LABORATORY STUDIES ON ROTAVIRUSES, ENTEROVIRUSES AND REOVIRUSES AS POLLUTANTS OF THE WATER ENVIRONMENT

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LIST OF ABBREVIATIONS

AGMK	African Green Monkey Kidney
BE	Beef Extract
BSA	Bovine Serum Albumin
BUdR	5-Bromodeoxyuridine
CH	Casein Hydrolysate
CPE	Cytopathic Effects
DEAE-dextran	Diethylaminoethyl-dextran
EBSS	Earle's Balanced Salt Solution
EM	Electron Microscope
FCS	Fetal Calf Serum
gm	gram
HEK	Human Embryonic Kidney
L	Litre
LAH	Lactalbumin Hydrolysate
MCC	Membrane Coating Components
MEM	Minimum Essential Medium (Eagle's)
mg	milligram
ml	millilitre
mm	millimetre
NB	Nutrient Broth
NCDV	Nebraska Calf Diarrhea Virus
nm	nanometre
PBS	Phosphate Buffered Saline (Dulbecco's)
PEG	Polyethylene Glycol
PFU	Plaque Forming Unit
PS	Protamine Sulfate
TPB	Tryptose phosphate Broth
YE	Yeast Extract
μg	microgram
μm	micrometre
xg	multiplied by the standard acceleration of gravity

ABSTRACT

Rotaviruses are now recognized as important causative agents of diarrhea in man and animals. There is also evidence to suggest that they can be transmitted by the consumption of sewage polluted waters. Routine in vitro cultivation of human rotaviruses is not yet possible. The simian agent SA-11 not only closely resembles human rotaviruses but it can also be readily cultivated in tissue culture. Since a plaque assay system for SA-11 virus has not been available, such a system was, therefore, needed before this virus could be used as a model in the development of techniques for the recovery of rotaviruses from the water environment.

Rotavirus SA-11 Plaque Formation

1. Incorporation of 5μg of trypsin per ml of the overlay (Eagle's minimal essential medium + 0.7% Ionagar #2) was necessary for plaque formation by SA-11 in MA-104 cells. Plaques (34 mm diameter) were produced after 5 days of incubation at 37°C.
2. In addition to trypsin, the incorporation in the overlay of α-chymotrypsin, elastase, subtilisin, pronase and pancreatin led to the formation of plaques by SA-11 in MA-104 cells. Addition of soybean trypsin-inhibitor to α-chymotrypsin-, pronase- or pancreatin-containing overlays completely inhibited virus plaque production. A similar effect was not seen with elastase or subtilisin.

3. Under a trypsin-containing overlay, the virus was able to form plaques in secondary monolayers of African green monkey kidney cells but it failed to produce plaques in BS-C-1, 1-132 and secondary monolayers of human embryonic kidney cells. A trypsin-containing overlay was also found to be necessary for the production of plaques by calf rotavirus (strain C-346) in MA-104 cells.

4. Initial trypsin treatment of the virus alone or its presence only during the early phases of virus-cell interaction was found to be insufficient for plaque production by the virus. Presence of trypsin in the agar overlay throughout the 5-day incubation period was essential for the optimal development of the virus plaques. Experiments using high (4 PFU/cell) and low (35 PFU/10^6 cells) multiplicities of infection suggest that trypsin added to fluid maintenance medium facilitates the cell-to-cell spread of progeny virus particles. Therefore, the enzyme incorporated in the agar overlay appears to play a similar role thereby assisting in the formation of SA-11 plaques.

Enteric Virus Concentration From Samples of Tap Waters

Discharges of raw or inadequately treated sewage result in viral pollution of the water environment. Finished waters from such sources may contain viruses because conventional water treatment methods are known to be relatively inefficient in their elimination. No systematic surveys, however, have been conducted in order to determine the true extent of this problem. This has been mainly due to the fact that simple and reliable techniques for the detection of small amounts of viruses in large volumes of tap waters have been generally unavailable.

1. Layers containing 3:1 mixture of talc and Celite 503 were found to be very efficient in the adsorption of entero-, reo-, and rota-viruses from experimentally-contaminated samples of tap water. Before experimental contamination and passage through the layer, adjustment of the sample pH to 6.0 and the addition of Earle's balanced salt solution (EBSS) to a final concentration of 1:100 were necessary for optimal virus adsorption to the layers. Under these conditions, between 90% to 96% of the input PFU could be adsorbed to the layers when working with 1-L volumes of tap waters.

2. Tryptose phosphate broth (TPB) and 3% beef extract (BE), both at pH 9.0, were shown to be highly efficient in the elution of the simian and calf rotaviruses. Furthermore, TPB was found to be as efficient as 10% fetal calf serum (FCS) in normal saline (pH 9.0) in the recovery of layer-adsorbed polio- and reoviruses and could, therefore be used as a general purpose eluent for the talc-Celite technique. When working with 1-L volumes of tap waters passage of 10-20 ml of eluent through the layers (47 mm diameter) resulted in the recovery of 87% to 94.5% of the added PFU.

3. When working with large layers (142 mm diameter). for processing large volumes of tap water (10-1,000 L), a 100-ml volume of eluent was needed for the elution of the layer-adsorbed viruses.

Overnight hydroextraction (4°C) of experimentally-contaminated eluents or eluates was highly efficient (87-98%) in virus recovery. In comparative tests, PEG hydroextraction was found to be simpler and superior to organic flocculation technique.

4. The combined efficiency of the talc-Celite and PEG hydroextraction techniques was tested in the recovery of enteric viruses from seeded samples of tap waters. Between 83-90% of the input virus could be recovered when working with 10-20-L samples and between 58-71% of the added virus could be recovered when working with 100-to 1,000-L volumes containing as little as 0.75 PFU/L.

5. The technique was found to work equally well with field isolates and with tap water samples containing either 0.05% or 0.1% of raw sewage.

GENERAL INTRODUCTION AND BACKGROUND

Viruses and Disease Production in Man

Before any discussion of viral pollution of the water environment and its possible impact on human health, it is considered essential to state the following points on the role of viruses in disease production in man.

1. Viruses are estimated to be responsible for more than 60% of the cases of infectious disease in man; there are more than 300 different types known to infect man; they collectively produce over 50 different disease syndromes (Horsfall, 1965).

2. Several viruses may cause clinically similar diseases and one type of virus may produce a variety of clinical conditions; for example, aseptic meningitis can be produced by 21 different enteroviruses and on the other hand, one particular type of adenovirus (type 3) can cause at least four clinically distinct ailments (Rhodes and van Rooyen, 1968).

3. Effective drugs against viral diseases are generally unavailable; safe and effective vaccines are available for the control of a very limited number of viral infections.

4. In comparison to certain human pathogenic bacteria (e.g. Salmonella typhi), much smaller quantities of a given virus may be needed to

initiate infection in a susceptible host (Plotkin and Katz, 1967; Loria et al., 1974; Westwood and Sattar, 1976).

5. In most cases, the body of the infected individual is able to arrest the process of infection while it is in the early or "sub-clinical" stages; but in a "clinical" case, depending on the type and severity of the disease, the outcome could be either complete recovery, transient or permanent damage or even death of the host.

6. Serious damage as a result of apparently mild infections due to certain viruses may become obvious several months to years later (Sells et al., 1975; Lake et al., 1976).

7. The presence of a given virus, existence of sufficient numbers of susceptible hosts and an effective mechanism of virus spread are the three factors necessary for outbreaks of viral disease in a given community.

Viruses differ in their affinity for various types of tissues and organs in the human body. Those that are capable of growing in the inside lining of the gut are referred to as "enteric" viruses. Enteric viruses, however, can and do grow in and affect other parts of the body such as the central nervous system, respiratory system, muscles, etc. This means that enteric viruses can and do produce a variety of mild and serious infections ranging from upset stomach to paralysis and death. More than 100 different types of enteric viruses of human origin are known to exist (Health and Welfare Canada, 1977; Sattar, 1978b; Melnick, 1978; Mahdy, 1979).

Viruses and Water Pollution

Because viruses are obligate parasites, it is impossible for them to multiply in the absence of susceptible living hosts. In view of this, viruses cannot cause any direct damage to the environment. Their presence and survival in polluted waters are, therefore, significant only from the point of view of human or animal health.

In their persistence in the water environment, viruses are much less hardy than certain pesticides, heavy metals and radioactive wastes. Unlike these pollutants, however, a single dose containing an extremely minute amount of virus may be sufficient to cause damage in a susceptible host.

Factors such as temperature, pH, UV irradiation, presence or absence of certain chemicals and biologicals (e.g. enzymes), association with particulate matter singly or in various combinations, determine the ability of enteric viruses to survive in the water environment. In view of this, and depending on the virus type, their survival in polluted waters may range from 2 days to 6 months or more (Akin et al., 1971).

The main sources of viral pollution of the water environment are: (a) liquid and solid municipal wastes, (b) farm wastes, (c) abbatoir and dairy wastes, and (d) land run-offs. In addition to these direct discharges, viruses in wastes disposed of on land or in air may also eventually end up in water environment (Gerba et al., 1975).

Until recently, emphasis was placed on surface water pollution with viruses. However, recent studies have shown that, at least under certain soil conditions, viruses disposed of on land could reach ground water reservoirs (Wellings et al., 1975; Dubois et al., 1979; Vaughn et al., 1978).

Since enteric viruses are discharged in large numbers in the feces of infected individuals, they represent the most important virus group with regards to water pollution with municipal and agricultural wastes. It must, however, be noted that a variety of non-enteric viruses, such as cytomegalo- (Cox and Hughes, 1974), rubella- (Green et al., 1965), and influenza viruses (Hinshaw et al., 1979) may also end up in sewage as a result of their discharges in various body secretions and excretions of infected individuals. Little is known about the quantities involved and their survival in sewage.

There are certain enteric viruses that can infect and grow in man as well as a variety of species of warm-blooded animals (Lundgren et al., 1968; Grew et al., 1970; Larkin, 1972; Metcalf, 1976). This could be significant where untreated agricultural and abbatoir wastes and storm waters from urban centres are discharged into the water environment.

Conventional methods of sewage as well as water treatment are relatively inefficient in the removal and inactivation of most of the enteric viruses (Berg, 1973). Because a number of communities depend on sewage-polluted sources for their recreational and potable water needs, fears have been expressed concerning the impact of this type of pollution on the health of such communities. Culp et al. (1973) have estimated that in 155 cities in the U.S. one of every 30 gallons of surface water entering treatment plants has already passed through the wastewater system of a community upstream. Therefore, as demands on available water resources increase further, the chances that enteric viruses will appear in community water supplies is also steadily increasing (Berg et al., 1976; U.S. Nat. Acad. Sci., 1977). In recent years, this issue has received considerable coverage both in the scientific (Cookson, 1974; Sobsey, 1975; Gerba et al., 1975; Martin, 1975; Okun, 1976;Pond, 1976; Goldfield, 1976; Shuval, 1977; Melnick et al., 1978; Sattar, 1978b;Mahdy, 1979) as well as the popular press (MacDonald, 1973; Harris and Brecher, 1974a 1974b, 1974c; Mostow, 1977; Labreche, 1978).

Viruses of human origin have also been isolated from potable waters in Canada (Sekla et al., 1978) and elsewhere (Coin et al., 1966; Foliguet et al., 1966; Shuval, 1970; Mack et al., 1972; McDermott, 1974; Petrilli et al., 1974; Clarke et al., 1975; Wellings et al., 1975; Nestor and Costin, 1976; Hoehn et al., 1977). Viruses recovered from finished waters of an urban community in the U.S. have been found to be unusually resistant to inactivation by chlorine (Bates et al., 1977). There have also been reports of virus isolations from treated waters used for swimming (McLean, 1967; Liebscher, 1970; D'Angelo et al., 1979).

Virus Transmission by the Water Route

Feces and/or urine are the two most important sources of viruses in water (Mahdy, 1979). However, ocular, respiratory, dermal and genital shedding of viruses may also lead to contamination of water by swimmers. This would suggest that virtually all human pathogenic viruses have the potential for transmission by the water route (Fox, 1976).

In nature viruses present in polluted waters can enter the human body directly via the mouth, the eyes, the nose, the ears, the genito-urinary tract and abrasions and breaks on the skin surface. Though the last four portals of entry are obviously important when swimming and bathing in virus-contaminated waters, it has been calculated that one also ingests between 10 to 50 ml of water during such activity (Shuval, 1976).

Transmission of viruses from sewage-polluted waters can also occur indirectly when such waters are used for the washing of food and ustensils, cultivation of shellfish (Gerba and Goyal, 1978) or the irrigation of vegetable crops (Tierney et al., 1977). Katzenelson et al., (1976a) have shown that spray irrigation with partially treated and improperly disinfected oxidation pond effluent carries a definite health risk, as the incidence of hepatitis A and enteropathogenic bacterial infections were found to be at least two to four times higher in agricultural communities using wastewater irrigation compared to those using other forms of irrigation.

Except for hepatitis A (Goldfield, 1976), acute non-bacterial gastroenteritis (Goldfield, 1976; Sliman, 1978) and adenoviruses (D'Angelo et al., 1979), the evidence for the role of potable and recreational waters in the transmission of enteric virus infections is mostly indirect and circumstantial. Consideration of the following factors may explain this lack of clear-cut evidence: (a) simple and efficient means of detecting small amounts of viruses in large volume samples have been generally unavailable, (b) absence of suitable animal models to assess the potential of potable waters in the transmission of human enteric viruses, (c) commonly used in vitro host systems are incapable of detecting more than 30% of the types of enteric viruses (Sattar, 1978a), (d) sewage-polluted waters generally contain a mixture of two or more enteric virus types; higher concentrations and/or faster replication of a second virus in the same sample may mask the presence of the virus incriminated in the outbreak suspected of being waterborne, (e) one particular type of enteric virus may manifest itself in a variety of clinical conditions and on the other hand, a given clinical condition could be produced by a variety of viruses, (f) low virus concentrations expected to be present in potable waters are more likely to result in subclinical infections; passage of the virus from the subclinical case to others in his immediate surroundings makes it extremely difficult to pin down the original vehicle of infection, (g) relatively mild infections spread through potable waters may result in noticeable damage weeks to months later (Sliman, 1978), (h) perhaps the most important factor in this respect is the lack of complete reporting of cases and outbreaks of waterborne infections (Goldfield, 1976).

In view of these difficulties, it is not possible either to determine the risk factor or to assess the true role of potable and recreational waters in the spread of enteric virus infections. However, in this regard, some estimates have been put forward in the literature. For example, Long and Bell (1972) have calculated that with 0.2% of the water being used for drinking, and assuming an infection rate of 30% (Plotkin and Katz, 1967), a water supply of 50 million U.S. gallons/day and containing as little as one virus plaque forming unit/50 gallons, could infect 600 persons daily. The following is a summary of the information available for the potential of potable and recreational waters in the transmission of viral infections.

Adenoviruses

Studies in the U.S. have demonstrated that fecal shedding of adenoviruses in young children exceeds that of enteroviruses (Cooney et al., 1972). Recently, Retter et al. (1979) have shown that nearly half of the adenoviruses detected in human stools by electron microscopy cannot be grown in presently available cell culture systems. Although they were found to be more susceptible to chlorine than enteroviruses, Fox (1976) believes that adenoviruses are hardy enough to make their waterborne spread a virtual certainty.

There are no published reports incriminating potable waters in the transmission of adenoviral infections. However, a number of outbreaks of such infections transmitted by swimming pool waters have been recorded (Bell et al., 1955; Ormsky and Aitchison, 1955; Foy et al., 1968; D'Angelo et al., 1979).

Enteroviruses

Though most enteroviruses are relatively stable and are excreted in large numbers in the feces of infected individuals, direct evidence for their transmission by the water route is not yet available.

Eight outbreaks of poliomyelitis believed to have been spread by potable waters have been reviewed by Mosley (1967). He, however, believes that the epidemiological evidence for only two of these outbreaks, one in Edmonton, Alberta (Little, 1954) and the other near Lincoln, Nebraska (Bancroft et al., 1957), suggests waterborne transmission.

Polio and other types of enteroviruses have been recovered from samples of potable waters (Coin et al., 1966; Foliguet et al., 1966; Shuval, 1970; Mack et al., 1972; McDermott, 1974; Petrilli et al., 1974; Clarke et al., 1975; Wellings et al., 1975; Nestor and Costin, 1976; Hoehn et al., 1977; Sekla et al., 1978). But in none of these studies has their presence in the water samples been related to cases of viral infections in the community.

Enterovirus isolates have been reported from water samples of pools (Kelly and Sanderson, 1961; McLean, 1967; Liebscher, 1970) and lakes (Hawley et al., 1973; Denis et al., 1974) used for wading and swimming. On the basis of virus isolations from clinical specimens as well Liebscher (1970), Hawley et al. (1973) and Denis et al. (1974) believe that the infections were contracted as a result of exposure to such virus-contaminated recreational waters.

Serum surveys have revealed major gaps in our immunity to certain enteroviruses and according to Fox (1976), "unless constant vigilance in protection of water supplies is maintained, non-polio enteroviruses could cause major outbreaks of serious disease". Goldfield (1976) believes that enterovirus contamination of the water environment is a foregone conclusion; in his view, "enteroviruses will eventually be shown throughout the world to be far more commonly transmitted by drinking water than is hepatitis A virus".

Hepatitis A Virus

The epidemiological evidence for the capacity of hepatitis A virus to spread through the consumption of sewage-polluted potable waters is well documented (Mosley, 1967; Goldfield, 1976; Craun, 1979a; 1979b). The unusually high chlorine resistance of this virus is well illustrated by the 1955 epidemic of infectious hepatitis in Delhi, India (Dennis, 1959).

An outbreak of hepatitis A by exposure to sewage-polluted recreational waters has also been reported (Bryan et al., 1974).

Certain strains of hepatitis A virus have recently been adapted to grow in cell cultures (Provost and Hilleman, 1979). This may eventually provide the means for the in vitro recovery of this agent from samples incriminated in such outbreaks.

Parvovirus-Like Agents

It is well established now that in addition to rotaviruses, another group of viral agents is also an important cause of gastroenteritis in man (Schreiber et al., 1977; Estes and Graham, 1979; Holmes, 1979). These viruses, named after the sites of the epidemics where they were first recognised, are the Norwalk (Ohio), Hawaii, and Montgomery County (Maryland) agents.

On the basis of the size (about 27 nm), density in cesium chloride and resistance to ether, acid and heat treatment, these agents are believed by some to belong to the parvovirus group (Kapikian et al., 1972; Dolin et al., 1972; Thornhill et al., 1977). Other investigators, however, consider them to be similar to caliciviruses (Estes and Graham, 1979; Holmes, 1979). Further characterization and the eventual classification of these agents must await their in vitro cultivation.

These agents seem to be mainly responsible for outbreaks of diarrhea in older children and adults (Greenberg et al., 1979). Wyatt et al. (1974), using human volunteers, have shown that infection with the Norwalk agent could confer cross-protection against the Montgomery County agent but not against the Hawaii agent.

Attempts to transmit diarrhea by these agents to experimental animals have as yet been unsuccessful. Wyatt et al. (1978b) have reported the production of subclinical infection in chimpanzee by the Norwalk agent.

There is mounting evidence now for waterborne outbreaks of diarrhea by these agents (Zweighoft *et al.*, 1978; Ouiverkerk *et al.*, 1978; Morens *et al.*, 1978). As is true for hepatitis A virus, attempts to recover these viruses from samples of incriminated waters will also depend on the development of methods for their routine *in vitro* cultivation. Recently, Rutala and Sobsey, (1978) have attempted to use the Minute Virus of Mice (a parvovirus) as a model for testing certain water concentration techniques for the recovery of these agents.

Reoviruses

Presence of antibodies to reoviruses in 80-100% of adults in a number of communities throughout the world shows them to be very common infectious agents of man (Leers and Rozee, 1966). In their being so widespread, little is known about the ability of reoviruses to cause disease in man. They are, however, the most frequently isolated viral agents from sewage, sludge and sewage-polluted surface waters (England, 1972; Subrahmanyan, 1977; Sattar and Westwood, 1978). This has led to the suggestion that they be considered as possible indicators of viral pollution of the water environment (Stanley, 1977).

