with a dilution of immunosorption purified horse antipoliovirus antibody. All antibodies used in the assay were obtained in lyophilized form from Cappel Laboratories. Sheep antipoliovirus antiserum was from day 41 antiserum. The negative virus control used PBS containing 1-10% Tween-80. Positive virus samples utilized monodisperse virus prepared according to the technique in section 6.4. Viability of virus during the adsorption incubation from 14 hours at 4°C was verified using the plaque assay as described in section 6.5. All antibody, enzyme, and PBS solutions were pre-filtered through 0.45 \(\mu\) filters or centrifuged before use to remove
Figure 6-1 - Immunoenzymatic Tube Assay

Sterile poly-styrene tube → Add 1 ml of 0.02 mg/ml horse antipoliovirus antibody in 0.05 M Na₂CO₃ pH 9.6 containing 0.02% NaN₃ → Remove. Add 0.9% NaCl containing 0.05% Tween-80. Incubate 2 minutes. Repeat 3x

↓

Adsorb 0.09 ml virus diluted in PBS with 0.05% Tween-80 and 0.02% NaN₃ (PBSTA). Incubate 1 hr., 37°C.

↓

Remove. Rinse 3x with 0.9 ml PBSTA.

↓

Add 0.9 ml sheep antipoliovirus antiserum diluted 1:150 in PBSTA. Incubate 30 min., 37°C.

↓

Remove. Rinse 3x with 0.9 ml PBSTA.

↓

Add 0.9 ml 0.01 mg/ml rabbit antisheep antibody - peroxidase conjugate in PBSTA. Incubate 30 min., 37°C.

↓

Remove. Rinse 3-5x with PBSTA.

↓

Add o-dianisidine reagent

↓

Measure change in optical density per hour at 460 nm.

PBS = Phosphate buffered saline
extraneous particulate matter. The enzyme use was horseradish peroxidase (Worthington). The dye reagent used consisted of 0.0112 g o-dianisidine hydrochloride dissolved in 1 ml of methanol, 10 ml 1/60 dilution of Triton X-100, 1 ml of 1/127 dilution of 30% hydrogen peroxide, 38 ml of deionized-distilled water and 50 ml 0.1 M phosphate buffer, pH 6.0. To the rinsed tubes, 0.9 ml of this reagent is added. The change in optical density at 460 nm was recorded.

6.14 Immunoenzymatic Filter Assay. The procedure for the immunoenzymatic technique is shown in Figure 6-2. The filters used were Sartorius 0.01 μm cellulose nitrate filters. All solutions were prefiltered through 0.025 μm cellulose nitrate filters before use to eliminate extraneous particulate matter and thus avoid filter clogging and high nonspecific background staining problems. Filters used in the assay were pretreated with 10% Tween-80 followed by 0.7% bovine serum albumin (A Grade, Calbiochem). All antibodies used in the procedure, with the exception of sheep antipoliovirus antiserum were obtained in lyophilized form from Cappel Laboratories. Horseradish peroxidase was obtained from Worthington. The poliovirus used was monodisperse and was prepared by the technique in section 6.4. Filters that received virus and immunoenzymatic reagents were stained, after rinsing in PBS, with diaminobenzidine reagent. Filters were air dried at 37°C and counted for reacted dye particles under the oil immersion lens of a Zeiss microscope. The dye reagent used for the immunoperoxidase assay was a modification of that of Kaplow (1965) and Avrameas (1971). Unless noted otherwise the stain consisted of 20 ml 0.1 M Tris buffer, pH 7.5, 10 mg diaminobenzidine hydrochloride and 1.0 ml 3.0% hydrogen peroxide (H2O2). Distilled-deionized water was used for making the dye reagent to avoid ion contamination which interferes with the assay. This dye reagent was incubated with the treated filters for up to 24 hours and removed by rinsing with distilled deionized water.