Rotaviruses

Acute gastroenteritis is one of the most common afflictions of man. In the developing world acute gastroenteritis, particularly in infants and children, has long been recognized as being among the major causes of morbidity and mortality. It is estimated that in the developing parts of Africa, Asia and Latin America 5 to 18 million childhood deaths per year are due to this illness (Rohde and Northrup, 1976). Even in more advanced areas of the world, acute gastroenteritis has been shown to be the second (after respiratory illness) most common clinical condition observed in man (Dingle et al., 1964).

Until about seven years ago, the etiology in the majority of the cases of acute diarrhea could not be determined. However, with the discovery of human rotaviruses (Bishop et al., 1973; Flewett et al., 1973; Middleton et al., 1974) it has now been established that these agents are responsible for nearly 70% of the cases of this ailment throughout the world (Steinhoff, 1978; Holmes, 1979). Recently, rotaviruses have also been incriminated in waterborne outbreaks of diarrhea (Lycke et al., 1978; Freij et al., 1978). A consideration of the following points further reinforces the potential of rotaviruses to spread through the waterborne route:

1. Rotaviruses are excreted in very large numbers in the feces of infected hosts. During the diarrheal phase in man, nearly 10^{10} virus particles/gm of feces have been reported (Flewett and Woode, 1978; McNulty, 1978).

2. The fecally excreted virus particles remain infectious for prolonged periods. Woode and Bridger (1975) have shown that rotaviruses remain infectious even after seven months of storage at room temperature (18-20°C).

3. Rotaviruses are relatively resistant to inactivation by a number of commonly used disinfectants. Even 3% "Chloros" (a sodium hypochlorite solution containing 11% available chlorine) was unable to inactivate them after several hours of exposure (Snodgrass and Herring, 1977b). The amount of active chlorine present here was many times higher than the levels used in conventional sewage and water disinfection.

4. Rotaviruses from one animal species appear to be able to produce infection in other types of animals (Bridger _et al._, 1975; Middleton _et al._, 1975; Mebus _et al._, 1976; Snodgrass and Herring, 1977a; Woode _et al._, 1976). This indicates that animal rotaviruses in agricultural wastes and land run-offs could be potentially dangerous to human health.

5. In the majority of early reports about epidemics of rotavirus infection, all the patients were infants or small children. Recently, however, epidemics of rotavirus infection have been described in adults (Kim _et al._, 1977; Tufvesson _et al._, 1977; Wenman _et al._, 1979) and contact of adults with infected children was not found to be necessary for contracting the illness (Meurman and Laine, 1977; Von Bonsdorff _et al._, 1978). It has also been shown that at least four serotypes of human rotaviruses exist (Flewett _et al._, 1978) and that infection by one does not confer immunity to the other serotypes (Yolken _et al._, 1978).

6. At least one type of animal rotavirus (Malherbe and Strickland-Cholmley, 1967) has been recovered from river water receiving wastes from an abbatoir.

7. Farrah et al. (1978a) have shown that there is a basic difference in the adsorptive behavior of polio- and rotaviruses to aluminum hydroxide and activated sludge flocs. They, therefore, suggest that water and wastewater treatment systems that are effective in the removal of enteroviruses may not be as efficient in the elimination of rotaviruses.

8. In a recent study (Hurst and Gerba, 1980) it has been shown that rotaviruses could retain their infectivity for several days in natural waters.

In spite of the mounting evidence for the potential of human rotaviruses to be transmitted through polluted waters, no techniques are as yet available for their efficient concentration and recovery from the water environment.

Other Enteric Viruses

Application of electron microscopy to the direct examination of stool samples has, in recent years, led to the discovery of a variety of hitherto unknown enteric viruses (Madeley, 1979; Holmes, 1979). Among these agents, astro-, calici- and coronaviruses have been implicated in outbreaks of diarrhea in man. Because of the present difficulties in their *in vitro* cultivation, no information is as yet available on their role in outbreaks of waterborne gastroenteritis.

Virus Recovery from the Water Environment

The ratio of fecal coliform bacteria to enteric viruses in human feces has been estimated to be about 65,000:1 (Kollins, 1966). Based on these estimates, the minimum expected density of enteric viruses in domestic sewage in temperate climates, is between 500 (Clarke and Kabler, 1964) and 700 (Kollins, 1966) infective units (IU)/100 ml. Discharges of such sewage into surface waters would result in a virus loading of between 0.1 (Kollins, 1966) and 3.0 (Chang, 1968) IU/100 ml. Because of these relatively small expected densities of enteric viruses in samples of sewage-polluted surface waters, between 50 and 100 liters (L) of raw water and several hundred L of finished water samples need to be examined for the study of viral pollution. This is in contrast to the unconcentrated 100 ml samples used for coliform estimations. Therefore, for the detection of enteric viruses, it is necessary to reduce the sample volume from several L to a manageable level of no more than a few ml. Loss or inactivation of virus during sample concentration is also to be avoided as much as possible. The need for the development of such concentration techniques has been emphasized (Health and Welfare Canada, 1977; U.S. Nat. Acad. Sci., 1977; Amer. Water Works Assoc., 1979).

A number of techniques have already been reported with particular emphasis on the recovery of enteroviruses from samples of liquid wastes and raw and finished waters (Hill et al., 1971; Sobsey, 1976; Wallis et al., 1979). Comparative testing of the virus recovering efficiency of many of these techniques was carried out in our laboratory (Westwood and Sattar, 1974). The results of these tests led to the development of the talc-Celite technique. The application of this technique in the recovery of enteric viruses from samples of raw sewage, treated and untreated effluents and surface waters has already been reported (Sattar and Westwood, 1976a, 1976b, 1978). The study to be described here was designed to extend the use of this technique for the recovery of entero-, reo- and rotaviruses from large samples of tap waters. The availability of such a technique would be useful from the following points of view:

1. Assessment of the virus removing and inactivating efficiency of water treatment systems.
2. Virological analysis of water samples incriminated in outbreaks of waterborne infections.
3. Monitoring of potable and recreational waters for viruses.
4. Surveys of water supplies to determine their virus content; this may lead to the setting up of meaningful virus standards for potable and recreational waters.

MATERIALS AND METHODS

Cells

BS-C-1 cells (Hopps et al., 1963), a continuous line derived from Cercopithecus aethiops kidneys was used for the growth and plaque assay of the entero- and reoviruses. A seed culture of these cells (at passage level 75) was initially obtained through the courtesy of Mr. D.A. McLeod of the Laboratory Centre for Disease Control (L.C.D.C.), Ottawa, Ontario.

MA-104 cells, a continuous line derived from Macacus rhesus kidneys by Microbiological Associates, Inc. (4733 Bethesda Avenue, Bethesda, Maryland, 20014) was used for the growth and plaque assay of the simian and calf rotaviruses. A seed culture of these cells was kindly supplied to us by Dr. H. Malherbe of the University of Texas at San Antonio.

L-132 cells (Davis and Bolin, 1960), a continuous line derived from human embryonic lungs was obtained through the courtesy of Dr. C.M. Johnson-Lussenburg of our Department.

Primary African green monkey kidney (AGMK) cells and primary human embryonic kidney (HEK) cells were obtained as suspensions from Connaught Laboratories Ltd. (1755 Steeles Avenue West, Willowdale, Ontario, Canada M2N 5T8).

Media

As stock cultures, the established cell lines were routinely cultivated as monolayers in 75 cm^2 plastic tissue culture flasks (Corning Glass Works, Science Products Division, N.Y. 14830) using Eagle's minimum essential medium (MEM) in Earle's base (Autopow; Flow Laboratories, Inc., 1710 Chapman Avenue, Rockville, Maryland 20852). Each 450 ml of the medium was supplemented with 25 mg gentamicin (Schering Corp., Kenilworth, New Jersey 07033), 13.5 ml of a 5.6% solution of sodium bicarbonate, 5 ml of a 200 mM solution of L-glutamine (Flow Laboratories) and 50 ml of virus - and mycoplasma - tested fetal calf serum (FCS) supplied by Microbiological Associates, Inc. Unless otherwise stated the maintenance medium consisted of MEM and 2% FCS.

Trypsinization

Each cell monolayer was trypsinized using 2 ml of a mixture of trypsin (0.25%) and Versene (0.05%) in Ca^{2+}- and Mg^{2+}- free phosphate buffered saline. A split ratio of 1:4 was generally used for the passage of cells and the cultures for plaque assay were put up in 25 cm^2 plastic tissue culture flasks (Lux Scientific Corp., 1157 Tourmaline Crescent, Newbury Park, Ca. 91320). The primary suspensions of AGMK and HEK cells were initially cultured in 75 cm^2 flasks (Corning). When the monolayers were formed, they were trypsinized and using a split ratio of 1:2 secondary cultures for plaque tests were put up in 25 cm^2 flasks.

Viruses

Laboratory-adapted Strains of Entero- and Reoviruses

Poliovirus 1 (Sabin) and reovirus 3 (Abney) were obtained from the L.C.D.C. Echovirus 6 and coxsackievirus B5 were kindly supplied to us by Dr. T.P. Subrahmanyan of the Central Public Health Laboratories (C.P.H.L.), Toronto, Ontario. Coxsackievirus A9 was a gift from Mr. P. Phipps of the Children's Hospital of Eastern Ontario, Ottawa, Ontario. All these viruses were plaque-purified in our laboratory prior to the preparation of virus stocks in BS-C-1 cells.

Field Isolates of Entero- and Reoviruses

The field isolates used in this study were echovirus 1, coxsackievirus B3, reovirus and one vaccine and one non-vaccine strain of poliovirus 1. These were recovered from samples of waste and surface waters during earlier studies in this laboratory (Sattar and Westwood, 1976a, 1977; Sattar, 1978a). Identification of these isolates was carried out on the basis of cytopathology, electron microscopic examination and serology. The poliovirus isolates were subjected to temperature marker (Lwoff and Lwoff, 1959) and serodifferentiation (McBride, 1959) tests by the Bureau of Biologics of the L.C.D.C. Further confirmation of the "wild" nature of the non-vaccine poliovirus strain was kindly carried out for us by Dr. N.A. Young of the U.S. National Institutes of Health. After their initial isolation in BS-C-1 cells, the field isolates had undergone one more passage in the same cell line prior to their use in this study.

Rotaviruses

Simian rotavirus SA-11 (strain H96) was kindly supplied to us by Dr. H. Malherbe and the calf rotavirus (strain C-486) was a gift of Dr. Babiuk of the University of Saskatchewan, Saskatoon.

In our initial attempts to grow the SA-11 virus in MA-104 cells, it was found that as little as 1% FCS (Microbiological Associates) in the maintenance medium could inhibit virus growth and as a result no cytopathic effects (CPE) could be seen even after 8 days of incubation. However, pronounced CPE in SA-11 infected monolayers became apparent by the second day of incubation when no serum was included in the maintenance medium. FCS had a similar inhibitory effect on the growth of the calf rotavirus (strain C-486).

Because of the inhibitory effect of FCS on the growth of SA-11 and calf rotavirus, it was necessary to wash the monolayers at least twice with Earle's balanced salt solution (EBSS) before virus inoculation. Maintenance medium (MEM without serum) was introduced into the cultures after one hour of adsorption of the virus at 37°C.

For the preparation of virus pools, infected cultures were kept at 37°C until nearly 75% of the monolayer was affected by virus cytopathic effects. The cultures then were frozen (-20°C) and thawed three times, followed by centrifugation at 1000 xg for 15 minutes. The supernatant was dispensed in 0.5 ml aliquots and kept frozen at -80°C.

Overlay Media

The basic ingredient of the overlay media was 2X MEM. Any one or a combination of the following was incorporated with it depending on the nature of the experiment.

Additives: The names of the additives used in this study and the relevant information pertaining to them are presented in Table 1.

Enzymes and Enzyme Inhibitor: The names of the enzymes and the inhibitor used in this study and the relevant information pertaining to them are presented in Table 2.

Solidifying Agents: Bacto Agar (Difco; lot no. 656451), Noble Agar (Difco; lot no. 612786), Oxoid Agar no. 1 (Oxoid; lot no. 133 4732), Oxoid Ionagar no. 2 (Oxoid; lot no. 076 1527) and methyl cellulose (BDH Chemical Ltd., Poole, England; lot no. 094 1090) were tested for their suitability as solidifying agents in the overlay medium. An appropriate amount of the solidifying agent was suspended in a measured volume of deionized water and autoclave sterilized.

TABLE 1

RELEVANT INFORMATION ON THE MATERIALS USED AS ADDITIVES TO THE OVERLAY MEDIA IN THIS STUDY

Item	Lot No.	Manufacturer	Preparation of Stock Solution
5-bromodeoxyuridine (BUdR)	R-384153	Grand Island Biological Co. GIBCO)., 3175 Staley Road, Grand Island, N.Y. 14072	1.0% in deionized water
Diethylaminoethyl-dextran (DEAE-dextran)	9404	Pharmacia, Uppsala, Sweden	1.0% in deionized water
Protamine sulfate (PS)	7220	Nutritional Biochemical Corp. (NBC) Cleveland, Ohio 44128	1.0% in deionized water
Beef extract (BE) in powder form	282-12544	Oxoid Ltd., London, England	20% in Earle's balanced salt solution (EBSS)
Bovine serum albumin V (BSA)	123C-1120	Sigma Chemical Co., St. Louis, Missouri 63178	20% in EBSS
Lactalbumin hydrolysate (LAH)	47708	GIBCO	20% in EBSS
Peptone	431149	Difco Laboratories, Inc., P.O. Box 1058A, Detroit, Mi. 48201	20% in EBSS
Yeast extract (YE)	652850	GIBCO	20% in EBSS
Nutrient broth (NB)	537645	Difco	1X in deionized water
Tryptose phosphate broth (TPB)	454688	Difco	1X in deionized water

[a] All above solutions were filter sterilized using 0.22μm membrane filter (Nalge Co., Division Sybron Corp., 75 Panorama Creek Drive, Rochester, N.Y. 14602) prior to storage at 4°C.

TABLE 2

RELEVANT INFORMATION ON THE ENZYME PREPARATIONS AND INHIBITOR USED IN THE STUDY OF THE EFFECT OF PROTEOLYTIC ENZYMES ON PLAQUE FORMATION BY ROTAVIRUS SA-11

Item	Lot No.	Specifications	Supplier	Preparation of Stock Solution and Storage
α-chymotrypsin	46C-8120	bovine pancreas; activity 40-50 units/mg solid	Sigma	0.1% in 0.001N HCl; 5°C
Elastase	85-405	porcine pancreas; activity 24.6 units/mg	Whatman Biochemicals Ltd., Springfield Mills, Maidstone, Kent, England.	0.1% in Earle's balanced salt solution (EBSS); -20°C.
Pancreatin	028-11825	porcine; in tablet form	Oxoid	a 1.0 gm tablet dissolved in 50 ml of distilled water at 37°C.; -20°C.
Papain	LS-0003126	papaya latex; 13.34μ/mg	Worthington Biochemical Corp., Freehold, New Jersey 07728	Fresh solution prepared before use in diluent specified by the supplier; 5°C.
Pepsin	46849	porcine mucosa; 1.0 mg sufficient to digest 60,000 mg of acid hemoglobin	General Biochemicals, 975 Laboratory Park, Chagrin Falls, Ohio 44022	0.1% in 0.001N HCl; 5°C.
Pronase	63-807	Streptomyces griseus, B grade; 45,000 proteolytic units/gm.	Calibochem, P.O. Box 12087, San Diego, California 92112	0.1% in EBSS; -20°C.
Subtilisin	23C-2520	Bacillus subtilis; 11 units/mg solid	Sigma	0.1% in EBSS; -20°C.
Thermolysin	001844	Bacillus thermoproteolyticus; 8810 units/mg protein	Calbiochem	0.1% in EBSS; -20°C.
Trypsin	70720	porcine; 1 gm sufficient to digest 250 gm of casein	GIBCO	0.1% in EBSS; -20°C.
Trypsin Inhibitor	48C-8045	Soybean; approximately 10,000 BAEE units/mg protein	Sigma	0.1% in EBSS; -20°C.

All above solutions were filter sterilized using $0.22\mu m$ diameter membrane filter (Nalge Sybron) prior to storage.

Plaque Assay Procedure

The growth medium was removed from confluent monolayers and they were then washed twice with about 5 ml of EBSS. A 0.5 ml volume of the appropriate inoculum was introduced into each culture using at least four flasks for each sample dilution tested. The inoculated cultures were placed at 37°C for one hour with periodic redistribution of the inoculum. At the end of the incubation period, the excess inoculum was removed and 5 ml of an overlay was introduced into each culture. For poliovirus 1, the overlay consisted of MEM supplemented with 2% FCS and 0.75% Noble agar. When working with the other enteroviruses and reovirus, 0.75% Bacto agar was used instead of Noble agar and the medium was further supplemented with 200μg/ml of DEAE-dextran and 100μg/ml of BUdR.

Virus-adsorbing Filters

The following 47 mm diameter filters were tested for their virus-adsorbing efficiency when processing experimentally contaminated tap water samples: Epoxy-fiberglass filter (Filterite Corp., Timonium, Md. 21093), fiberglass-asbestos (type M-780, series AA; Cox Instrument Div., Lynch Corp., Detroit, Mich.) and cellulose-diatomaceous earth "charged-modified" resin (Zeta Plus 50S, AMF, CUNO Division, Meriden, Conn.). Also tested were the epoxy-filter glass tubes (63.5 x 24.5 mm) of 8μm porosity (Balston Inc., Lexington, Mass. 02173).

Talc-Celite Layers

Preparation of Suspension

Talcum powder (magnesium silicate; J.T. Baker) and Celite 503 (Flux-calcinated diatomite; J.T. Baker) were purchased from Canlab (Canadian Laboratory Supplies, Toronto, Ontario M8Z 2H4). Before use both of these powders were washed three times in distilled water. After the final wash, they were left in the incubator at 37°C for drying. A mixture of 10 gm of talc and 3.3 gm of Celite was added to one liter of distilled water. The suspension was sterilized by autoclaving at 121°C for 20 minutes.

Preparation of Layers

Layers prepared for the processing of small volumes (up to one liter) were held in 47 mm diameter glass filter holders (Millipore Corporation, Ashby Road, Bedford, Ma. 07130). For large volume samples (10 to 1000 liters) the layers were prepared in either a specially made Plexiglass (Sattar and Westwood, 1976b) or a 142 mm diameter stainless steel membrane filter holder (Sartorius membrane filter, Gmblt. 34 Göttingen/FRG). A filter paper (Whatman number 114) of the appropriate diameter was moistened in sterile distilled water and placed in the holder. The talc-Celite suspension (using 30 ml for the small and 120 ml for the large layers) was poured into the holder and suction was applied for removal of the water. This resulted in the deposition of the talc-Celite mixture as a smooth and uniform layer on the filter paper. The layer was carefully covered with a second moistened filter paper. An AP25 prefilter disc (Millipore Corp.) was then placed on the sandwiched layer.

Water and Sewage Samples

Finished and Raw Water

All the finished water samples used in this study were collected from the tap in our laboratory. This represented a mixture of treated surface water (Ottawa River), from two municipal plants. The type of treatment is the same at both plants and consists of prechlorination, addition of alum and activated silica, slow mixing, settling, filtration (anthracite and sand), pH correction with lime, fluoridation and post chlorination. Ottawa River water collected at the plants prior to treatment represented the samples of raw water. The relevant characteristics of raw and finished waters from these plants are represented in Table 3.

Sewage

Raw sewage was collected as grab samples at the main inlet of the Green Creek Pollution Control Center in Ottawa.

Processing of Water Samples

Three major steps were involved in the conditioning of the water samples before processing. These are described as follows:

TABLE 3

RELEVANT PHYSICAL, CHEMICAL AND BACTERIOLOGICAL CHARACTERISTICS OF RAW AND FINISHED WATERS FROM THE BRITANNIA AND LEMIEUX ISLAND WATER PURIFICATION PLANTS, OTTAWA, ONTARIO*

	Raw water	Finished water
(mg/L unless otherwise noted)		
Physical		
Temperature, °C.	10.3	12.6
Color (Hazen Units)	43.0	4.0
Turbidity (N.T.U.)	2.7	0.45
Chemical		
pH	7.2	8.6
Total alkalinity, $CaCO_3$	19.0	23.0
Total Hardness, $CaCO_3$	33.3	54.5
Calcium Hardness, $CaCO_3$	24.7	41.8
Magnesium Hardness, $MgCO_3$	12.5	13.0
Specific Conductivity, Micromhos/cm	83.0	130.0
Chloride, Cl	3.0	4.5
Suspended Solids	3.5	0.4
Dissolved Solids	61.0	76.0
Total Solids	67.0	82.0
Bacteriological		
Standard Plate count/ml at 37°C	925.0	2.0
Total Coliforms 100/ml	118.0	0.0
Fecal Coliforms 100/ml	24.0	0.0

* Data supplied by the Regional Municipality of Ottawa-Carleton and are given as mean values for the samples collected from both the plants during 1977.

Dechlorination

The treated water used here generally contained 0.5 mg/L of residual chlorine. Before experimental contamination of the water sample with the virus under study it was, therefore, necessary to neutralize the chlorine. This was carried out by adding a 0.4% solution of sodium thiosulfate ($Na_2S_2O_3$; Fisher Scientific) to the sample to a final concentration of 1:100.

Addition of EBSS

EBSS (Earle, 1943), which acted as a source of cations, was added to the sample to give a final concentration of 1:100.

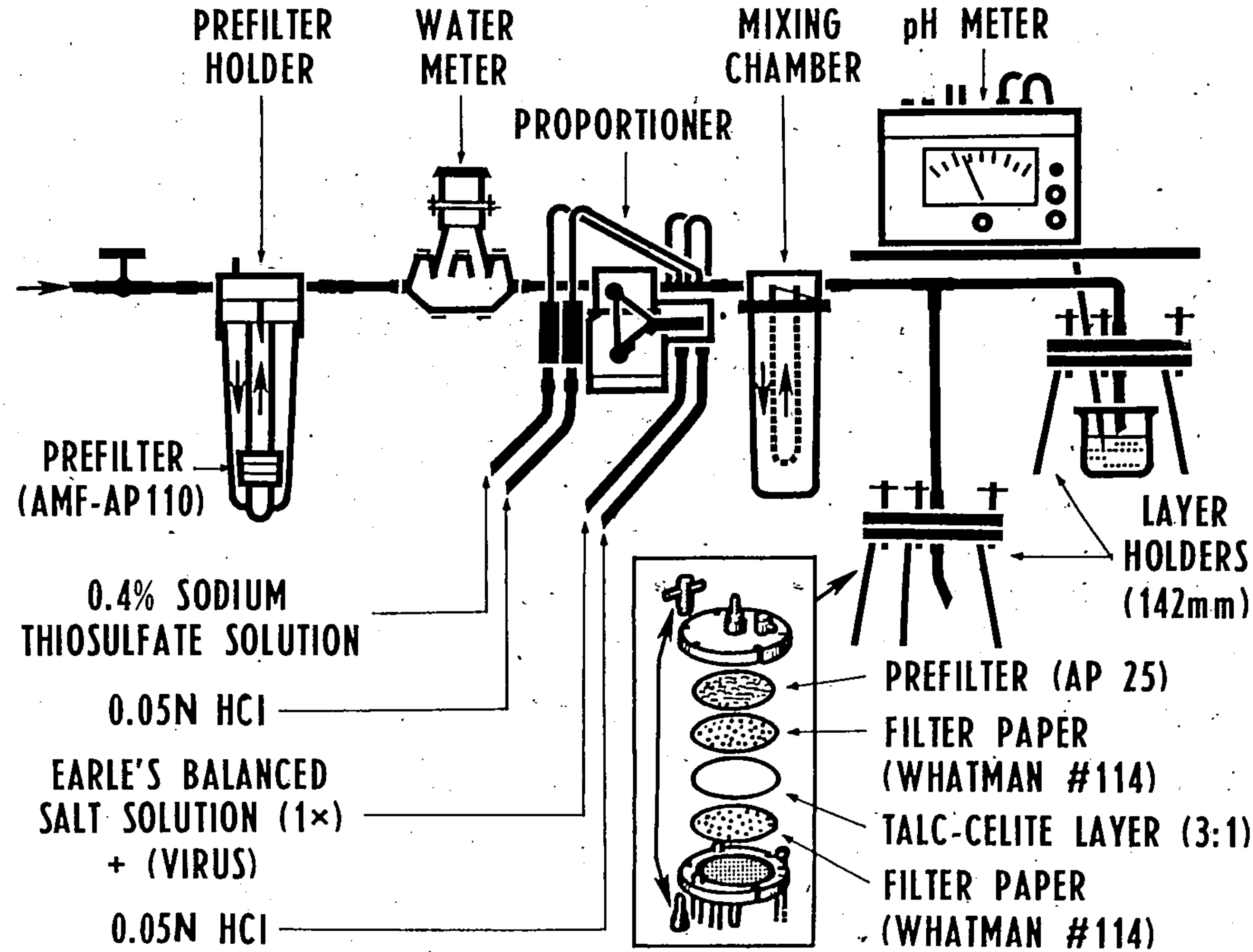
Acidification

The sample pH was adjusted to 6.0 with the help of a freshly prepared 0.05N HCl solution.

The conditioned sample was then contaminated with an appropriate amount of virus under test and passed through the layer.

Conditioning of 10-100 L samples was performed in stainless steel pressure vessels used to hold the sample before filtering. For the conditioning of samples greater than 100L a fluid proportioner (Johanson and Son, Clifton, New Jersey) was found to be necessary. Separate injection pumps were used for the addition of $Na_2S_2O_3$, EBSS and HCl. Virus for the experimental contamination of the samples was suspended in EBSS and injected along with it. The details of such a set up are presented in Figure 1.

Figure 1: Set-up for the processing of
100-1,000-litre samples of
potable waters.


PREFILTER
HOLDER
WATER
METER
PROPORTIONER
MIXING
CHAMBER
pH METER
PREFILTER
(AMF-AP110)
LAYER
HOLDERS
(142mm)
0.4% SODIUM
THIOSULFATE SOLUTION
0.05N HCl
EARLE'S BALANCED
SALT SOLUTION (1×)
+ (VIRUS)
0.05N HCl
PREFILTER (AP 25)
FILTER PAPER
(WHATMAN #114)
TALC-CELITE LAYER (3:1)
FILTER PAPER
(WHATMAN #114)

In the tests with large volume samples of potable waters the use of clarifying filters was not found to be necessary. However, as shown in Figure 1, when sample preclarification was required, it could be achieved by using a 5 cm portion of an AP 110 cartridge filter (AMF Corp.). Any particle-associated viruses retained on the clarifying filter could be recovered by exposing it first to the eluent and then passing the same material through the layers.

Eluting Agents

The names of the virus eluting agents used in this study and the relevant information pertaining to them are presented in Table 4.

Second-Step Concentration of Eluates

Organic Flocculation

The procedure used here was the same as described by Katzenelson et al. (1976b), except that the pH of 3% beef extract in deionized water was adjusted to 9.0 with the help of 10N NaOH before adding the virus (poliovirus 1, Sabin). Working with 100 ml samples, the pH was brought down to 3.5 with 2N HCl added dropwise under continuous stirring for 30 minutes. This was followed by centrifugation for 10 minutes at 3,000 xg. The supernatant was removed, and the sediment was redissolved in 10 ml (1/10 the original volume) of 0.15 M Na_2HPO_4 (pH 9.0). The pH of both the supernatant and the dissolved sediment was adjusted to 7.2 before inoculation into BS-C-1 cells for plaque assay.

TABLE 4

RELEVANT INFORMATION ON THE VIRUS-ELUTING AGENTS USED IN THIS STUDY

Item	Lot. No.	Manufacturer	Concentration used	pH
Protein-rich substances				
Fetal calf serum (FCS)	At least 6 different lots used	Microbiological Associates	10% in saline	8.0 or 9.0
Beef extract (BE) in paste form	168 5556	Oxoid	3% in deionized water	8.0 or 9.0
Beef extract (BE) in powder form	282 12544	Oxoid	3% in deionized water	8.0 or 9.0
Casein hydrolysate (CH)	49606	GIBCO	3% in deionized water	8.0 or 9.0
Lactalbumin hydrolysate (LAH)	47708	GIBCO	3% in deionized water	9.0
Nutrient broth (NB)	537645	Difco	1X in deionized water	9.0
Tryptose phosphate broth (TPB)	454688	Difco	1X in deionized water	9.0
Single amino acids				
Arginine	7177	NBC	3% in deionized water	9.0
Asparagine	5084	NBC	3% in deionized water	9.0
Glutamine	R473353	GIBCO	3% in deionized water	9.0
Glycine	Not available	Eastman Kodak Co., Rochester, N.Y. 14650	3% in deionized water	9.0
Glycine-NaOH	-		0.1M in deionized water	9.5 or 11.0

pH adjustment was done with 5N NaOH.
All eluting agents, except FCS, were autoclave sterilized.

Hydroextraction with Polyethylene Glycol (PEG)

A known amount of the virus under study was added to a 100 ml volume sample of the eluting agent under study (pH 9.0) either before (eluent) or after (eluate) it was passed through talc-Celite layers. The virus-contaminated sample was then poured into a dialysis sac (2.7 cm diameter; 4.8 nm pore size - Fisher Scientific Co.) and the sac was sealed and placed in a 250 ml glass beaker. Enough PEG (6,000 or 20,000 molecular weight) powder (J.T. Baker Chemical Co.) was poured into the beaker to surround the sac completely and it was placed at 4°C. Hydroextraction was allowed to proceed for either 4 hours or overnight (16 to 19 hours). After four hour hydroextraction, the outside of the sac was thoroughly washed in tap water, the material inside was carefully removed and collected. The pH of the concentrate was adjusted to 7.2 with 0.1N HCl and it was passed through a 0.2μm membrane filter (Nalge Corp.) before plaque assay.

When hydroextraction was allowed to proceed overnight, nearly all the liquid from inside the sac was removed. The material remaining in the sac was resuspended in 10 ml of EBSS and plaque-assayed after membrane filtration.

PART I

Development of a Plaque Assay Technique for Simian Rotavirus SA-11 and Study of the Role of Trypsin in SA-11 Plaque Formation

INTRODUCTION

Although they had been isolated from a number of animal species earlier (Adam and Kraft, 1963; Malherbe and Strickland-Cholmley, 1967; Mebus et al., 1969) rotaviruses were recognized as causative agents of diarrhea in human infants only about 7 years ago (Bishop et al., 1973; Flewett et al., 1973; Middleton et al., 1974). That rotaviruses can cause diarrhea in human adults as well has now been shown (Von Bonsdorff et al., 1976, 1978; Meurman and Laine, 1977; Kim et al., 1977; Wenman et al., 1979). They have also been detected in cases of intussusception (Konno et al., 1978) and Salmi et al. (1978) suggest that rotavirus diarrhea could lead to either Reye's syndrome or encephalitis.

Rotaviruses are spherical double-stranded RNA viruses with a particle diameter of about 70 nm (Flewett and Woode, 1978). Strains isolated from humans and animals possess a common group specific antigen located on the inner capsid layer of the virion (Kapikian et al., 1976; Woode et al., 1976; Thouless et al., 1977). The species-specific antigen of these viruses has been shown to reside on the outer capsid layer (Woode, 1976; Thouless et al., 1977). Up to now four serotypes of human rotaviruses have been recognized (Flewett et al., 1978).

Rotaviruses resemble reo- and orbiviruses in their morphology, morphogenesis and in possessing a segmented genome. They are, however, antigenically unrelated to the members of these two virus groups

(Kapikian et al., 1975; Woode and Bridger, 1975). Because of these similarities and differences, rotaviruses are now placed in a separate genus of the family Reoviridae (Melnick, 1979).

Although some strains have recently been adapted to grow in cell culture (Wyatt et al., 1980), easy and reliable means of in vitro cultivation and quantitation of human rotaviruses in general have not yet become available.

Among the known animal rotaviruses, the cell-culture-adapted simian agent SA-11 (Malherbe and Strickland-Cholmley, 1967) shows the closest resemblance to the rotaviruses isolated from humans (Kapikian et al., 1976; Schoub et al., 1977). Therefore, until easy and reliable means for human rotaviruses cultivation and quantitation in vitro become available, SA-11 continues to serve as a model for their study. Because of the absence of a simple and sensitive plaque assay system for SA-11, some earlier investigators (Malherbe and Strickland-Cholmley, 1967; Farrah et al., 1978a; 1978b) with this virus resorted to the use of cumbersome and less sensitive means of infective virus quantitation. The development of an appropriate plaque assay system for SA-11 virus was, therefore, considered necessary before it could be effectively used in the testing of the techniques for the recovery of rotaviruses from the water environment. In this section of the thesis, the development of such a titration system for SA-11 in MA-104 cells is described. Results of the experiments to study the role of trypsin in SA-11 plaque production are also presented here.

EXPERIMENTAL AND RESULTS

A. Plaque Formation by Rotavirus SA-11

It has been known for some time that the in vitro infectivity of a number of viruses is markedly enhanced in the presence of proteolytic enzymes such as trypsin (Gifford and Klapper, 1967; Spendlove et al., 1970; Appleyard and Maber, 1974: Tobita and Kilbourne, 1974; Klenk et al., 1975; Lazarowitz and Choppin, 1975). This phenomenon has now been shown to apply to porcine (Theil et al., 1977), bovine (Babiuk et al., 1977; Almeida et al., 1978; Clark et al., 1979) and avian (McNulty et al., 1979) rotaviruses as well. Matsuno et al. (1977) found that presence of trypsin in the overlay was also essential to plaque formation by the Lincoln strain of the Nebraska calf diarrhea virus (NCDV). Wyatt et al. (1978a), on the other hand, reported that trypsin or other proteolytic enzymes were not needed for the plaque formation of the UK strain of bovine rotavirus. It has also been reported that the simian rotavirus SA-11 does not depend on proteolytic enzymes either for enhanced growth in cell cultures (Schoub and Bertran, 1978) or for the formation of plaques (Wyatt et al., 1978a).

Need for Trypsin in the Overlay

Although SA-11 grows and produces pronounced CPE in MA-104 cells, when an overlay consisting of MEM (without serum) and 0.75% Bacto Agar was used, the virus failed to form detectable plaques in the cells even after 7 days of incubation at 37°C. However, when trypsin at a final concentration of 5μg/ml was incorporated in the overlay, small (about 1 mm in diameter) plaques could be seen after 5 days of incubation. Longer incubation did not lead to any further increase in plaque size, but resulted in the rapid thinning and deterioration of the monolayers. Increase in the amount of trypsin in the overlay also produced premature and non-specific cell degeneration.

Effect of Agar Type on SA-11 Plaque Formation

In an effort to further improve the size and appearance of the plaques, different types of agar were tested as solidifying agents for the overlay. These included Bacto Agar, Noble Agar, Oxoid Agar No. 1 and Ionagar No. 2. As can be seen from the data in Table 5, presence of Ionagar No. 2 in the trypsin-containing overlay gave plaques of the largest (3-4 mm diameter) size in monolayers of MA-104 cells after 5 days of incubation. Figure 2 shows the appearance of such plaques along with a virus-inoculated culture which had received the same overlay but without trypsin. On the basis of these observations, Ionagar No. 2 was chosen as the solidifying agent for the overlay in subsequent experimentation.

That trypsin itself and not some impurity in the enzyme preparation was responsible for the formation of plaques was demonstrated in the following experiment. After the virus inoculated cultures received the trypsin-containing overlay, they were left for about 20 minutes at room temperature for the overlay to solidify. Then discs (7.0 mm diameter), punched out of AP25 (Millipore) prefilters and impregnated with soybean trypsin inhibitor (100µg/disc), were introduced and placed at the center of the overlay. A disc soaked in EBSS was deposited on a second similarly inoculated and overlayed culture. The bottles were then incubated for 5 days at 37°C. As can be seen from Figure 3, plaque formation was inhibited in the area where the diffusion of trypsin inhibitor from the disc had taken place. An increase or decrease in

TABLE 5

EFFECT OF THE TYPE OF AGAR ON ROTAVIRUS SA-11 PLAQUE FORMATION IN MA-104 CELLS

Type of Agar[a]	No. of plaques /0.5 ml[b]	Plaque diameter (mm)[c]
Bacto	32	1
Oxoid #1	34	1
Noble	32	1
Ionagar #2	38	3.5

[a] Overlay consisted of MEM, 5μg/ml of trypsin and 0.7% of agar under test.

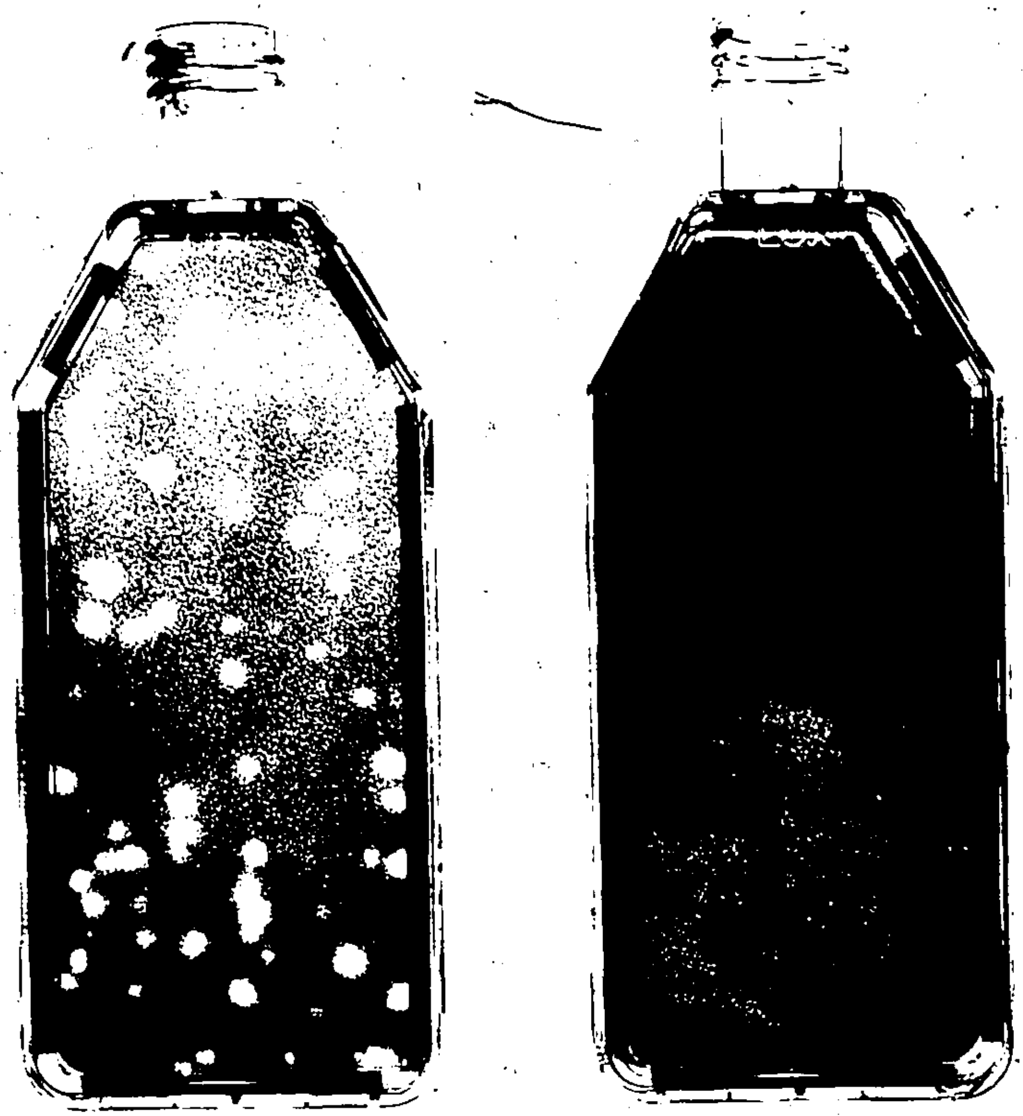
[b] Each flask was inoculated with 0.5 ml of stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

[c] About 25 plaques were counted to determine average diameter.