6.15 Photomicroscopy. Filters from the immunoenzymatic assay were photographed to verify reacted dye particle counts. A Zeiss microscope was used under the oil immersion lens, with an EFM adaptor allowing attachment of a Nikon M-35S camera. The film used was Ilford ASA 50 black and white (Pan F 135-20). Total magnification power of the system was 750 X. Blue or green filters were used to maximize the contrast between the reacted dye particles and the background. The film was developed using Kodak Microdol X (13 min, 21 + 2°C). The stop bath was 1% reagent grade acetic acid (30 seconds), and the fixer was Kodak fixer (10 minutes). Contact sheets were made with the aid of an enlarger, and developed in the following sequence: 1:2 Dektol for 1% minutes; 1% acetic acid for 5 seconds, and Kodak fixer for 2 minutes.
Figure 6-2 - Immunoenzymatic Filter Assay (Virus Visualization Procedure)

Pretreated filter (containing virus rinse 2x with PBS

Add 1-2 drops sheep anti-polio antiserum (5 mg/ml) incubate 15-30 min., 25°C

Rinse 3-5x with PBS or TBS

Add 1-2 drops rabbit anti-sheep antibody (5 mg/ml) incubate 15-30 min., 25°C

Rinse 3-5x with PBS or TBS

Add 1-2 drops of sheep anti-peroxidase (5 mg/ml) incubate 15-30 min., 25°C

Rinse 3-5x with PBS or TBS

Add 1-2 drops peroxidase (5 mg/ml) incubate 15-30 min., 25°C

Rinse 3-5x in PBS or TBS

Transfer filter to cellulose pad in petri dish (soaked in diaminobenzidine reagent)

Incubate in dark (1-20 hours)

Rinse filter with distilled water and examine under microscope-virus indicated by brown spots on filter.

PBS = Phosphate buffered saline, TBS = Tris buffered saline.
7.0 Results and Discussion

7.1 Sheep Antipoliovirus Antibody Production

As no commercial preparations of antipoliovirus antibody were readily available, antiviral antiserum was produced in our laboratory. This was the only immunological reagent that had to be custom-made.

The production of antipoliovirus antibody in sheep requires relatively pure virus to serve as the antigen. The objective of the first step of this process was to produce purified virus of high titer. The recovery of poliovirus infectivity at various stages of the antigen preparation procedure is shown in Figure 7-1. Overall recovery of virus infectivity prior to the formaldehyde inactivation step was 36 percent. The antiserum produced with this antigen exhibited very high neutralizing titer. The increase in virus neutralization titer with time after initial inoculation is plotted in Figure 7-2. By 41 days after inoculation the titre of the antiserum stabilized at 1:110,000. This high titer reflects the high degree of purity of the antigen used for immunization. The fact that nearly identical VNT's were obtained using poliovirus and day 41 antiserum in both monkey (Vero) and human (Hep-2) cells indicates that essentially none of the activity of the antiserum was directed at the Vero cells in which the virus antigen was produced.

These results suggest that antibody of very high activity and specificity for poliovirus was present in our preparation of sheep antipoliovirus antiserum. Since this high-activity/high-specificity antiserum was used for all subsequent immunoenzymatic assays, the sensitivity of the assays was considerably enhanced, and cross-reactivity of the antiserum with cellular components isolated as part of virus preparations (which would give spuriously high results) could be expected to be minimal.

7.2 Gel Diffusion

Gel diffusion tests were performed on antibodies that were purchased commercially to determine the presence of immunological activity. This was done to assure that effort expended in developing the immunoenzymatic assays was not spent using inactive reagents. Gel diffusion tests were also performed on sheep antipoliovirus antiserum as an independent way of confirming the high activity and high specificity that was noted in Section 7.1. All antibodies submitted to this test produced single precipitin bands when diffused in an agarose gel toward their respective antigens. Since these precipitation zones formed from microgram amounts of material, relatively high immunological activity was indicated for all preparations tested. As in Section 7.1, the higher the activity and specificity of the immunological reagents used in the assays, the greater will be the sensitivity of the immunoenzymatic assay, and the lower will be the detection limit (the lowest quantity of virus that can be detected with statistical significance).
Figure 7-1. Recovery of Poliovirus Infectivity During Antigen Preparation

Vero Roller Bottle Cultures Infected at High Multiplicity

1. Freeze-thaw 3X
2. Centrifuge 10-speed

*Initial Total: 9.1x10^10 PFU*

- Supernatant (9.1x10^10 PFU)
- Pellet

3. Centrifuge (supernatant)
4. Centrifuge (pellet)

- COX 0.45u filter
- Retentate: 6.6x10^10 PFU
- Filtrate: 1.6x10^8 PFU

5. Centrifuge (retentate)
6. Centrifuge (filtrate)

- PM-30 membrane filter
- Freon extraction (2X) (phase separation by 10-speed centrifugation)

*Final Total: 4.0x10^10 PFU*

7. Adjust aqueous phase to pH 3.5, add AlCl₃ to 0.005M
8. Adsorb to COX 0.45u filter (to remove excess protein and concentrate antigen)
9. Rinse with 10 ml. PBS
10. Elute with 7 ml. 0.05M glycine, pH 11.5
11. Adjust eluate to pH 7

*Final Total: 4.0x10^10 PFU*

12. Inactivate virus in 1/800 dilution (in distilled/deionized water) formaldehyde, pH 7.0-7.6/37°C/24 hours
13. Plaque: <1 PFU
FIGURE 7-2
DEVELOPMENT OF ANTIPOLIOVIRUS ANTIBODY IN SHEEP SERUM
VIRUS NEUTRALIZATION TITRE VS TIME

VIRUS NEUTRALIZATION TITRE

Virus Neutralization Titre

TIME AFTER INITIAL INOCULATION (DAYS)

INOCULATION GIVEN

0 10 20 30 40 50

0 1:50,000 1:100,000 1:150,000

v = INOCULATION GIVEN

FIGURE 7-2
DEVELOPMENT OF ANTIPOLIOVIRUS ANTIBODY IN SHEEP SERUM
VIRUS NEUTRALIZATION TITRE VS TIME
7.3 Reduction of Nonspecific Background Staining

Development of methods to reduce nonspecific background staining for eventual inclusion as part of the filter assay of Section 7.7 received a large amount of attention. It is necessary to treat the filter with some sort of coating agent to minimize the binding of excess antibody and enzyme to the filter. Otherwise, background reactions in the filter assay would obscure the presence of virus-antibody-enzyme complexes and reduce the sensitivity of the technique.

A condensed version of the results from these experiments is shown in Table 7-1. Experiments with various types of filters and different types of pretreatment indicated that pretreatment with 0.01% Siliclad or small (0.5 ml) quantities of 1% Tween-80 did not significantly reduce nonspecific background staining compared to untreated filters. Treatment with larger (4 ml) quantities of 1% Tween-80, or soaking the filters in 10% Tween-80 or 0.7% albumin reduced background staining to more acceptable levels. The greatest reductions in background staining were obtained by soaking the filters with 10% Tween-80, and then treating them with 0.7% albumin. The filter staining obtained in these pretreatment studies did not appear to be very sensitive to the length or temperature of incubation of immunoenzymatic reagents with the filters.

The Tween-80/albumin combination was sufficiently successful that it was utilized in subsequent immunoenzymatic filter assays. Coincidentally, many of the experiments in this set of studies involved the Sartorius 0.01 μm cellulose nitrate filters, which were later selected as the standard filter for the immunoenzymatic filter assays. Thus, the results of this section are directly applicable to Section 7.7.

7.4 Virus Retention on Filters

Quantitating virus using immunoenzymatic procedures involves first immobilizing the virus on some sort of surface. One of the most feasible means for accomplishing this is by filtration. Experiments were carried out to determine what types of filters, in terms of composition and pore size, are more efficient in retaining virus. Virus retention experiments were carried out under several different conditions to differentiate the extent to which virus retention is a function of filter pore size, and the extent to which it is a function of electrostatic attraction between the filter and the virus. Filtration of virus in 0.01 M Tris buffer, pH 8.0 measures primarily the effect of pore size on virus retention. Filtration of virus in 0.05 M glycine buffer, pH 3.5 (containing 5X10^-4 A1C13) measures the effects of both pore size and electrostatic attraction.