Figure 2: Plaque formation by simian rotavirus SA-11 in MA-104 cells after five days of incubation at 37°C.

(A) monolayer with 5μg of trypsin per ml of the overlay

(B) virus-inoculated monolayer with overlay containing no trypsin



A B

Figure 3: Inhibition of the action of trypsin on the plaque forming ability of rotavirus SA-11 in MA-104 cells.

(A) On the overlay containing 5μg of trypsin per ml a 7 mm diameter filter paper disc impregnated with EBSS was placed at the spot indicated by an "X".

(B) On the overlay containing 5μg of trypsin per ml a 7 mm diameter filter paper disc impregnated with 100μg of soybean trypsin inhibitor was placed at the spot indicated by an "X".

A B

the concentration of the inhibitor in the disc produced a corresponding widening or reduction in the zone of plaque inhibition. It thus provided simple and direct evidence for the specific need for trypsin in plaque formation by this virus.

Effect of Protein-Supplemented Overlay on SA-11 Plaque Formation

The presence of protein supplements in the overlay is considered helpful in the proper and prolonged maintenance of cell monolayers. Since serum could not be used here, BE, YE, LAH and peptone were incorporated separately in the trypsin-containing overlay at a final concentration of 0.5%. The results of these experiments are summarized in Table 6. Presence of BE and YE in the overlay resulted in no plaque formation by the virus. Plaques were formed in the presence of peptone or LAH but, when compared to cultures with unsupplemented overlay, there was no detectable improvement in their size and number.

Absence of SA-11 plaques under overlays supplemented with BE or YE could have been due to non-specific virus inhibitors associated with them. It is also possible that the presence of these protein-rich substances in the overlay had interfered with the proteolytic activity of trypsin essential to virus plaque development. That the latter was the case was shown in the following experiment: The virus was diluted to the desired level in EBSS containing 0.5% of either BE or YE and kept overnight at 4°C. The material was then inoculated into MA-104 cells and left at 37°C for 1 hour. At the end of the incubation period, the bottles were divided into two lots. The

TABLE 6

EFFECT OF PROTEIN-RICH SUPPLEMENTS IN THE OVERLAY ON PLAQUE FORMATION BY ROTAVIRUS SA-11 IN MA-104 CELLS

Type of Supplement[a]	No. of plaques/0.5 ml[b]	Plaque diameter (mm)[c]
None	36	3.5
Beef extract (BE)	0	-
Yeast extract (YE)	0	-
Lactalbumin hydrolysate (LAH)	30	3.5
Peptone	34	3.5

[a] Overlay consisted of MEM, 5μg/ml trypsin, 0.7% Ionagar #2 and 0.5% of supplement under test.

[b] Each flask was inoculated with 0.5 ml of stock virus diluted to 10^{-5} in EBSS. Plaques were counted after five days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

[c] About 25 plaques were counted to determine average diameter.

monolayers in bottles from one lot were washed twice with EBSS and those from the other lot were kept unwashed. All of them then received the same trypsin-containing overlay and incubation. Plaques could be detected only in the bottles which were washed before receiving the overlay.

Effect of Overlay Containing DEAE-Dextran, Protamine Sulfate or 5-BUdR

Matsuno et al. (1977) reported that along with trypsin, DEAE-dextran was needed in the overlay for optimal plaque productions by the Lincoln strain of NCDV in MA-104 cells. The results of similar experiments with SA-11 virus and MA-104 cells as presented in Table 7, show that the presence of DEAE-dextran up to 50μg/ml in the overlay did not contribute to the improvement of either plaque size or number. Addition of 100μg or more of the substance per ml of the overlay in fact resulted in a decrease in the size and number of SA-11 plaques.

Presence of protamine sulfate in the overlay has been shown to enhance plaque formation by certain viruses (Wallis and Melnick, 1968). Addition of 50 to 300μg of protamine sulfate per ml of the overlay had no noticeable effect on plaque formation by SA-11 virus (Table 7). Similar results were obtained when 25 to 200μg of 5-BUdR was incorporated per ml of the overlay (Table 7).

Effect of Virus Diluent-Suspending Medium

Up to this stage EBSS was used as virus diluent as well as the suspending medium during virus adsorption to cell monolayers. It has,

TABLE 7

EFFECT OF CHEMICAL ADDITIVES IN OVERLAY ON ROTAVIRUS SA-11 PLAQUE FORMATION IN MA-104 CELLS

Additive and conc. in µg/ml of overlay[a]	No. of plaques / 0.5 ml[b]	Plaque diameter (mm)[c]
None	38	3.5
DEAE-dextran		
50	32	3.5
100	30	2.0
200	28	2.0
300	28	2.0
Protamine sulfate		
50	36	3.5
100	38	3.5
200	34	3.5
300	34	
5-bromodeoxyuridine		
25	38	3.5
50	36	3.5
100	38	3.5
200	38	3.5

[a] Overlay consisted of MEM, 5µg/ml trypsin. 0.7% Ionagar #2 and the desired conc. of additive under test.

[b] Each flask was inoculated with 0.5 ml of stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

[c] About 25 plaques were counted to determine average diameter.

however, been shown that presence of protein-rich additives in the diluent and during virus adsorption to cells enhances the plaquing efficiency (Hamblet <u>et al.</u>, 1967). To test this for SA-11, BSA, TPB or NB was added to EBSS to a final concentration of 0.5%. From the data presented in Table 8, it can be seen that the presence of these protein-rich additives in virus diluent-suspending medium affected neither the size nor the number of SA-11 plaques in MA-104 cells.

Effect of Incubation Temperature

In order to see what incubation temperature would be optimal for SA-11 plaque production, separate lots of virus inoculated and overlayed cultures were placed at 33, 35, 37 and 39°C for 5 days. At the end of this period, plaques produced were examined and counted. As can be seen from the data in Table 9, incubation at 37°C was found to be optimal with regards to both plaque size and number.

Effect of Overlay with Methyl Cellulose

Agars of various types are known to contain sulfated polysaccharides which can inhibit plaque formation by viruses (Takemoto, 1966). To avoid this possibility, use of methyl cellulose as a solidifying agent in overlays has been recommended (Hotchin, 1955). The use of methyl cellulose as a substitute for Ionagar No. 2 in our system was tested. When an overlay medium consisting of MEM, 5μg/ml of trypsin in 1.0% methyl cellulose was used, SA-11 plaque formation was completely inhibited. Variation in the amount of trypsin from 2.5 to 10μg/ml of the overlay did not allow plaque formation in the presence of methyl cellulose. Even when the methyl cellulose-containing overlay was supplemented with 10-

TABLE 8

EFFECT OF VIRUS DILUENT ON PLAQUE FORMATION BY ROTAVIRUS SA-11 IN MA-104 CELLS

Diluent	No. of plaques /0.5 ml[a]	Plaque size (mm)[b]
Earle's balanced salt solution (EBSS)	34	3.5
0.5% tryptose phosphate broth in EBSS	34	3.5
0.5% nutrient broth in EBSS	28	3.5
0.5% bovine serum albumin in EBSS	30	3.5

[a] Each flask received 0.5 ml of stock virus diluted to 10^{-5} in the diluent under test. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in three separate experiments.

[b] About 25 plaques were counted to determine average diameter.

TABLE 9

EFFECT OF INCUBATION TEMPERATURE ON PLAQUE FORMATION BY ROTAVIRUS SA-11 IN MA-104 CELLS

Incubation temperature (°C.)	No. of plaques /0.5 ml[a]	Plaque diameter (mm)[b]
33	44	2.0
35	36	2.5
37	42	3.5
39	24	2.5

[a] Each culture received 0.5 ml of stock virus diluted to 10^{-5} in EBSS and after virus adsorption (37°C) and overlaying were incubated at the appropriate temperature for five days. Numbers represent the mean of a total of 10 counts obtained in two separate experiments.

[b] About 25 plaques were counted to determine average diameter.

to 25μg/ml of DEAE-dextran or 10 to 50μg/ml protamine sulfate no plaque formation occurred. Any further increase in the amounts of these cationic polymers made the overlay cytotoxic.

SA-11 Plaque Formation in Other Types of Cell Cultures

The ability of the SA-11 virus to form plaques in cell cultures other than that of MA-104 line was investigated. The cells tested were AGMK, HEK, BS-C-1 and L-132. Because primary cultures do not produce uniform and satisfactory monolayers for virus plaquing, the AGMK and HEK cells were used as secondary monolayers. After adsorption with SA-11, the cells were covered with overlay either with (5μg/ml) or without trypsin and incubated. No plaques could be seen in monolayers of HEK, BS-C-1 or L-132 cells even when the incubation period was extended to eight days. As can be seen from Figure 4, plaques (5-6 mm diameter) were produced in the AGMK cells only when trypsin was present in the overlay. This is in contrast to the finding of Wyatt *et al.* (1978a) who reported that the presence of trypsin or other proteolytic enzymes in the overlay was not necessary for SA-11 plaque formation in primary cultures of AGMK cells.

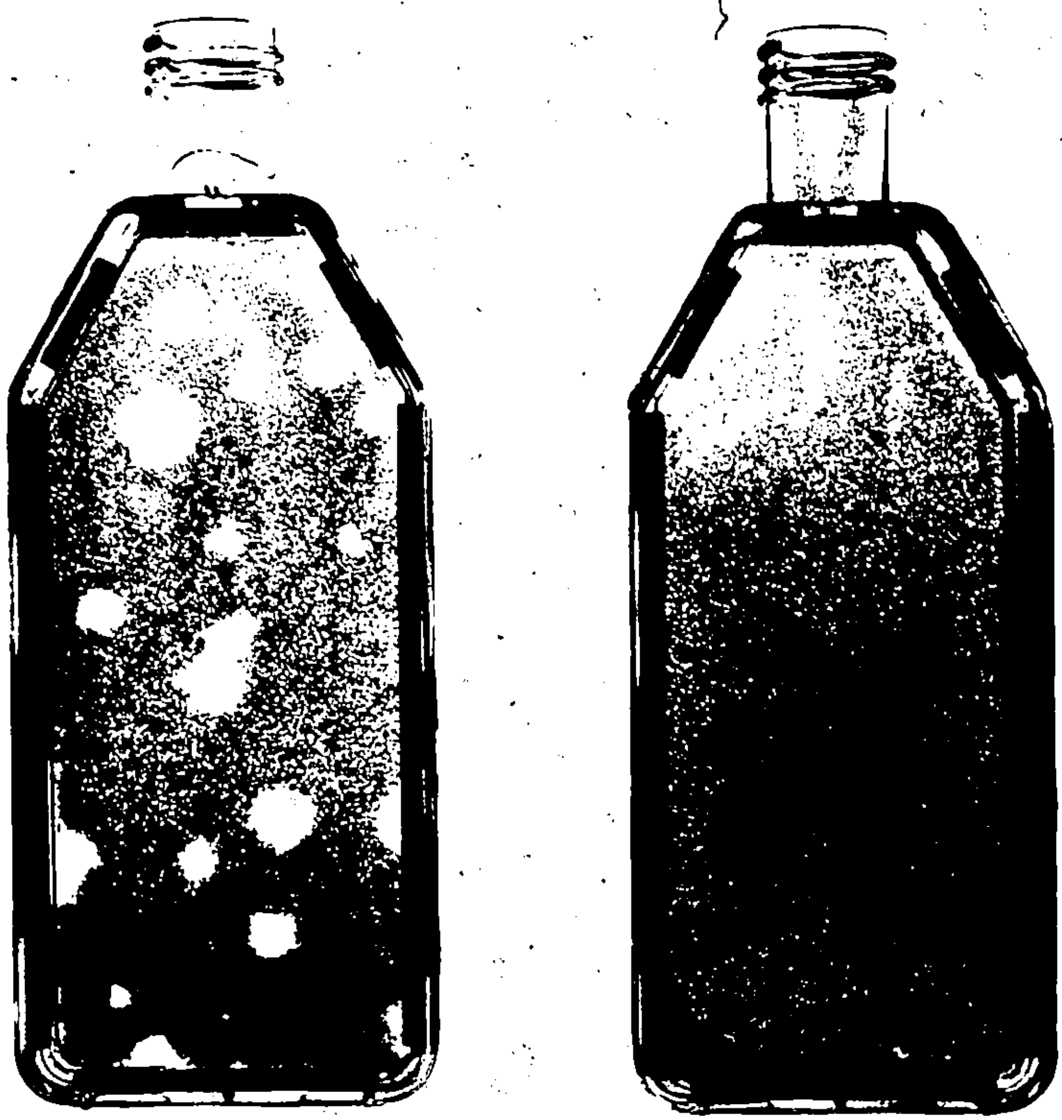
Other Proteolytic Enzymes and Rotavirus SA-11 Plaque Formation

In the foregoing experiments it was established that presence of trypsin in the overlay was necessary for plaque formation by SA-11 in MA-104 cells. Would other proteolytic enzymes produce a similar effect? To test this, eight other proteolytic enzyme preparations (Table 2)

Figure 4: Plaque production by simian rotavirus SA-11 in AGMK cells after five days of incubation at 37°C.

(A) monolayer with 5μg of trypsin per ml of the overlay

(B) virus-inoculated monolayer with overlay containing no trypsin



A B

were selected and their effect on SA-11 plaque formation was compared to that of trypsin. The results of these experiments are presented in Table 10. When incorporated in the overlay in concentrations per ml indicated, α-chymotrypsin (10μg), elastase (0.5μg), subtilisin (0.5μg), pronase (2.5μg) and pancreatin (25μg) were as efficient as trypsin in assisting SA-11 plaque formation. No plaques were produced when pepsin (25μg), papain (10μg) or thermolysin (10μg/ml) was added to the overlay; use of higher concentrations of these three enzymes resulted in rapid thinning and early degeneration of the monolayers.

Pancreatin is a mixture of enzymes including trypsin (Osol and Farrar, 1960). Similarly, pronase, derived from Streptomyces griseus is a mixture (Hiramatsu and Ouchi, 1963) which includes a trypsin-like enzyme (Jurasek et al., 1969). To see whether the effect of these enzyme mixtures on SA-11 plaque formation was in fact due to the trypsin or trypsin-like activity contained in them, the following experiments were performed: Trypsin inhibitor in various concentrations was added to overlays containing the enzyme preparations previously shown to affect SA-11 plaque formation. Such overlays were then introduced into virus-inoculated cultures. The results of these experiments are summarized in Table 11. Addition of the trypsin inhibitor to the pancreatin-, pronase-, or α-chymotrypsin-containing overlays completely inhibited SA-11 plaque formation. However, virus plaque formation was not noticeably affected when the trypsin inhibitor was incorporated in the elastase- or subtilisin-containing overlays.

TABLE 10

EFFECT OF DIFFERENT PROTEASES ON THE PLAQUE FORMATION ABILITY OF ROTAVIRUS SA-11 IN MA-104 CELLS

Enzyme Preparation	Conc. in μg/ml in overlay[a]	No. of Plaques /0.5 ml[b]	Plaque diameter (mm)[c]
Trypsin	5	38	3.5
Pronase	5	36	3.5
Pancreatin	25	36	3.5
α-chymotrypsin	10	30	3.5
Elastase	0.5	36	3.5
Subtilisin	0.5	30	3.5
Papain	10	0	-
Pepsin	50	0	-
Thermolysin	5	0	-

[a] Overlay consisted of MEM, 0.7% Ionagar #2 and the specified concentration of the enzyme under test. Concentrations above the ones mentioned lead to premature degeneration of the monolayers.

[b] Each flask was inoculated with 0.5 ml of the stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in 3 separate experiments.

[c] About 25 plaques were measured to determine the average plaque diameter.

TABLE 11

EFFECT OF TRYPSIN INHIBITOR ON THE DIFFERENT PROTEASES FOUND TO HELP IN THE FORMATION OF PLAQUES BY ROTAVIRUS SA-11 IN MA-104 CELLS

Enzyme and conc. in μg/ml in overlay[a]	Trypsin inhibitor conc. in μg/ml in overlay[a]	No. of Plaques 0.5 ml[b]	Plaque Diameter (mm)[c]
Trypsin (5)	0	38	3.5
	5.0	0	-
Pronase (5)	0	36	3.5
	5.0	0	-
Pancreatin (25)	0	36	3.5
	10.0	0	-
α-chymotrypsin (10)	0	30	3.5
	5.0	10	<1.0
	7.5	0	-
Elastase (0.5)	0	36	3.5
	1.0	32	3.5
Subtilisin (0.5)	0	30	3.5
	1.0	30	3.5

[a] Overlay consisted of MEM, 0.7% Ionagar #2 and the specified concentration of the enzyme under test and trypsin inhibitor when required.

[b] Each flask was inoculated with 0.5 ml of the stock virus diluted to 10^{-5} in EBSS. Plaques were counted after 5 days of incubation at 37°C. Numbers represent the mean of a total of 12 counts obtained in 3 separate experiments.

[c] About 25 plaques were measured to determine the average plaque diameter.

Reproducibility of Results Obtained by Plaque Assay Technique

For retrospective statistical analysis of SA-11 plaque assay system, data from ten separate titrations performed on the same virus pool during an eight-month period, were selected (Table 12). These were subjected to a one-way analysis of variance as described in Appendix I. As can be judged from mean square values obtained, the plaque assay gave reproducible results. This also showed that there was no decrease in the plaque titre over the 8 month period due to either virus storage (-80°C) or the increase in the cell passage level.

TABLE 12

PLAQUE COUNTS OBTAINED IN SEPARATE TITRATIONS OF ROTAVIRUS SA-11 IN MA-104 CELLS

Serial No.	Date of titration	Plaque counts obtained	Mean
1	12/09/78	42,35,35,31,28	34.2
2	08/10/78	42,38,34,34,29	35.4
3	28/10/78	46,42,37,35,32	38.4
4	28/11/78	40,40,34,32,26	34.4
5	12/12/78	38,38,32,29,27	32.8
6	17/01/79	43,40,36,32,30	36.2
7	30/02/79	38,38,37,32,30	35.0
8	26/03/79	43,37,35,32,30	35.4
9	20/04/79	41,38,35,33,32	35.8
10	21/05/79	40,36,35,30,27	33.6

The virus was diluted to 10^{-5} in EBSS and 0.5 ml of this dilution was inoculated into each culture of MA-104 cells. After one hour of adsorption at 37°C, each flask received 5 ml of an overlay containing MEM, 5μg of trypsin/ml and 0.7% Ionagar #2. Plaques were counted after five days of incubation at 37°C.

B. Role of Trypsin in Rotavirus SA-11 Plaque Formation

Enzymatic removal of the outer capsid of reoviruses has been shown to result in the enhancement of their infectivity (Spendlove et al., 1970). The enhancement of the in vitro infectivity of myxoviruses in the presence of proteolytic enzymes has been ascribed to the cleavage of viral proteins (Appleyard and Maber, 1974; Tobita and Kilbourne, 1974; Klenk et al., 1975; Lazarowitz and Choppin, 1975).

From the results of the experiments described up to this stage it has been established that the presence of certain types of proteolytic enzymes in the overlay is essential for the formation of plaques by SA-11 in MA-104 cells. Matsuno et al. (1977) and Smith et al. (1979) have also reported similar findings. However, the mechanism(s) by which such enzymes facilitate rotavirus plaque formation is not yet clearly understood. In order to elucidate this, the stage in rotavirus plaque formation at which such enzymes played their role needed to be determined. This was, therefore, the purpose of the experiments to be described here.