Table 7-2 summarizes the results from the virus retention experiments testing a variety of filters. Without exception, where identical filters have been tested with both 0.01 M Tris buffer, pH 8.0 and 0.05 M glycine buffer, pH 3.5, the latter leads to greater virus retention. However, since the conditions of the immunoenzymatic filter assay will take place at
Table 7-1. Testing of Various Methods for Reducing Nonspecific Background Staining

<table>
<thead>
<tr>
<th>Filter Type</th>
<th>Pretreatment</th>
<th>Dye Used</th>
<th>Enz/Ab Incubation</th>
<th>Background Top of Filter</th>
<th>Background Bottom of Filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>0.5ml 1% Tween 80</td>
<td>Diaminobenzidine</td>
<td>30 37</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>0.01% Siliclad</td>
<td>Diaminobenzidine</td>
<td>20 37</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Millipore 0.025μm</td>
<td>0.01% Siliclad</td>
<td>Diaminobenzidine</td>
<td>20 37</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Sartorius 0.05μm cellulose nitrate</td>
<td>0.01% Siliclad</td>
<td>Diaminobenzidine</td>
<td>20 37</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>4ml 1% Tween 80</td>
<td>Diaminobenzidine</td>
<td>20 37</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>10% Tween 80 soak for 1 hr., - 3 drops albumin filtered</td>
<td>Diaminobenzidine</td>
<td>10 25</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>0.7% Albumin soak (1hr) + 10 drops albumin filtered</td>
<td>Diaminobenzidine</td>
<td>10 25</td>
<td>0-+</td>
<td>0-+</td>
</tr>
<tr>
<td>Sartorius 0.01μm cellulose nitrate</td>
<td>10% Tween 80 soak (1hr) + 5 drops 10% Tween 80 filtered + 1.0 ml of 0.7% albumin filtered</td>
<td>Diaminobenzidine</td>
<td>10 25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Filters undergo entire immunoenzymatic procedure, minus virus.
b. In addition to the filters shown, 16 other filter/treatment combinations were tested and found unacceptable.
c. Results recorded as relative intensity of color on a scale of 0 → ++.
Table 7-2. Retention of Poliovirus on Membrane Filters

<table>
<thead>
<tr>
<th>Filter Type</th>
<th>Pore Size</th>
<th>Pretreatment</th>
<th>Buffer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Virus Retention&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleopore</td>
<td>0.08 μm</td>
<td>None</td>
<td>0.01 M Tris, pH 8.0</td>
<td>3</td>
</tr>
<tr>
<td>Millipore VM</td>
<td>0.05 μm</td>
<td>None</td>
<td>0.01 M Tris, pH 8.0</td>
<td>73</td>
</tr>
<tr>
<td>Nucleopore</td>
<td>0.03 μm</td>
<td>None</td>
<td>0.05 M Glycine, pH 3.5</td>
<td>37</td>
</tr>
<tr>
<td>Millipore VS</td>
<td>0.025 μm</td>
<td>None</td>
<td>0.01 M Tris, pH 8.0</td>
<td>90</td>
</tr>
<tr>
<td>Sartorius cellulose nitrate</td>
<td>0.05 μm</td>
<td>None</td>
<td>0.01 M Tris, pH 8.0</td>
<td>54</td>
</tr>
<tr>
<td>Sartorius cellulose acetate</td>
<td>0.01-0.02 μm</td>
<td>None</td>
<td>0.01 M Tris, pH 8.0</td>
<td>100</td>
</tr>
<tr>
<td>Sartorius cellulose nitrate</td>
<td>0.01 μm</td>
<td>None</td>
<td>0.05 M Glycine, pH 3.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01 M Tris, pH 8.0</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05 M Glycine, pH 3.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% Tween-80 (45 min.) and 0.7% albumin</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% Tween-80 (30 min.) and 0.7% albumin</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% Tween-80 (15 min.) and 0.7% albumin</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10% Tween-80 (5 min.) and 0.7% albumin</td>
<td>45</td>
</tr>
<tr>
<td>a. 0.01 M Tris buffer, pH 8.0 (containing 1% heat-inactivated FCS) permits retention by physical size only. 0.05 M Glycine buffer, pH 3.5 (containing 5 X 10^-4 M AlCl₃) allows additional retention by electrostatic attraction.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Based on filtering a 2.0 x 10⁴ PFU/ml virus suspension.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
a pH between 7.0 and 8.0, we must initially judge the filters according to their potential for retaining virus with the Tris buffer. Under these conditions the filters that show ninety percent or greater virus retention are the 0.025 μm Millipore, the Sartorius 0.01-0.02 μm cellulose acetate and the Sartorius 0.01 μm cellulose nitrate. The cellulose acetate filters, although having 100% virus retention capability, had slow flow rates which limit the usefulness of these filters. Of these three filters the Sartorius 0.01 μm cellulose nitrate exhibits the best combination of virus retention and desirable flow characteristics. The effect of Tween-80 and albumin pretreatment of the 0.01 μm cellulose nitrate filter on virus retention was examined since this was the method of choice for reducing non-specific staining. Numbers from duplicate runs indicate that pretreatment does reduce virus retention, but that long (30-60 min.) soaking times in Tween-80 lead to some recovery of virus retention. The reduction in virus retention that is observed with pretreatment of 0.01 μm cellulose nitrate filters may be a result of an increase in diameter of filter pores due to conformational changes in the cellulose nitrate. It may also be a result of a decrease in electrostatic attraction of the treated filter for the virus, or a combination of this with changes in pore size.