Effect of Trypsin Treatment of the Virus

Recently it was suggested that the in vitro enhancement of infectivity of a bovine rotavirus after trypsin treatment was due to the direct action of the enzyme on the virus particles (Barnett et al., 1979). It was, therefore, decided to test the effect of such treatment on the plaque forming ability of SA-11. To the virus, which had been grown in the absence of trypsin, the enzyme was added to give a final concentration of either 10 or 100μg/ml. The mixture was incubated for 30 minutes at 37°C. At the end of this period, sufficient trypsin inhibitor was added and appropriate dilutions of the treated virus were used for plaque assay with or without trypsin in the overlay agar. From the data summarized in Table 13, it can be seen that when the trypsin-treated virus was tested with overlay containing no trypsin plaque formation did not take place. Trypsin treatment of the virus alone, therefore, was not enough to allow it to form plaques. Furthermore, in the presence of trypsin-containing agar overlay, the plaque titer of the trypsin-treated virus was found to be similar to that of the untreated virus controls.

It is well-recognized that break-up of infectious virus clumps leads to an increase in infectivity titer (Hamblet et al., 1967). As has been suggested earlier (Clark et al., 1979; Smith et al., 1979) trypsin treatment of clumped rotavirus particles could lead to their disaggregation and give the appearance of enhanced infectivity. Although we did not see an increase in the plaque titer after trypsin treatment of the virus it was considered necessary to ascertain that our virus pool was devoid of aggregates which were not being dissociated by the enzyme treatment applied. An appropriate dilution of the stock virus was prepared in EBSS. After removing an aliquot which was to act as control, the rest was passed through an 80 nm pore diameter polycarbonate membrane filter (Bio-Rad); the membrane was previously

TABLE 13

EFFECT OF TRYPSIN TREATMENT OF ROTAVIRUS SA-11 PRIOR TO INOCULATION IN MA-104 CELL MONOLAYERS

Trypsin concentration in virus suspension[a] (μg/ml)	Trypsin in agar overlay[b]	No. of plaques/0.5 ml[c]
0	+	38
10	-	0
10	+	42
100	-	0
100	+	42

[a] Stock virus was treated with the desired amount of trypsin for 30 minutes at 37°C. Trypsin inhibitor was added to the treated virus before making appropriate dilutions for plaque assay.

[b] In trypsin-containing overlay the concentration of the enzyme was 5μg/ml.

[c] Numbers represent the mean of a total of twelve counts obtained in three separate experiments.

treated with a 3.0% solution of tryptose phosphate broth to minimize virus adsorption to its matrix (Wallis et al., 1972a). As can be seen from the data in Table 14, there was no major difference in the plaque titer of the virus before and after such membrane filtration, indicating the absence of virus aggregates.

Effect of Trypsin on Virus Adsorption

If trypsin exerts its effect only in the early phases of virus-cell interaction, then its presence in the initial inoculum alone should contribute to rotavirus plaque formation. This view was tested by allowing the virus to adsorb to the host cells in the presence of trypsin. At the end of one hour at 37°C the trypsin-containing inoculum was either removed by thorough washing of the cultures or by their subsequent incubation (30 minutes at 37°C) in the presence of trypsin inhibitor. The cultures then received an agar overlay with or without trypsin and incubated for plaque formation to proceed. Table 15 presents the data from these experiments.

The presence of trypsin during the initial phases of virus-cell interaction, but its absence in the overlay did not result in the production of virus plaques. Plaques produced when trypsin was present in the initial inoculum as well as in the overlay did not differ in any way from those that developed when trypsin was added only to the overlay.

TABLE 14

MEMBRANE FILTRATION[a] TO DETECT THE PRESENCE OF AGGREGATES IN THE ROTAVIRUS SA-11 STOCK

	Number of plaques/0.5 ml[b]
Unfiltered virus stock	32
Virus stock after passage through a membrane pretreated with 5 ml of 3% solution of tryptose phosphate broth	30

[a] Unipore polycarbonate membranes of 80 nm pore size were used.

[b] The figures represent the mean of a total of eight counts obtained in two separate experiments.

TABLE 15

EFFECT OF TRYPSIN ON INITIAL VIRUS-CELL INTERACTION

	Trypsin in overlay[c]	Number of plaques/0.5 ml[d]
No trypsin treatment	+	36
Trypsin treatment of cell monolayers prior to virus inoculation[a]	-	0
	+	38
Trypsin treatment during the virus adsorption period[b]	-	0
	+	40

[a] MA-104 monolayers were incubated at 37°C for 30 minutes with 1 ml of EBSS containing 10μg of trypsin. They were then exposed to 10μg of trypsin inhibitor for 30 minutes. After thorough washing with EBSS they were used for virus inoculation.

[b] Rotavirus SA-11 was suspended in EBSS with 10μg of trypsin/ml and allowed to adsorb on cell monolayers for one hour at 37°C. Then the cultures were either thoroughly washed with EBSS or incubated for 30 minutes with trypsin inhibitor.

[c] The agar overlay was used either without (-) trypsin or with (+) 5μg of trypsin/ml.

[d] Figures represent the mean of a total of twelve counts obtained in three separate experiments.

The effect of prior treatment of the host cells with trypsin was also investigated. MA-104 monolayers were first exposed to trypsin for 30 minutes at 37°C. The cultures were then treated with trypsin inhibitor and thoroughly washed before inoculation with the virus. After virus adsorption, the cultures received overlays with or without trypsin. The results, also seen in Table 15, show that prior trypsin treatment of the cells also did not influence the plaque forming ability of the virus.

Effect of Centrifugation

Centrifugation of cell monolayers after virus inoculation has been shown to enhance the infectivity as well as the plaque forming ability of certain viruses (Osborn and Walker, 1968; Bryden et al., 1977). In the following experiments we investigated the effect of centrifugation with and without trypsin on the plaque forming ability of SA-11. Table 16 presents the results of these experiments. Centrifugation alone was not sufficient for SA-11 plaque-formation; trypsin in the overlay agar was still necessary. However, centrifugation of the virus inoculated cultures during the adsorption period gave nearly four times the number of plaques when compared to cultures which had been subjected to stationary incubation for one hour at 37°C.

Addition of trypsin to the virus inoculum followed by centrifugation did not produce any further improvement in the plaque count. Centrifugation of the cell monolayers prior to the introduction of the

TABLE 16

EFFECT OF CENTRIFUGATION OF MA-104 MONOLAYERS ON THE PLAQUE FORMING ABILITY OF ROTAVIRUS SA-11

	Trypsin in inoculum[a]	Trypsin in overlay[b]	Number of plaques/0.5 ml[c]
Stationary incubation for one hour at 37°C	-	+	8
Centrifugation of monolayers at 1,600xg for 30 minutes prior to virus inoculation followed by stationary incubation	+	+	10
	+	-	0
	-	+	7
	-	-	0
Centrifugation of monolayers at 1,600xg for 30 minutes after virus inoculation	+	+	38
	+	-	0
	-	+	34
	-	-	0

[a] Virus inoculum was suspended in EBSS without (-) or with (+) 5μg of trypsin/ml.

[b] The agar overlay used was either without (-) trypsin or supplemented with (+) 5μg of trypsin/ml.

[c] Figures represent the mean of a total of twelve counts obtained in three separate experiments.

virus inoculum gave the same number of plaques as those in cultures subjected to stationary incubation.

Plaque Inhibitors in Agar

Certain types of agar are known to contain substances which can interfere with virus replication (Wallis and Melnick, 1968). The presence of trypsin in the overlay may be leading to the degradation of such inhibitors and thus permitting the development of SA-11 plaques. To test this possibility, the following experiments were conducted. One hundred ml of a 1.4% solution of Ionagar No. 2 in deionized water was autoclave sterilized. After cooling it down to about 45°C, trypsin was added to it to give a final concentration of 100μg/ml. The mixture was incubated for 24 hours at 37°C. It was then heated at 100°C for 30 minutes to melt the agar as well as inactivate the enzyme. This enzyme-treated agar was subsequently used in the overlay for SA-11 inoculated MA-104 cultures. No plaque formation occurred in the cultures which had received the overlay containing the trypsin-pretreated agar. This demonstrated that plaque production by SA-11 under trypsin-containing overlay could not be ascribed to the degradation of trypsin-sensitive plaque inhibitors in the agar.

Effect of Trypsin Addition or Inhibition at Various Stages During Plaque Development

If the presence of trypsin in the overlay is necessary throughout the period of plaque development then a delay in its addition to or early inhibition in the overlay should affect plaque formation by the virus. To test this, the following experiments were carried out. MA-104 monolayers inoculated with the virus received agar overlay to which no trypsin was added. The cultures were then placed at 37°C. The following day, one set of cultures from this lot received 5.0 ml of a second overlay containing 5.0μg of trypsin per ml. This procedure was repeated with additional sets of cultures on the second, third and fourth day of incubation.

The results of these experiments are presented in Table 17. When trypsin was added to the overlay at the end of the first day of incubation, there was a corresponding delay in the development of the plaques. However, any further delay in the addition of trypsin to the overlay not only postponed the formation of the plaques but also resulted in the appearance of only half their number when compared to the controls.

Similarly, the effect of inhibiting the action of trypsin during various stages of plaque development was tested by first overlaying the virus inoculated monolayers with the agar containing 5.0μg of trypsin/ml. At the end of the first, second, third and fourth days of incubation, separate sets of the cultures received 5.0 ml of a second overlay containing 5.0μg of trypsin inhibitor/ml.

Inhibition of the action of trypsin in the overlay after the first or second day of incubation resulted in the delayed formation of fewer and smaller plaques (Table 18). When the inhibitor was

TABLE 17

EFFECT OF DELAYED ADDITION OF TRYPSIN ON PLAQUE PRODUCTION BY ROTAVIRUS SA-11 IN MA-104 CELLS

Day of addition of trypsin to overlay[a]	Day plaques counted	No. of plaques/0.5 ml[b]	Average plaque diameter (mm)[c]
0	5	36	3.5
1	6	36	4.0
2	7	20	4.0
3	8	20	4.5
4	9	15	4.5

[a] Rotavirus-inoculated monolayers were incubated at 37°C for one hour. Except for controls, the remaining cultures received an overlay without trypsin. At the appropriate time during the incubation, the cultures received a second overlay with 5μg of trypsin/ml.

[b] Numbers represent the mean of a total of twelve counts obtained in three separate experiments.

[c] About 25 plaques were counted to determine average diameter.

TABLE 18

EFFECT OF THE INHIBITION OF TRYPSIN IN OVERLAY ON THE DEVELOPMENT OF ROTAVIRUS SA-11 PLAQUES IN MA-104 CELLS

Day of addition of trypsin inhibitor[a]	Day plaques counted	No. of plaques/0.5 ml[b]	Average plaque diameter (mm)[c]
0	5	0	-
1	6	20	1.0
2	7	28	1.5
3	8	34	3.5
4	9	36	3.5

[a] Virus inoculated monolayers received an agar overlay with 5µg of trypsin/ml. Except for controls, at the appropriate time during the incubation period, the other cultures received a second overlay containing 5µg of trypsin inhibitor/ml.

[b] Numbers represent the mean of a total of twelve counts obtained in three separate experiments.

[c] About 25 plaques were measured to determine the average diameter.

added after the third or fourth day of incubation, there was a corresponding delay in plaque development but their number and size was equal to those in the controls.

Single-Cycle Virus Replication in the Presence of Trypsin

The findings of the forgoing experiments demonstrated that optimal plaque formation by SA-11 could occur only when trypsin was present in the agar overlay throughout the five-day incubation period. This suggested that the enzyme may in some way enhance the intracellular replication of the virus. To test this, the following experiments were carried out.

Monolayer cultures of MA-104 cells were inoculated with SA-11 using a multiplicity of infection of about 4.0 PFU/cell. After one hour of adsorption at 37°C, the monolayers were washed three times with EBSS to remove the unadsorbed virus. Each culture then received 5.0 ml of the fluid maintenance medium either with (10μg/ml) or without trypsin. They were placed back at 37°C and at appropriate intervals representative bottles were removed from the incubator and stored at -20°C. At the end of the experiment, the material from the stored cultures was processed and plaque assayed as described in "Materials and Methods". The data from these experiments are summarized in Table 19. Presence of trypsin in the maintenance medium affected neither the rate of replication nor the final titre of the virus.

TABLE 19

VIRUS YIELD AT DIFFERENT POSTINOCULATION INTERVALS IN THE SINGLE CYCLE GROWTH OF SA-11 VIRUS IN MA-104 CELLS[a]

Time postinoculation (hr)	Virus yield[b] (PFU/ml)	
	Medium with trypsin	Medium without trypsin
6	2.4×10^{4}	2.5×10^{4}
10	6.7×10^{6}	6.8×10^{6}
16	8.2×10^{7}	8.2×10^{7}
20	8.4×10^{7}	8.6×10^{7}

[a] MA-104 monolayers were inoculated with a multiplicity of 4 PFU/cell. After one hour of incubation at 37°C, excess inoculum was removed, monolayers washed thrice with EBSS and then received the maintenance medium with (10µg/ml) or without trypsin.

[b] At the specified intervals of time, representative cultures from each lot were taken out of the incubator, frozen (-20°C) and thawed three times. The material was centrifuged at 1,000 xg for 15 minutes and the supernatant was plaque assayed.

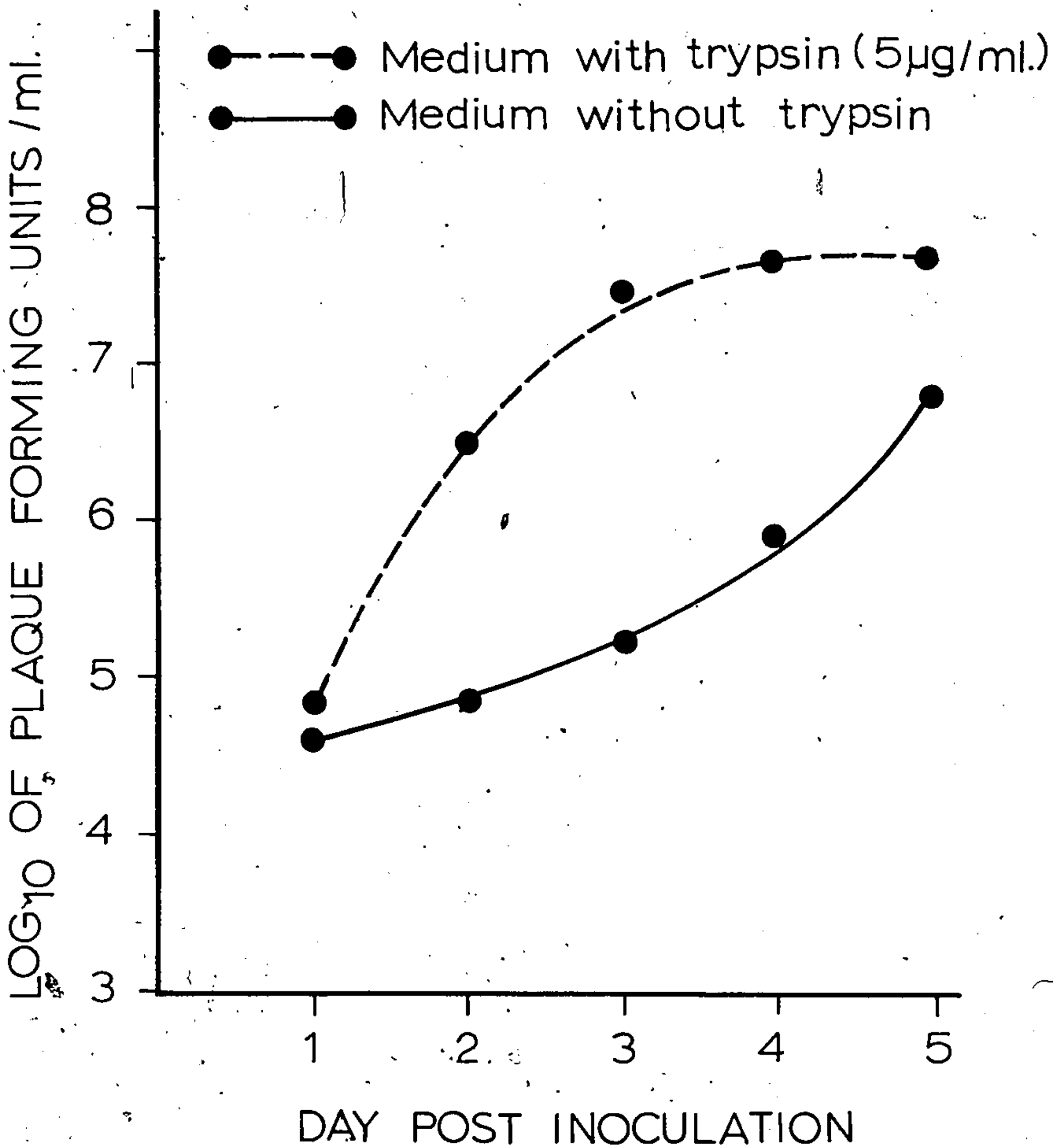
Multiple-Cycle Virus Replication in the Presence of Trypsin

Babiuk et al. (1977) and Almeida et al. (1978), using the immunofluorescent focus technique, found that presence of trypsin in the maintenance medium resulted in a faster and better spread of bovine rotavirus in the monolayer. It was, therefore, of interest to see if such spread in the presence of trypsin affected the rate of build-up and the final yield of infectious SA-11 particles during repeated cycles of virus replication in a given culture. The following experiments were designed to answer this question.

To each MA-104 monolayer, containing approximately 1.0×10^6 cells, an inoculum with 35.0 PFU of SA-11 was added. After a one-hour virus adsorption period at 37°C, each culture received 5.0 ml of the fluid maintenance medium either with or without trypsin. At appropriate intervals representative bottles were removed from the incubator and stored at -20°C. At the end of the experiment, the material from the stored cultures was processed and plaque assayed. As can be seen from the data presented in Figure 5, there was no noticeable difference in the virus titre in the trypsin-free and trypsin-containing cultures at the end of 24 hours of incubation. A significant difference in the titre was, however, noticed after 48 hours when the trypsin-containing cultures showed a titre of 3.0×10^6 PFU/ml reaching a maximum of 4.8×10^7 PFU/ml by the end of 120 hours; in contrast to this, the trypsin-free cultures had 7.2×10^4 PFU/ml after 48 hours and the maximum titre reached at 120 hours was 6.4×10^6. The rapid build-

Figure 5: Effect of trypsin on virus yield in multiple cycle replication of SA-11 in MA-104 cells.

EFFECT OF TRYPSIN ON VIRUS YIELD IN MULTIPLE CYCLE REPLICATION OF SA-11 IN MA-104 CELLS.



up of infectious virus after 24 hours in the presence of trypsin was strongly suggestive of better release and faster dissemination of the progeny virus in the culture during successive cycles of replication.

C. Applicability to Other Rotaviruses

The plaque assay for the simian rotavirus SA-11 (strain H-96) as described above was applied to the titration of the calf rotavirus (strain C-486). Large (3-4 mm diameter) and uniform plaques were obtained in MA-104 cells after 5 days of incubation at 37°C. Addition of DEAE-dextran (100μg/ml) to the overlay did not lead to any improvement in either the size or the number of plaques. This is in contrast to an earlier report (Matsuno et al., 1977) on NCDV (Lincoln strain) plaque formation in MA-104 cells.

DISCUSSION

The Need for Trypsin in the Overlay

The results of this study show that trypsin is essential for plaque formation by simian rotavirus SA-11 (strain H96) and calf rotavirus (strain C-486) in cultures of MA-104 cells. This is in contrast to an earlier report by Wyatt et al. (1978a) but in agreement with the observation of Matsuno et al. (1977) who reported that the Lincoln strain of NCDV could produce plaques in MA-104 cells only when trypsin was incorporated in the overlay. Also, the need for trypsin in the overlay for the formation of plaques by the SA-11 virus has recently been confirmed by Smith et al. (1979).

In our experiments with SA-11 and calf rotavirus, the need for DEAE-dextran in the overlay was not indicated. In fact, the presence of this substance in the overlay in amounts greater than 100μg/ml resulted in a slight reduction in the size and number of the plaques. Similar results were reported recently by Smith et al. (1979), but they found that DEAE-dextran at an optimal concentration of 100μg/ml of the overlay consistently gave better plaques. The observation of Matsuno et al. (1977) that the presence of DEAE-dextran in the overlay increased the number of NCDV in MA-104 cells, could not be confirmed in this study.

Incorporation of protamine sulfate in the trypsin-containing overlay did not alter either the number or the size of SA-11 plaques. This may have been due to the digestion of protamine sulfate by trypsin (Wallis and Melnick, 1968).

Addition of halogenated pyrimidines to virus-infected cell cultures has been shown to overcome interferon production (Holmes et al., 1964). In vitro potentiation of the replication of both DNA and RNA viruses have also been reported in the presence of halogenated pyrimidines (St. Jeor and Rapp, 1973; Paul et al., 1974; Green and Baron, 1975). Incorporation of BUdR in the overlay produced no noticeable alteration in the plaque forming ability of SA-11 in MA-104 cells.

The absence of SA-11 plaques in the presence of beef extract or yeast extract was not found to be due to non-specific virus inhibitors associated with them. Instead, these protein-rich substances appeared to interfere with the proteolytic activity of trypsin which was essential to virus plaque development.

A suitable virus diluent is among the factors considered necessary for optimal plaque production (Hamblet et al., 1967; Valle, 1971). Proteinaceous substances in virus diluents not only exert a protective effect on virus infectivity, but they have also been known to disaggregate virus clumps (Hamblet et al., 1967). Addition of protein-rich substances to the diluent (EBSS), however, was not found to alter either the number or size of SA-11 plaques.

The use of methyl cellulose in the overlay led to a total inhibition of SA-11 plaque formation. This inhibitory effect could not be reversed by an increase in the amount of trypsin in the overlay or the addition of cationic polymers such as DEAE-dextran or protamine sulfate.

Large plaques, 5-6 mm diameter, were formed by SA-11 in secondary monolayers of AGMK cells, but the virus failed to form plaques in BS-C-1, L-132 and secondary cultures of HEK. Similar findings have been reported recently by Estes et al. (1979a). Although the calf rotavirus (strain C-486) grows to a high titer in BS-C-1 cells, it also failed to form plaques in this cell line.

The results of this study also show that, apart from trypsin, certain other proteolytic enzyme preparations could assist SA-11 in its plaque formation in MA-104 cells. Among these were pancreatin, chymotrypsin, pronase, subtilisin and elastase. No plaques by SA-11, however, could be seen when papain, pepsin or thermolysin were incorporated into the overlay.

Pancreatin, prepared from the pancreas of the hog or the ox, is a mixture of enzymes containing mainly amylase, lipase and trypsin (Osol and Farrar, 1960). Similarly, pronase represents a mixture of alkaline proteinases, aminopeptidases and carboxypeptidases (Narabashi et al., 1968). Jurasek et al. (1969) have also isolated a trypsin-like enzyme from commercial preparations of pronase and showed this enzyme to resemble bovine trypsin in its catalytic mechanism as well as its mode of substrate binding. Therefore, absence of virus plaques upon the addition of trypsin inhibitor to pancreatin- or pronase-containing overlayers points to trypsin or a trypsin-like enzyme as the active ingredient in these mixtures. Soybean trypsin inhibitor is known to interfere with the activity of bovine chymotrypsin as well (Wu and Laskowski, 1955). This may explain the absence of virus plaques upon its addition to chymotrypsin-containing overlays. Soybean trypsin inhibitor, however, did not inhibit the formation of plaques when added to subtilisin- or elastase-containing overlays. It could be that the difference in the substrate binding sites of these enzymes compared to trypsin was responsible for the failure of trypsin inhibitor to affect their activity. It is known that soybean trypsin inhibitor does not inhibit at least the activity of elastase (Gertler and Birk, 1970).

It is interesting to note that all those enzymes which were found to facilitate SA-11 plaque formation in this study are serine proteases (Hartley, 1960). A number of such enzymes are present in the pancreatic secretions. This may explain why rotaviral infections are generally limited to the digestive tract (Theil et al., 1978).

The Role of Trypsin

The following conclusions could be drawn based on the results of this investigation on the rôle of trypsin in plaque production by rotavirus SA-11 in MA-104 cells: (a) initial trypsin treatment of the virus alone is not enough for it to produce plaques, (b) presence of trypsin during the initial phases of virus-cell interaction alone is also insufficient for plaque production, (c) plaque formation with trypsin in the overlay was not due to the enzymatic degradation of plaque-inhibitors in Ionagar #2, (d) centrifugation of the monolayers after virus inoculation followed with the use of trypsin-containing agar overlay gave nearly four times the number of plaques when compared to similar cultures subjected to stationary incubation, (e) presence of trypsin in the agar overlay throughout the five-day incubation period was essential for the production and optimal development of the plaques, (f) with a high multiplicity of infection leading to a single-cycle of virus growth, the presence of trypsin in the fluid maintenance medium appears to affect neither the rate of replication nor the final yield of infectious virus; on the other hand, during a multiple-cycle growth of the virus in a culture with trypsin-containing maintenance medium, the build-up of infectious virus is faster and the final yield higher when compared to that in a trypsin-free culture.

The findings, therefore, suggest that the continued presence of trypsin in the agar overlay facilitates the release and the subsequent spread of the progeny virus thereby assisting in the development of the plaques. A similar role for proteolytic enzymes during reovirus plaque production has been suggested (Wallis et al., 1966). However, the exact mechanism by which such enzymes assist virus release and spread in the culture remains to be elucidated.

Interferons can be inactivated by trypsin (Isaac et al., 1957), and this may lead to the enhanced spread of the virus in the presence of the enzyme (Babiuk et al., 1977; Almeida et al., 1978). It should be possible to test this if rotaviruses can be adapted to grow in cell lines which are non-interferon producing. The establishment of such a line from human embryonic lung tissue has recently been reported (Taguchi et al., 1979). As has been stated earlier, presence of halogenated pyrimidines in virus-infected cell cultures has been shown to reduce interferon production (Holmes et al., 1964). In this study, however, addition of BUdR to the overlay without trypsin did not lead to SA-11 plaque formation.

Enzymatic removal of the outer capsid of reovirus has been shown to result in the enhancement of their infectivity (Spendlove et al., 1970). But the studies of Bridger and Woode (1976) suggest that this phenomenon may not apply to rotaviruses because the absence of the outer capsid renders them non-infectious. Moreover, the preliminary EM examination of trypsin-treated rotaviruses in our laboratory did not show the removal of the outer capsid. This is in agreement with the earlier observations of Rodger et al. (1977).

If cleavage of virus polypeptides by trypsin was proven to be the true basis for the enhancement of infectivity of the virus (Barnett et al., 1979), the continued presence of trypsin in the overlay would still be necessary for the many cycles of virus entry and replication needed for the production of a single plaque.

Presence of trypsin has been reported to stimulate the division of cultured cells (Carney and Cunningham, 1977). From the observations made in this study, addition of trypsin in the agar overlay (which was always used without any serum), there was no evidence for the enhancement of cell growth.

Recently it has been shown (Brugmans et al., 1979) that a certain proportion of the trypsin used in the dispersion of monolayers is carried over by intracellular association with cells; in the absence of serum, such cells were shown to maintain proteolytic activity for a certain period. Such carry-over of trypsin may explain why in certain studies (Wyatt et al., 1978a) presence of the enzyme in the overlay was not found to be necessary for rotavirus plaque formation.

It has been reported that gentamicin may interfere with the activity of trypsin and certain other proteolytic enzymes (Asch and Farnham, 1979). We regularly employed gentamicin as an antibacterial agent in our cell culture media and agar overlays and failed to notice any apparent interference with the action of trypsin. Substitution of gentamicin with a mixture of penicillin and streptomycin made no difference to the plaque forming ability of SA-11 in the presence of trypsin.

PART II

Talc-Celite Layers and Polyethylene Glycol Hydroextraction in the Concentration of Entero-, Reo-, and Rotaviruses from Samples of Tap Waters

INTRODUCTION

In the last 15 years a great deal of effort has been put into the development of methods for recovering infectious viruses from the water environment (Hill et al., 1971; Shuval and Katzenelson, 1972; Sobsey, 1976; Gerba et al., 1978; Wallis et al., 1979). However, only a few of these reported techniques have been found suitable for working with large volumes of virus-polluted waters. These methods will be reviewed here with special emphasis on the ones that have shown promise in the concentration of viruses from tap water samples of greater than 100 L.

Use of Membrane and Cartridge Filters

The membrane filter adsorption-elution technique has received considerable attention and wide use in the concentration of viruses from various types of waters (Cliver, 1965, 1967c; Wallis et al., 1979). The technique involves two main steps; first the adsorption of the virus to the membrane filter surface, and second the removal of the membrane-adsorbed virus by elution with a small volume of a suitable fluid. The adsorption step is principally related to the unique surface properties of the virion and the chemical composition of the membrane itself. Although the exact mechanism of virus adsorption to such membrane surfaces is not yet clearly understood, Mix (1974) has suggested that it may be based on electrostatic forces between the virion and the membrane matrix.

Several factors have been found to influence the adsorption of viruses to membrane filters. Cliver (1965) observed that viruses adsorbed poorly to cellulose triacetate filters when the porosity exceeded the virion diameter by as much as 3 times, while viruses adsorbed efficiency to cellulose nitrate membrane filters even when the porosity exceeded the virion diameter by as much as 285 times. Cliver (1965) also noted that treatment of membranes with serum or gelatin interfered considerably with virus adsorption. It was later reported (Cliver, 1967) that enteroviruses could be concentrated 1,000-fold from 1-L volumes of dechlorinated tap water by adsorption to Millipore HA membrane filters and elution with phosphate-buffered saline containing 30% chicken serum.

Wallis and Melnick (1967a) showed that enteroviruses from crude cell culture harvests could be concentrated on Millipore cellulose nitrate membranes (0.45μm porosity). Also, they showed that virus adsorption to the membrane matrix was significantly enhanced when divalent salts ($MgCl_2$) were present in the virus suspending medium. Conversely, they noticed that the presence of organic and proteinaceous substances in the virus suspending medium interfered with virus adsorption to membrane filters. These interfering substances were referred to as membrane coating components (MCC) and they were shown to be removed by treating the sample first with an anion-resin. The Millipore cellulose nitrate membranes were subsequently applied by Wallis and Melnick (1967b) to the concentration of viruses from raw sewage. Their use was then extended to working with samples of raw and treated waters.

Wallis et al. (1972b, 1972c) described the development of a portable device to be used in the field for virus concentration from water. A 293 mm diameter (0.45μm porosity) cellulose nitrate membrane (Millipore HA) was used for virus adsorption. Presence of divalent cations and the adjustment of sample pH to 5.5 was found necessary for virus adsorption to the membrane. The adsorbed virus was eluted with 1 L of glycine buffer at pH 11.5. Using a smaller diameter membrane, the eluate could be further concentrated and from nearly 1,100 L of tap water 78% of added poliovirus 1 could be recovered. A further refinement of the system was reported by Homma et al. (1973) when the use of divalent cation (Mg^{++}) was replaced with the addition of smaller amounts of trivalent cations (Al^{+++}). Later the system was applied to isolating naturally occurring viruses from samples of waste- and seawaters (Homma et al., 1973; Metcalf et al., 1974).

The findings of Sobsey et al. (1973) that under acidic conditions viruses could be adsorbed to filters in the absence of added cations resulted in a modified version of the portable virus concentrator. Tap water was acidified to pH 3.5 and no cations were added. Using a fiber glass cartridge depth filter and a membrane filter (0.65μm porosity; Cox) in series, 378 L of tap water were processed with the recovery of 77% of added poliovirus 1.

Farrah et al. (1976a) tested a variety of filters for virus adsorption. All filters tested adsorbed greater than 90% of poliovirus 1 added to tap water at pH 3.5, but the Filterite fiberglass pleated filters (Duo-Fine series) were found to be the most resistant to clogging. Another advantage was that the Filterite filters could be regenerated after autoclaving or treatment with 0.1N NaOH without loss of virus recovering efficiency (Farrah et al., 1977a).

A serious limitation to the system when processing large volumes (over 378 L) of tap water was the adsorption of humic acid and other organics to the filters and their subsequent elution along with the adsorbed virus (Farrah et al., 1976b). These substances were found to interfere with the second-step concentration of the eluates (Sobsey et al., 1976) using the membrane adsorption-elution method. As a result, alternate methods of second-step concentration had to be developed (Farrah et al., 1977b).

Recently, Farrah et al. (1978a) reported the concentration of polioviruses 1 from tap water by entrapment of aluminum hydroxide flocs on pleated cartridge (Filterite) filters. In this technique lowering of the sample pH was found to be unnecessary. The floc generated with the addition of aluminum chloride to the sample was found to adsorb the added virus. Retention of the floc on the filter and its subsequent elution with glycine buffer (pH 11.0), followed by second-step concentration of the eluate, was reported to recover 70% of the added poliovirus.

Virus concentration systems similar to the ones developed by Wallis and Melnick were also reported by other investigators (Hill et al., 1972; Jakubowski et al., 1974; Hill et al., 1976; Jakubowski et al., 1978). Tube filters were used as the virus adsorbing material. In these systems also, lowering of sample pH to 3.5-4.0 and the addition of cations have been found to be necessary to enhance virus adsorption to the filters.

Kessick and Wagner (1978) have shown that most filters now used in the recovery of viruses from the water environment (e.g. Cox and Filterite filters) are negatively charged in the pH range of 2-7, with the least negative charge occurring at low pH values. Since most viruses are negatively charged near neutral pH (Briton and Lauffer, 1959) this might explain the need for sample acidification or the addition of cations for maximum virus recovery from the water environment when working with these filters.

Recently, Sobsey and Jones (1979) have shown that filters which are positively charged in the pH range of 5 to 9 could efficiently adsorb poliovirus from tap water without acidification of the sample or the addition of cations. If these positively charged filters are shown to be efficient in the concentration of other groups of enteric viruses and suitable for working with other types of waters, their use would offer definite advantages over the negatively charged filters.

Use of Particulate Materials

Concentration of viruses from water using a variety of particulate materials has also been described (Hill et al., 1971; Sobsey, 1976). Magnetic iron oxide (Rao et al., 1968), polyelectrolyte 60 (Wallis et al., 1970; Berg, 1971; Sattar and Westwood 1976b), Bentonite (Moore et al., 1974) and a variety of silicate minerals (Schaub et al, 1974; Lo and Sproul, 1977) are among such substances used.

The use of talc, a hydrous magnesium silicate ($H_2Mg_3 (SiO_3)_4$), in the concentration of viruses from the water environment was first reported by Patterson and Kalter (1973) and Kalter and Millstein (1974). These observations were confirmed and further extended by Westwood and Sattar (1974). It was also shown that the incorporation of Celite 503 (diatomaceous earth) with talc could overcome certain disadvantages in the use of talc alone (Sattar and Westwood, 1976a). Such talc-Celite layers were then applied to the recovery of viruses from samples of sewage, effluents and surface waters (Sattar and Westwood, 1976b, 1978; Sattar and Ramia, 1978). Because of the promise shown by this technique in these earlier studies, it was decided to see if its application could be extended to working with samples of tap waters as well.

EXPERIMENTAL AND RESULTS

A. Talc-Celite Layers in the Concentration of Laboratory-Adapted Strains of Enteric Viruses from Experimentally-contaminated One-litre Samples of Tap Waters

Poliovirus 1 (Sabin)

i) Effect of pH and Suspending Medium on Virus Adsorption to the Layers.

A number of techniques (Hill et al., 1971; Sobsey, 1976; Gerba et al., 1978; Wallis et al., 1979) for virus recovery from the water environment require a sample pH of 3.5 for efficient virus adsorption to the filtration matrix. This low pH level has been shown to be deleterious to a number of viruses expected to be present in sewage and sewage-polluted waters (Fields and Metcalf, 1975; Farrah et al., 1978b; Estes et al., 1979b; Sobsey and Jones, 1979). During earlier work in this laboratory, it was found that reduction of pH of sewage and surface water samples to 6.0 produced optimal virus adsorption to talc-Célite layers (Westwood and Sattar, 1974; Sattar and Westwood, 1976a). The following experiments were, therefore, conducted to see if the above observation would hold true when working with samples of tap water as well.

The pH of 1-L volume of tap water was adjusted to 6.0 and an equivalent volume of the same water was left at its original pH of 7.6. After experimental contamination with poliovirus 1 (Sabin), both of these samples were passed through 47 mm diameter talc-Celite layers. The filtrates were collected and plaque assayed. At the original sample pH nearly 94% of the input PFU passed through the layer unadsorbed, whereas, on acidification of the sample to pH 6.0 only about half of the added virus was lost in the filtrate.

Homma et al. (1973) and Farrah et al. (1976a) have shown that virus adsorption to membrane filters could be enhanced if acidic sample pH was combined with the addition of certain salts as sources of cations. In our experience, addition of aluminum chloride ($AlCl_3$) to pH (6.0)-adjusted water samples to a final concentration of either 5 x 10^{-4} M or 2 x 10^{-5} M (Farrah et al., 1978c) resulted in the formation of floccules. This led to a considerable decrease in the rate of flow of the samples through the layers.

The use of EBSS (Earle, 1943) and Dulbecco's phosphate buffered saline (PBS; Dulbecco's and Vogt, 1954), both of which contain a variety of salts, was tried in order to find a suitable substitute for $AlCl_3$. The result of these tests are given in Table 20. Presence of EBSS at a final concentration of 1:100 in a poliovirus-contaminated tap water sample at pH 6.0, resulted in the loss of only 6.6% of the input virus in the filtrate. Although PBS is similar to EBSS in its composition, the presence of PBS in the sample led to a loss of about 16.0% of the added virus in the filtrate.

TABLE 20

EFFECT OF SUSPENDING MEDIUM ON POLIOVIRUS 1 (SABIN) ADSORPTION TO TALC-CELITE LAYERS

		Earle's balanced salt solution (EBSS)		Dulbecco's phosphate buffered saline (PBS)	
Experiment no.	Input virus PFU/L	Total PFU in filtrate	% PFU in filtrate	Total PFU in filtrate	% PFU in filtrate
1	$9.0x10^4$	$0.1x10^4$	1.0	$1.3x10^4$	14.4
2	$6.0x10^4$	$0.6x10^4$	10.0	$0.9x10^4$	10.0
3	$3.4x10^4$	$0.3x10^4$	9.0	$0.8x10^4$	23.5
Mean	$6.13x10^4$	$0.3x10^4$	6.7	$1.0x10^4$	16.0
Standard deviation			±5.0		±7.0

After the adjustment of pH to 6.0 and the addition of either EBSS or PBS to give a final concentration of 1:100, 1-L volumes of experimentally-contaminated samples of tap water were passed through talc (300 mg)-Celite (100 mg) layers. Plaque assays on filtrates were carried out in monolayers of BS-C-1 cells.

Based on the results of these experiments, all subsequent testing of the talc-Celite technique was carried out after adjustment of sample pH to 6.0 and the addition of EBSS to a final concentration of 1:100.

ii) Effect of Prefilter AP25

The use of a prefilter is necessary if rapid clogging of the layers with particulate matter in the samples is to be avoided. In the experiments done above, a prefilter was used on the top of the layer. It was considered important to test if the presence of prefilters on the layers was in any way interfering with or contributing to the virus-adsorbing efficiency of the layers.