Both alginate and 0.01 μm cellulose nitrate filters demonstrate 100% retention of poliovirus-antipoliovirus antibody complexes (data not shown). Alginate filters were deemed to possess great potential for retention of virus due to their gel-like surface when wet, and were deemed suitable for further investigation with regard to their use in immunoenzymatic virus assays.

The results indicated that the Sartorius 0.01 μm cellulose nitrate filter should be the filter used for the immunoenzymatic filter assay due to its acceptable virus retention capability and its good flow characteristics. Combined with its previously investigated (Section 7.3) positive feature of low background staining with Tween-80/albumin treatment, it is a clear first choice for the filter assay. The alginate filter also received further attention due to its virus retention potential.

7.5 Properties of Alginate Filters

Because alginate filters retained viruses well and, therefore, seemed promising for application to immunoenzymatic assay of viruses, their properties were further investigated. During virus retention experiments in Section 7.4, it was noticed that filtrates from PBS washings of alginate filters contained visible turbidity. In order to determine whether dissolution of the alginate gel layer would be sufficiently severe to prohibit the use of this filter in immunoenzymatic assays, a series of alginate filters were exposed to a range of salt and serum concentrations (at constant pH) that were expected to occur during the immunoenzymatic filter assay. These experiments were designed to determine whether acceptable working concentrations of salt and protein could be found for these filters that would fall within the constraints of the immunoenzymatic assay.
The experiments with alginate filters indicate that loss of alginate from the filter occurs with increasing salt or serum concentrations. These experimental results are summarized in Figure 7.3. Increasing the salt concentration from 0.01 to 0.1 M led to a tripling of the turbidity of the filtrate to 15 times that of the control. The lowest salt concentration tested (0.007 M) led to turbidity readings 34 times that of the unfiltered control. A similar trend was observed for the effect of serum concentration, as measured by both turbidity and carbohydrate determinations. A concentration of 0.1% serum led to a turbidity 5 times that of the unfiltered control. Increasing the serum concentration from 0.1 to 1.0 percent lead to a 28-fold increase in orcinol-reactive material in the filtrate, and a 7-fold increase in turbidity. A one-twentieth dilution of PBS (0.007 M salt) was chosen as the diluent for serum in these experiments since it contributed only a small amount of salt-induced turbidity (see inset, Figure 7-3).

Conditions during the immunoenzymatic procedure involved 0.14 M salt and 0.5% serum. As seen in Figure 7-3, this corresponds to measurable turbidity (12 NTU) in the filtrate being contributed by protein that was present in antiserum and antibody reagents in the assay. The salt concentration in the PBS washings which were used to remove excess protein during the filter assay contributed high levels of turbidity (>30 NTU). This indicated that significant degradation of the alginate filters would occur if they were used in the immunoenzymatic procedure. Therefore, the properties of alginate filters were deemed to be not compatible with the conditions of the assay, and they were not considered further for use in the assay.

7.6 Immunoenzymatic Tube Assay

Binding virus to the inside of a test tube according to the method of Section 6.13 potentially allows quantitation of virus with somewhat greater rapidity than the filter method of Section 6.14. With the tube assay procedure, the intensity of color developed in the tubes with the appropriate immunoenzymatic reagents and dye reagent is related to virus concentration.