Poliovirus-contaminated samples of conditioned tap water were passed through the layers with and without prefilters (AP25). Filtrates were plaque assayed to determine the amount of unadsorbed virus. Table 21 shows the results of these experiments. In the presence of prefilters, only 3.4% of the input virus was lost in the filtrate, whereas in its absence virus loss was nearly ten times as much.

iii) Elution of Layer-Adsorbed Virus

After having established the conditions optimal for efficient virus adsorption to the talc-Celite layers, it was necessary to work out a suitable way for the recovery of the layer-adsorbed

TABLE 21

EFFECT OF AP25 PREFILTER ON THE POLIOVIRUS 1 (SABIN) ADSORBING EFFICIENCY OF TALC-CELITE LAYERS

Experiment no.	Input virus PFU/L	Layer without prefilter: Total PFU in filtrate	Layer without prefilter: % PFU in filtrate	Layer with prefilter: Total PFU in filtrate	Layer with prefilter: % PFU in filtrate
1	$8.0x10^4$	$3.2x10^4$	40.0	$0.36x10^4$	4.5
2	$8.0x10^4$	$3.0x10^4$	37.5	$0.40x10^4$	5.0
3	$4.0x10^4$	$1.3x10^4$	32.5	$0.16x10^4$	4.0
4	$2.0x10^4$	$0.6x10^4$	20.0	Undetectable	0.0
Mean	$5.5x10^4$	$2.0x10^4$	35.0	$0.23x10^4$	3.4
Standard deviation			±4.5		±2.0

After the adjustment of pH to 6.0 and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated samples of tap water were passed through talc (300 mg)-Celite (100 mg) layers with and without prefilters. Plaque assays on filtrates were performed in monolayers of BS-C-1 cells.

virus. Buffers at pH 11.5 were found to be efficient in the recovery of enteroviruses adsorbed to membrane and cartridge filters (Hill et al., 1971; Sobsey, 1976 ; Gerba et al., 1978; Wallis et al., 1979). However, certain strains of enteroviruses (Shaffer, 1977) and members of other enteric virus groups are rapidly inactivated at this pH level (Fields and Metcalf, 1975; Farrah et al., 1978b; Estes et al., 1979b; Sobsey and Jones, 1979). Elution with buffers at pH 10.5 gave maximum virus recovery only if the eluent was passed at least five times through the filter (Farrah et al., 1976a). Proteinaceous materials can interfere with the adsorption of virus particles to membrane filters presumably by competing with virus for adsorption sites (Wallis and Melnick, 1967a). Because of this, a variety of protein-containing solutions are also known to possess virus eluting properties (Konowalchuk and Speirs, 1971; Hill et al., 1974; Bitton, 1975; Katzenelzon et al., 1976b; Farrah et al., 1978b).

Earlier work in this laboratory had shown 3% BE solution and 10% FCS in saline to be efficient in the elution of sludge-adsorbed viruses (Sattar and Westwood, 1976c). The following experiments, therefore, were conducted to compare the efficiency of these two eluents with glycine buffer (pH 11.0) in the recovery of poliovirus 1 (Sabin) adsorbed to talc-Celite layers.

After passing a 1-L volume of conditioned and experimentally-contaminated tap water sample through the layers, virus elution was attempted with subsequent passage of 20 ml of the eluent under test. Eluates were plaque assayed to determine the amount of virus recovered. Results are summarized in Table 22. With 94.5% recovery of the input poliovirus, 10% FCS at pH 9.0 was found to be the best eluent. This pH level is not known to be deleterious to most enteric viruses. Moreover, the presence of protein in the suspending medium may exert a protective effect on virus infectivity.

Other Laboratory-Adapted Strains of Enteric Viruses

i) Virus Adsorption to the Layers

The technique was also tested for its efficiency in the recovery from tap water samples of other representative members of enterovirus (echo 6, coxsackie A9 and coxsackie B5), reovirus (reo 3, Abney strain) and the rotavirus (simian rota SA-11, strain H96; and calf rotavirus, strain C-486) groups.

A 1-L volume of conditioned tap water was contaminated with a known number of PFU of the virus under study. It was then passed through a 47 mm diameter talc-Celite layer. The results of these experiments,shown in Table 23,clearly indicate that at a sample pH of 6.0 and in the presence of EBSS at a final concentration of 1:100, the talc-Celite layers could efficiently adsorb members of at least three different enteric virus groups.

TABLE 22

COMPARISON OF FIVE ELUENTS IN THE RECOVERY OF POLIOVIRUS 1 (SABIN) ADSORBED TO TALC-CELITE LAYERS

Experiment no.	Input virus PFU/L	3% beef extract in distilled water (pH 8.0)		3% beef extract in distilled water (pH 9.0)		10% fetal calf serum in saline (pH 8.0)		10% fetal calf serum in saline (pH 9.0)		0.1 M glycine buffer (pH 11.0)	
		Total PFU in eluate	% recovery	Total PFU in eluate	% recovery	Total PFU in eluate	% recovery	Total PFU in eluate	% recovery	Total PFU in eluate	% recovery
1	$10.0x10^4$	$4.6x10^4$	46.0	$8.2x10^4$	82.0	$4.8x10^4$	48.0	$9.6x10^4$	96.0	$8.0x10^4$	80.0
2	$10.0x10^4$	$4.1x10^4$	41.0	$8.2x10^4$	82.0	$5.4x10^4$	54.0	$8.6x10^4$	86.0	$7.6x10^4$	76.0
3	$4.6x10^4$	$2.6x10^4$	56.5	$4.2x10^4$	91.0	$2.8x10^4$	61.0	$4.4x10^4$	96.0	$3.8x10^4$	83.0
4	$4.0x10^4$	$2.4x10^4$	60.0	$3.6x10^4$	90.0	$2.4x10^4$	60.0	$4.0x10^4$	100.0	$3.6x10^4$	90.0
Mean	$7.2x10^4$	$3.4x10^4$	50.9	$6.1x10^4$	86.3	$3.9x10^4$	55.8	$6.7x10^4$	94.5	$5.8x10^4$	82.3
Standard deviation			±9.0		±5.0		±6.0		±6.0		±6.0

After the adjustment of pH to 6.0 and the addition of EBSS (1:100), 1-L volumesof experimentally-contaminated samples of tap water were passed through talc (300 mg)-Celite (100 mg) layers. For elution of adsorbed virus a 20 ml volume of eluent under test was then passed through the layer. Virus plaque assays were performed in monolayers of BS-C-1 cells.

TABLE 23

EFFICIENCY OF ADSORPTION OF DIFFERENT LABORATORY-ADAPTED ENTERIC VIRUS STRAINS TO TALC-CELITE LAYERS

Virus	Input virus PFU/L	Total PFU in filtrate	% PFU lost in filtrate	Standard deviation
Echovirus 6	$4.5x10^4$	$0.25x10^4$	6.0	±4.95
Coxsackievirus B5	$5.0x10^4$	$0.33x10^4$	6.67	±2.81
Coxsackievirus A9	$1.6x10^4$	$0.16x10^4$	10.0	±2.50
Reovirus 3 (Abney)	$3.8x10^4$	$0.32x10^4$	8.68	±1.55
Simian Rotavirus SA-11 (H96)	$5.9x10^4$	$0.40x10^4$	6.8	±1.10
Calf Rotavirus (C-486)	$4.0x10^4$	$0.35x10^4$	8.65	±2.00

After adjustment of pH to 6.0 and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated sample was passed through a talc (300 mg)-Celite (100 gm) layers. To determine the amount of virus lost in the filtrate, plaque assays were performed in either BS-C-1 (entero and reo) or MA-104 (rota) cell monolayers. Results represent the mean values from four experiments for each virus tested.

ii) Virus Recovery

1. Protein Solutions

As can be seen from the data in Table 24, between 87 and 92% of the input PFU of echovirus 6, coxsackievirus B5, coxsackievirus A9 and reovirus 3 (Abney) could be recovered from 1-L samples of tap water. Because of its high eluting efficiency, 10% FCS was used in the rest of the study for the recovery of entero- and reovirus adsorbed to the talc-Celite layers. However, later on it was recognized that the use of this eluent could not be extended to working with rotaviruses. This was due to the presence of rotavirus inhibitory activity in certain lots of commercial fetal calf serum (Clark et al., 1979; Estes et al., 1979a;Schlafer et al., 1979). A suitable eluent for working with this virus group was, therefore, sought.

The results summarized in Table 25 show that the mean recoveries of the adsorbed simian rotavirus SA-11 with BE, CH, NB and TPB were 90, 79, 82 and 93% respectively. In the first two experiments, LAH gave virus recoveries of 62.5 and 66.0%. Because these recoveries were consistently lower than those obtained with the other eluents, LAH was eliminated from subsequent experimentation.

TABLE 24

RECOVERY OF DIFFERENT LABORATORY-ADAPTED ENTERIC VIRUS STRAINS ADSORBED TO TALC-CELITE LAYERS

Virus	Input virus PFU/L	Total PFU recovered	% recovery	Standard deviation
Echovirus 6	4.5×10^4	4.12×10^4	92.0	±4.27
Coxsackievirus B5	5.0×10^4	4.60×10^4	92.0	±4.0
Coxsackievirus A9	1.6×10^4	1.40×10^4	87.0	±6.28
Reovirus 3 (Abney)	3.8×10^4	3.50×10^4	90.0	±2.87

After adjustment of the sample pH to 6.0, and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated tap water samples were passed through layers of talc (300 mg)-Celite (100 mg). Layer-adsorbed virus was eluted with 20 ml of 10% FCS in saline (pH 9.0). The eluate was plaque assayed in monolayers of BS-C-1 cells. Results represent the mean value from four experiments for each virus tested.

TABLE 25

COMPARISON OF DIFFERENT ELUENTS IN THE RECOVERY OF ROTAVIRUS SA-11 ADSORBED TO TALC-CELITE LAYERS

Expt. no.	Input virus PFU/L	3% Beef extract		3% Casein hydrolysate		3% Lactalbumin hydrolysate		Nutrient broth		Tryptose-phosphate broth	
		Total PFU in eluate	% Recovery	Total PFU in eluate	% Recovery	Total PFU in eluate	% Recovery	Total PFU in eluate	% Recovery	Total PFU in eluate	% Recovery
1	$8.0x10^4$	$7.4x10^4$	92.5	$6.0x10^4$	75.0	$5.0x10^4$	62.5	$7.0x10^4$	87.5	$7.4x10^4$	92.5
2	$7.6x10^4$	$7.2x10^4$	95.0	$6.6x10^4$	87.0	$5.0x10^4$	66.0	$7.0x10^4$	79.0	$7.3x10^4$	96.0
3	$3.8x10^4$	$3.2x10^4$	84.0	$3.0x10^4$	79.0	N.D.	-	$3.0x10^4$	79.0	$3.4x10^4$	89.0
4	$3.8x10^4$	$3.4x10^4$	89.0	$2.8x10^4$	74.0	N.D.	-	$3.2x10^4$	84.0	$3.6x10^4$	95.0
Mean	$5.8x10^4$	$5.3x10^4$	90.0	$4.6x10^4$	79.0	$5.0x10^4$	64.3	$5.05x10^4$	82.3	$5.40x10^4$	93.0
Standard deviation			±4.76		±5.91		±2.47		±4.15		±3.12

After the adjustment of pH to 6.0, and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated samples of tap water were passed through talc (300 mg)-Celite (100 mg) layers. For elution of adsorbed virus a 10 ml volume of eluent under test was then passed through the layer. All eluents were prepared in deionized water and their pH adjusted to 9.0. Virus plaque assays were performed in monolayers of MA-104 cells.

2. TPB as a Universal Eluent

The eluting efficiency of TPB in the recovery of layer-adsorbed SA-11 virus prompted us to test this eluent in the recovery of layer-adsorbed polio and reovirus. The results in Table 26 show that rehydrated TPB could be used as an efficient alternative to FCS and BE in the recovery of layer-adsorbed viruses.

3. Single Amino Acid Solutions

It has been reported (Farrah and Bitton, 1978) that certain solutions containing single amino acids could be used in the elution of poliovirus adsorbed to membrane filters. Due to the obvious disadvantages involved in the use of more complex protein solutions, the efficiency of a number of single amino acid solutions in the elution of layer-adsorbed viruses was tested. As is evident from Table 27, the basic amino acids arginine (83%) and glycine (80%) were superior to the acidic amino acids glutamine (40%) and asparagine (29%) in their rotavirus SA-11 eluting efficiency. This is in agreement with the earlier observations of Farrah and Bitton (1978) on poliovirus elution. In our own experience (Table 27), poliovirus 1 recoveries with arginine and glycine were 82 and 80%, respectively.

That the low virus recovery with an acidic amino acid was due to virus inactivation was ruled out in

TABLE 26

COMPARISON OF 10% FETAL CALF SERUM (FCS) IN SALINE AND TRYPTOSE PHOSPHATE BROTH (TPB) IN THE RECOVERY OF POLIOVIRUS 1 (SABIN) AND REOVIRUS 3 (ABNEY) ADSORBED TO TALC-CELITE LAYERS

		10% FCS in saline (pH 9.0)			TPB (pH 9.0)		
Virus	Input virus PFU/L	Total PFU in eluate	% recovery	Standard deviation	Total PFU in eluate	% recovery	Standard deviation
Poliovirus 1 (Sabin)	$6.15x10^4$	$5.57x10^4$	90.75	±2.63	$5.65x10^4$	92.75	±1.41
Reovirus 3 (Abney)	$4.0x10^4$	$3.67x10^4$	92.75	±3.46	$3.55x10^4$	88.0	±4.33

After adjustment of the sample pH to 6.0, and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated samples of tap water were passed through talc (300 mg)-Celite (100 mg) layers. For elution of adsorbed virus, a 10 ml volume of eluent under test was then passed through the layer. Virus plaque assays were performed in monolayers of BS-C-1 cells. Results represent the mean values from four experiments for each virus.

TABLE 27

COMPARISON OF DIFFERENT SINGLE AMINO ACID SOLUTIONS IN THE RECOVERY OF POLIOVIRUS 1 (SABIN) AND ROTAVIRUS SA-11 (H96) ADSORBED TO TALC-CELITE LAYERS

Virus	Input virus PFU/L	Eluting agent (pH 9.0)	Total PFU in eluate	% recovery	Standard deviation
Rotavirus SA-11 (H96)	7.90×10^4	3% Arginine	6.5×10^4	83	±1.61
		3% Glycine	6.3×10^4	80	±2.02
		3% Glutamine	3.1×10^4	40	±1.53
		3% Asparagine	2.3×10^4	29	±1.90
Poliovirus 1 (Sabin)	6.15×10^4	3% Arginine	5.0×10^4	82	±2.66
		3% Glycine	4.9×10^4	80	±1.0

After adjustment of the sample to 6.0 and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated samples of tap water were passed through layers of talc (300 mg)-Celite (100 mg). Layer-adsorbed virus was eluted with 10 ml of the eluent under study. Eluates were plaque assayed in either BS-C-1 (polio) or MA-104 (SA-11) cell monolayers. Results represent the mean values of three experiments with SA-11 virus and four experiments with poliovirus 1 (Sabin).

the following experiment: rotavirus SA-11 adsorbed to the talc-Celite layer was first eluted with a solution of glutamine followed by a subsequent elution with TPB. As shown in Table 28,glutamine solution was able to recover not more than 40% of the input PFU, whereas the treatment of the same layer with TPB eluted an additional 54% of the added virus. This indicated that the amino acid was not inactivating the virus, but leaving most of it still adsorbed to the layers. A similar phenomenon was observed upon the extension of the use of acidic amino acid solution to the elution of layer-adsorbed poliovirus 1 (Table 28).

4. Use of Antifoam C

The use of FCS, BE or TPB resulted in excessive frothing during the process of elution of the layer-adsorbed virus. In order to minimize frothing and possible virus inactivation in the eluate, the use of Antifoam C was tested. When Antifoam C (Sigma) was added to the eluent to give a final concentration of 1:1000, formation of froth was suppressed to a large degree. The use of this substance was not found to have any adverse effect on the virus eluting efficiency of FCS, BE or TPB. Also, its addition to the virus diluent did not interfere with virus adsorption to cell monolayers.

TABLE 28

SEQUENTIAL ELUTION OF POLIOVIRUS 1 (SABIN) AND ROTAVIRUS SA-11 (H96) ADSORBED TO TALC-CELITE LAYERS

Virus	Input virus PFU/L	Eluting agent (pH 9.0)	Total PFU in eluate	% recovery	Standard deviation
Rotavirus SA-11 (H96)	$6.5x10^4$	3% Glutamine	$2.60x10^4$	40.0	±1.53
		Tryptose phosphate broth (TPB)	$3.50x10^4$	54.0	±3.25
Poliovirus 1 (Sabin)	$4.4x10^4$	3% Glutamine	$2.0x10^4$	46.0	±2.71
		TPB	$2.13x10^4$	49.0	±2.63

After adjustment of the sample pH to 6.0 and the addition of EBSS (1:100), 1-L volumes of experimentally-contaminated samples of tap water were passed through layer of talc (300 mg)-Celite (100 mg). Layer-adsorbed virus was first eluted with 10 ml of 3% glutamine (pH 9.0) and then the layer was again eluted with 10 ml of TPB (pH 9.0). Eluates were plaque assayed in either BS-C-1 (polio) or MA-104 (SA-11) cell monolayers. Results represent the mean values for three experiments with SA-11 virus and four experiments with poliovirus 1.

Testing of a Variety of Filters in the Concentration of Seeded Poliovirus 1 (Sabin) from Samples of Tap Water

The adjustment of sample pH to 6.0 and the addition of EBSS were shown to work efficiently in the concentration of viruses using talc-Celite layers. The purpose of the following experiments was to test if such sample conditioning could be extended to working with some of the other techniques described for virus concentration from the water environment.

The results presented in Table 29 show that at sample pH 6.0 and in the presence of EBSS (1:100), about 71% and 81% of the input virus was detectable in the filtrate with the Cox and Filterite membrane filters respectively. Also, about 75% of the input virus was detectable in the filtrate at sample pH of 6.0 when using the Balston filter (Jakubowski et al., 1974).

With Zeta Plus (50S) filter (Sobsey and Jones, 1979) about 25% of the input virus was present in the filtrate if the tap water sample was processed at its original pH of 7.6. At pH 6.0, however, nearly 8% of the input virus was detected in the filtrate without the addition of cations.

TABLE 29

EFFECT OF TAP WATER pH AND PRESENCE OF EARLE'S BALANCED SALT SOLUTION (EBSS) ON THE ADSORPTION OF POLIOVIRUS 1 (SABIN) TO A VARIETY OF FILTERS

Filter	Sample volume (L)	Input virus PFU/sample	Sample pH	EBSS[a]	Total PFU in filtrate	% virus PFU lost in filtrate	Standard deviation
Cox	1.0	3.9×10^5	6.0	+	2.9×10^5	71.0	±5.77
Filterite	1.0	4.5×10^5	6.0	+	3.6×10^5	81.0	±10.4
Zeta Plus (50S)	1.0	1.2×10^5	7.6	-	0.3×10^5	25.0	±2.5
			6.0	-	0.1×10^5	8.0	±1.5
Balston	20.0	4.5×10^5	6.0	+	3.3×10^5	75.0	±4.62

To determine the amount of virus lost in the filtrate, plaque assays were performed in monolayers BS-C-1 cells. Results represent the mean values from four experiments.

[a] Earle's balanced salt solution added to sample to a final concentration of 1:100.

B. Second-Step Concentration of Eluates

Adsorption-elution techniques used for virus-concentration from the water environment require between 100 and 2,000 ml of a suitable eluent for the recovery of matrix-adsorbed virus. A further 10- to 200-fold reduction in the volume of the eluate becomes necessary before its inoculation into cell cultures. A number of techniques have been reported for the second-step concentration of eluates. These include two-phase separation (Shuval et al., 1967, 1969), use of smaller diameter membrane filters (Wallis et al., 1972b, 1972c) PEG hydroextraction (Cliver, 1967a; Wellings et al., 1975), organic flocculation (Katzenelson et al., 1976b), and aluminum hydroxide flocculation followed by hydroextraction (Farrah et al., 1977b). Of these techniques organic flocculation and PEG hydroextraction appeared to be suitable for testing.

Organic Flocculation

In the initial experiments, working with 100 ml of 10% FCS in saline, we noticed that when the pH of the virus-contaminated sample (pH 9.0) was brought down to 3.5, the suspension turned slightly cloudy without the generation of floccules that could be sedimented by centrifugation. Since 3% BE solution could be used as a substitute for 10% FCS solution in virus elution, the technique was applied to experimentally-contaminated samples of 3% BE in deionized water (pH 9.0).

As can be seen from the data presented in Table 30, 66% of the input virus could be recovered in the precipitate; the supernatant was found to contain nearly 27% of the added virus.