Figure 7-4 presents results from duplicate runs of immunoenzymatic tube assays, using immunoadsorbed antibody (VNT = 1:66,000) prepared according to the method of Section 6.3. Immunoadsorbed antibody was used because it was of relatively high purity compared to unpurified antipoliovirus antiserum, and was expected to lead to lower optical density contributions from background reactions. The relationship between the rate of increase in optical density and virus concentration was not linear. The change in optical density per hour was proportional to the logarithm of the virus concentration. This may be due to kinetics being a limiting factor in adsorption of the virus to the tube. In other words, since adsorption of virus first depends on contact, the lower the virus concentration of the sample put into the tube, the lower is the probability of a successful collision of a virus particle with the tube wall that results in virus
FIGURE 7-3
LOSS OF ALGINATE FROM ALGINATE FILTERS AS A FUNCTION
OF SALT OR PROTEIN CONCENTRATION

CARBOHYDRATE DETERMINATION
AND TURBIDITY
VS.
ANTISERUM CONCENTRATION

TURBIDITY vs. SALT CONCENTRATION (NaCl)

TURBIDITY
CARBOHYDRATE DETERMINATION
(Grinol Method)

CONTROL
(Not filtered)

OPTICAL DENSITY OF FILTRATE AT 230 nm (Grinol Method)

ANTISERUM CONCENTRATION (Percent in 1:20 PBS)
FIGURE 7-4
IMMUNOENZYMATIC TUBE ASSAY
RATE OF CHANGE IN OPTICAL DENSITY VS. VIRUS CONCENTRATION

RATE OF INCREASE IN OPTICAL DENSITY AT 460 nm (ΔO.D./hr)

VIRUS CONCENTRATION (PFU/ml)

2 x 10^7 2 x 10^6 2 x 10^5 2 x 10^4 2 x 10^3 2 x 10^2
adsorption. The slope of the assay curve was approximately 0.1 optical density units per hour per order of magnitude of virus. If 0.2 units per hour is taken as the practical sensitivity limit of the technique, due to background reactions and variability of the method, then the limit of detection of this method is about 100 PFU/ml of poliovirus.

The immunoenzymatic tube method shows good potential for use in detecting virus at low levels and above. The sensitivity of the method will have to be improved if it is to detect very low levels of virus (Several PFU).

7.7 Immunoenzymatic Filter Assay

Trapping virus on a small-pore membrane filter and assaying according to the procedure of Section 6.14 allows visualization of virus particles as dark-colored clumps of precipitated reacted dye. Each reacted dye clump represents a single virus particle or virus aggregate.

Figure 7-5 presents results from duplicate runs of immunoenzymatic filter assays. The filters used were 0.01µm cellulose nitrate filters. Immunoadsorbed antibody was found to give results similar to those obtained with unpurified day-41 sheep antipoliovirus antiserum. The relationship between reacted dye particle counts and input virus is arithmetically linear, yielding a nonlinear curve when plotted on semilogarithmic graph paper. In comparing this with the results of Section 7.6, this is to be expected, as there should be no loss of virus occurring for kinetic reasons in the filter assay.

Under oil immersion, there are theoretically 4875 optical fields in the viewing area (about 10 mm diameter) of a 13 mm filter. With a statistical limit of counting 10 particles per 10 fields, the limit of detection for this method is 2.7 X 10^4 particles. This means that the filter assay cannot detect fewer than 130 PFU with a 13 mm filter. Interestingly, the smaller the filter diameter used, the lower should be the detection limit, since the virus is being concentrated over a smaller area. Working with very small diameter filters, an area that has not been explored, could be an effective strategy for very directly improving the sensitivity of this method.

The immunoenzymatic method has good potential for detecting virus at levels greater than 100-200 PFU, according to the system that we have devised. Further improvements in sensitivity may prove worthwhile.
Figure 7-5
IMMUNOENZYMATIC FILTER ASSAY
Particle Counts Per Filter vs. Input Virus

Number of Reacted Dye Particles ($10^5$) Per Filter
(Based on Counts of 10 Optical Fields)

Input Virus (PFV)

51
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