Hydroextraction with Polyethylene Glycol (PEG)

i) Four-Hour Hydroextraction

A 100 ml volume of poliovirus 1 (Sabin)-contaminated 10% FCS in saline was placed in a dialysis sac and hydro-extracted with either PEG 6,000 or PEG 20,000 for 4 hours at 4°C. The volume of the material remaining in the sacs was carefully measured before plaque assay. Results of these experiments are summarized in Tables 31 (PEG 6,000) and 32 (PEG 20,000).

On an average, with PEG 6,000, 91.5% of the input poliovirus PFU could be recovered with a 5.5-fold concentration of the eluent; whereas PEG 20,000 gave an 87.0% virus recovery with 4.0-fold sample concentration.

PEG 20,000 is more expensive than PEG 6,000. Because it did not offer any significant advantages over PEG 6,000, all subsequent experiments were carried out with the low molecular weight PEG.

TABLE 30

ORGANIC FLOCCULATION IN THE CONCENTRATION OF POLIOVIRUS 1 (SABIN) FROM 3% BEEF EXTRACT IN DEIONIZED WATER

Experiment no.	Input virus PFU/100 ml	PFU in supernatant	% PFU in supernatant	PFU in precipitate	% PFU in precipitate
1	$8.0x10^4$	$2.0x10^4$	25.0	$5.8x10^4$	72.5
2	$9.0x10^4$	$2.4x10^4$	27.0	$6.4x10^4$	71.0
3	$8.8x10^4$	$2.6x10^4$	30.0	$4.8x10^4$	54.0
4	$0.8x10^4$	$0.3x10^4$	32.5	$0.5x10^4$	62.0
5	$0.8x10^4$	$0.2x10^4$	27.5	$0.5x10^4$	62.0
6	$0.7x10^4$	$0.2x10^4$	31.5	$0.4x10^4$	63.0
Mean	$4.7x10^4$	$1.3x10^4$	27.0	$3.0x10^4$	66.0
Standard deviation					±7.0

The pH of 100 ml of experimentally-contaminated sample was brought to 3.5. The resulting precipitate was sedimented by 10 minutes' centrifugation at 3,000 xg. The sediment was redissolved in 10 ml of phosphate buffer. Plaque assays were carried out in BS-C-1 cells.

TABLE 31

FOUR-HOUR POLYETHYLENE GLYCOL 6,000 HYDROEXTRACTION IN THE CONCENTRATION OF POLIOVIRUS 1 (SABIN)

Experiment no.	Input virus PFU/100 ml	Volume of concentrate (ml)	Concentration factor	Total PFU in concentrate	% recovery
1	1.36×10^4	33	3X	1.35×10^4	99.0
2	1.80×10^4	17	6X	1.50×10^4	83.0
3	1.80×10^4	11	9X	1.53×10^4	85.0
4	0.48×10^4	25	4X	0.39×10^4	82.0
5	0.24×10^4	20	5X	0.24×10^4	100.0
6	0.16×10^4	16	6X	0.16×10^4	100.0
Mean	0.97×10^4	20.3	5.5X	0.86×10^4	91.5
Standard deviation					±9.0

A 100 ml volume of 10% fetal calf serum in saline (pH 9.0) was contaminated with a known amount of the poliovirus. It was placed in a dialysis bag and hydroextracted for four hours at 4°C. The volume of the material remaining in the bag was carefully measured and it was plaque assayed in BS-C-1 cells.

TABLE 32

FOUR-HOUR POLYETHYLENE GLYCOL 20,000 HYDROEXTRACTION IN THE CONCENTRATION OF POLIOVIRUS 1 (SABIN)

Experiment no.	Input virus PFU/100 ml	Volume of concentrate (ml)	Concentration factor	Total PFU in concentrate	% recovery
1	1.40×10^4	24	4X	1.18×10^4	84.0
2	1.48×10^4	18	5X	1.27×10^4	86.0
3	0.24×10^4	32	3X	0.20×10^4	83.0
4	0.24×10^4	34	3X	0.23×10^4	96.0
5	0.17×10^4	25	4X	0.15×10^4	88.0
6	0.17×10^4	20	5X	0.144×10^4	85.0
Mean	0.62×10^4	25.5	4X	0.52×10^4	87.0
Standard deviation					±5.0

A 100 ml volume of 10% fetal calf serum in saline (pH 9.0) was contaminated with a known amount of the poliovirus. It was then placed in a dialysis bag and hydroextracted for four hours at 4°C. The volume of the material remaining in the bag was carefully measured and it was plaque assayed in BS-C-1 cells.

ii) Overnight Hydroextraction

Although eluent concentration with PEG hydroextraction for four hours gave efficient virus recoveries, there was considerable variation in the reduction of sample volume (Tables 31, 32). To overcome this, overnight (16-19 hours) hydroextraction (4°C) with PEG 6,000 was attempted. This resulted in the removal of nearly all the liquid from inside the sac. The brown viscous material remaining inside the sac was resuspended in 10 ml of EBSS and plaque-assayed. The results of experiments with four laboratory-adapted strains of enteroviruses (polio 1, echo 6, coxsackie A9 and coxsackie B5) and one reovirus (reo 3) are presented in Table 33.

Additional experiments were performed to test the efficiency of this technique in the concentration of polio-, reo-, and rotaviruses suspended in either 3% BE or TPB. These results are also included in Table 33. With a 10-fold concentration, recovery of input PFU for the tested viruses ranged between 87.0 and 97.0%.

iii) Overnight Hydroextraction of Experimentally-Contaminated Samples of Eluates

Field samples of raw and finished waters contain a variety of chemicals, some of which are retained by the filtration matrix during sample processing (Farrah et al., 1976b). Passage of eluents through the matrix for virus elution also results in the release and accumulation of these chemicals in the eluate. Concentration of eluates containing such chemicals could make it either virus inhibitory or cytotoxic.

TABLE 33

OVERNIGHT POLYETHYLENE GLYCOL 6,000 HYDROEXTRACTION IN THE CONCENTRATION OF LABORATORY-ADAPTED ENTERIC VIRUS STRAINS

Virus	Eluting agent	No. of experiments	Input virus PFU/100 ml	Total PFU in concentrate (10 ml)	% recovery	Standard deviation
Poliovirus 1 (Sabin)	10% FCS	6	1.20×10^4	1.00×10^4	90.0	±8
Echovirus 6	10% FCS	6	2.20×10^4	2.00×10^4	92.0	±7
Coxsackievirus A9	10% FCS	4	0.28×10^4	0.24×10^4	88.0	±4
Coxsackievirus B5	10% FCS	6	0.33×10^4	0.29×10^4	87.0	±10
Reovirus 3 (Abney)	10% FCS	8	0.42×10^4	0.40×10^4	97.0	±12
Rotavirus SA-11 (H96)	3% BE	4	0.22×10^4	0.21×10^4	94.0	±1.9
	TPB	4	0.22×10^4	0.22×10^4	96.0	±2.9
	10% FCS	2	0.22×10^4	0	0	-
Calf Rotavirus	TPB	4	0.39×10^4	0.37×10^4	95.0	±5.2
	10% FCS	2	0.39×10^4	0	0	-

A 100ml volume of either 10% FCS in saline (pH 9.0), 3% BE in deionized water (pH 9.0) or TPB (pH 9.0) was experimentally-contaminated with the virus. Hydroextraction with PEG was carried out overnight at 4°C. The material remaining in the dialysis sac was resuspended in 10 ml of EBSS and plaque assayed in either BS-C-1 (entero- and reovirus) or MA-104 (rotaviruses) cell monolayers. Figures represent mean values for the number of experiments indicated.

The experiments conducted up to this stage involved experimentally-contaminated samples of the eluent. However, it was considered necessary to demonstrate that the technique was also efficient in virus recovery from experimentally-contaminated samples of eluates.

Field samples of raw or finished waters were processed using talc-Celite layers as has been described earlier. A 100 ml volume of 10% FCS in saline (pH 9.0) was then passed through the layer and collected as the eluate. A known amount of the virus under test was added to it before overnight hydroextraction with PEG 6,000. The concentrate remaining in the dialysis sac was resuspended in 10 ml of EBSS and plaque-assayed. Results of these experiments with two laboratory-adapted (Sabin polio 1, and echo 6) and five field isolates of enteric viruses (one vaccine polio 1, one non-vaccine polio 1, echo 1, coxsackie B3 and reo) are presented in Table 34.

As can be seen from these data, virus recovery from the concentrated eluate compared favorably with the results of the experiments with the eluent; between 87 and 98% of the input PFU of the seven enteric virus strains could be recovered with a 10-fold concentration of the eluate.

TABLE 34

OVERNIGHT POLYETHYLENE GLYCOL 6,000 HYDROEXTRACTION IN THE CONCENTRATION OF ENTERIC VIRUSES FROM EXPERIMENTALLY-CONTAMINATED SAMPLES OF ELUATES

Virus	No. of experiments	Type of sample passed through the layers	Input virus PFU in 100 ml of eluate	Total PFU in concentrate (10 ml)	% recovery	Standard deviation
Lab.-adapted strains						
polio 1 (Sabin)	3	purified water	$1.40x10^4$	$1.27x10^4$	91.0	±8.0
	3	raw water	$1.40x10^4$	$1.33x10^4$	95.0	±4.0
echo 6	3	purified water	$1.20x10^4$	$1.13x10^4$	94.0	±10.0
	3	raw water	$1.20x10^4$	$1.10x10^4$	93.0	±17.0
Field isolates						
polio 1 (vaccine strain)	6	raw water	$1.70x10^4$	$1.55x10^4$	93.0	±6.0
polio 1 (non-vaccine strain)	6	raw water	$0.76x10^4$	$0.67x10^4$	91.5	±8.0
echo 1	4	raw water	$0.48x10^4$	$0.42x10^4$	87.0	±7.0
coxsackie B3	6	raw water	$2.20x10^4$	$2.10x10^4$	96.0	±8.0
reo	4	raw water	$0.25x10^4$	$0.24x10^4$	98.0	±7.0

Samples of purified (500 litres) or raw waters (40 litres) from the Ottawa River were passed through the talc-Celite layers. The layers were then eluted with 100 ml of 10% fetal calf serum in saline (pH 9.0). A known amount of virus under test was added to the eluate and it was subjected to hydroextraction. The material remaining in the dialysis sac was resuspended in 10 ml of EBSS and plaque assayed in BS-C-1 cells. Figures represent mean values for the number of experiments indicated.

C. Talc-Celite Layers in the Concentration of Laboratory-Adapted Strains of Entero-, Reo-, and Rotaviruses from Large Volumes of Tap Water

Effect of Sample Volume and Input PFU on Poliovirus 1 (Sabin) Recovering Efficiency of the Layers

The initial experiments to establish the potential of the technique were conducted using 1-L sample volumes with fairly high levels of virus contamination. In the field situation, however, the technique would be expected to process much larger sample volumes of finished waters which may contain very low virus levels. Therefore, it was essential to test the virus-recovering efficiency of the technique in an experimental set-up which would closely resemble the field situation. To achieve this, 142 mm diameter layers were used for the processing of tap water samples ranging in volume from 10 to 1,000 L and containing from a high input of 4.0×10^4 to a low input of 1.2 poliovirus PFU/L. Virus elution was carried out by the subsequent passage of 100 ml of the eluting agent (10% FCS in saline, pH 9.0) through the layers. A 10-fold reduction in the volume of the eluate was achieved by overnight PEG hydroextraction. The results of these experiments are presented in Table 35.

There was approximately a 23% reduction in the virus recovering efficiency of the technique when the sample volume was increased from 20

TABLE 35

RELATIONSHIP OF TAP WATER SAMPLE SIZE AND POLIOVIRUS 1 (SABIN) INPUT DOSE TO THE VIRUS RECOVERING EFFICIENCY OF TALC-CELITE LAYERS

Sample size	Virus input PFU/sample	Total/PFU in final concentrate (20 ml)	% recovery	Standard deviation
10 L	$3.95x10^5$	$3.35x10^5$	86	±4.75
20 L	$6.10x10^5$	$5.23x10^5$	86	±9.10
20 L	$4.50x10^3$	$3.75x10^3$	83	±5.0
100 L	$2.10x10^5$	$1.40x10^5$	61	±2.31
100 L	$1.20x10^2$	$0.81x10^2$	58	±2.38
1,000 L	$3.0x10^5$	$1.83x10^5$	60	±4.55
1,000 L	$3.40x10^3$	$2.20x10^3$	64	±5.73

After adjustment of pH to 6.0 and the addition of EBSS (1:100), experimentally-contaminated samples were passed through 142 mm diameter layers of talc (1.2 gm)-Celite (0.4 gm). 100 ml of 10% FCS in saline (pH 9.0) was then passed to elute the virus. The eluate was subjected to overnight hydroextraction with PEG 6,000 at 4°C. Final concentrate was subjected to plaque assay in BS-C-1 cells. Results represent an average of four experiments at each sample volume and virus dose.

to 100 L. However, a further 10-fold increase in the sample volume did not result in any additional drop in its efficiency. In other words, when 1,000-L volumes of poliovirus 1 (Sabin)-contaminated samples were passed through the layers, 60-64% of the input PFU could be recovered. These results also indicate that in experiments with 1,000-L volumes, the virus-recovering efficiency of this technique remained the same when the amount of input virus in the sample ranged from as high as 300 PFU/L to as low as 1.2 PFU/L (Figure 6).

Recovery of Other Laboratory-Adapted Strains of Enteric Viruses

The efficiency of the technique was also tested in the recovery of other laboratory-adapted strains of enteric viruses from large volumes of tap water. Either 20 or 100-L volumes of conditioned and experimentally-contaminated water samples were passed through the large layers of talc-Celite (142 mm diameter). 10% FCS in saline (pH 9.0) was the eluting agent in the recovery of the entero- and reoviruses tested, while TPB (pH 9.0) was the eluting agent in the recovery of SA-11 virus. A 10-fold reduction in the volume of the eluate was achieved by overnight PEG hydroextraction. The results are presented in Table 36.

When 20 L samples were tested, 81% of echovirus 6, 89% of coxsackievirus B5, 90% of reovirus 3 (Abney), and 81-85% of SA-11 virus could be recovered. In the concentration of 100 liter samples the virus recovery was 62-64% for reovirus 3 and 59-61% for SA-11 virus.

Figure 6: Relationship of tap water sample size and poliovirus (type 1, Sabin) input dose to the virus recovering efficiency of talc-Celite (1.2 gm/0.4 gm) layers.

RELATIONSHIP OF TAP WATER SAMPLE SIZE AND POLIOVIRUS (TYPE I, SABIN) INPUT DOSE TO THE VIRUS RECOVERING EFFICIENCY OF TALC-CELITE LAYERS.

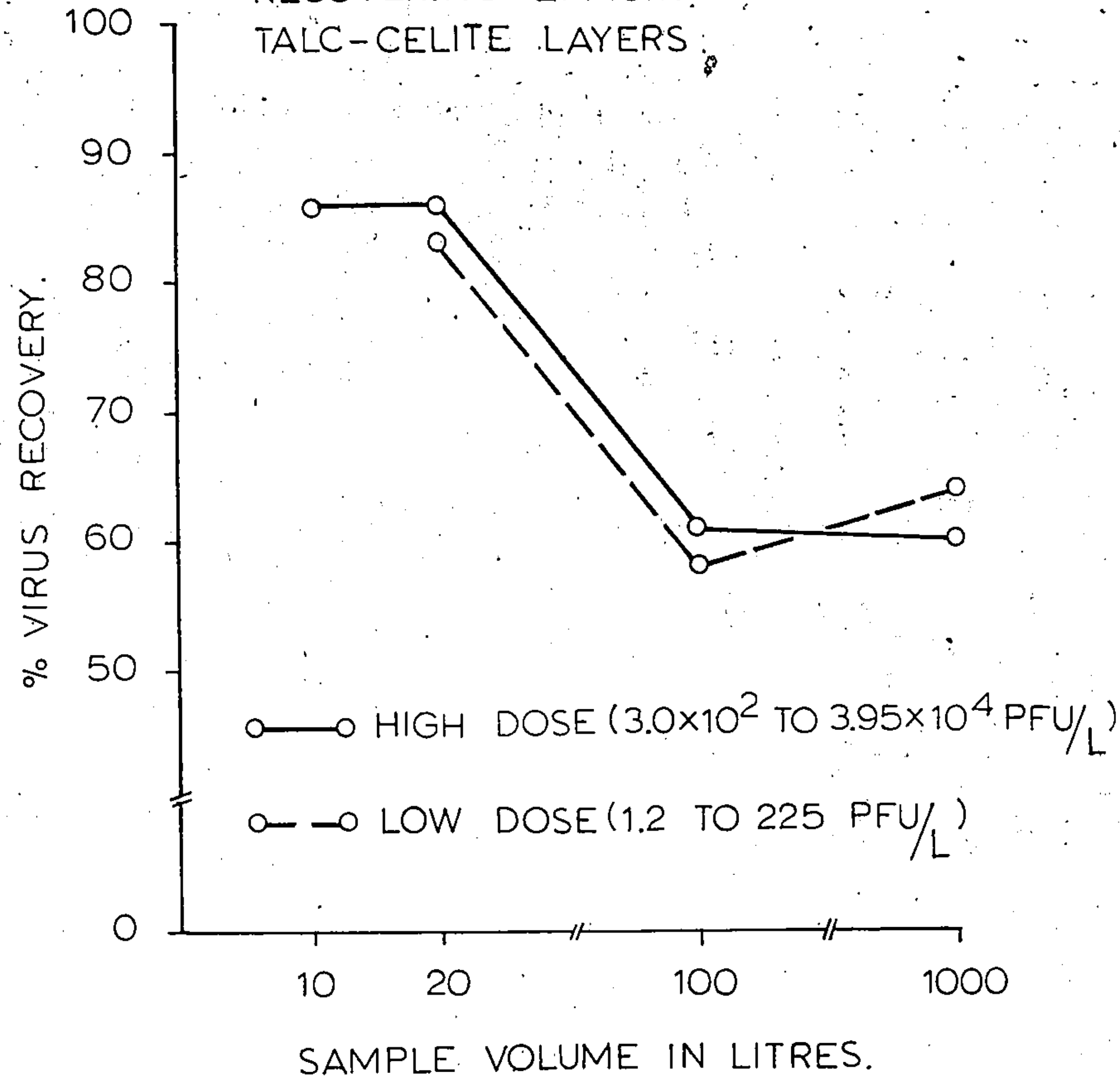


TABLE 36

TALC-CELITE LAYERS IN THE RECOVERY OF LABORATORY-ADAPTED ENTERIC VIRUS STRAINS FROM 20-100 L VOLUMES OF TAP WATERS

Virus	Sample volume (L)	Input PFU per sample	Total PFU recovered	% recovery	Standard deviation
Echovirus 6	20.0	$6.80x10^5$	$5.50x10^5$	81.0	±4.57
Coxsackievirus B5	20.0	$1.80x10^5$	$1.60x10^5$	89.0	±4.43
Reovirus 3 (Abney)	20.0	$3.70x10^5$	$3.40x10^5$	90.0	±5.23
	100.0	$7.50x10^5$	$4.80x10^5$	64.0	±1.83
	100.0	$0.75x10^2$	$4.70x10^2$	62.0	±3.85
Rotavirus SA-11 (H96)	20.0	$5.60x10^5$	$4.70x10^5$	85.0	±2.31
	~~100.0~~	$6.80x10^5$	$4.70x10^5$	71.0	±5.13
	100.0	$1.36x10^2$	$0.68x10^2$	59.0	±3.0

After adjustment of the sample pH to 6.0 and the addition of EBSS (1:100), an appropriate volume of the experimentally-contaminated sample was passed through a 142 mm diameter of talc (1.2 g)-Celite (0.4 g) layer. For echo 6, coxsackie B5 and reo 3 the virus was eluted with 100 ml of 10% FCS in saline (pH 9.0). SA-11 virus was eluted with 100 ml of TPB (pH 9.0). The eluate was hydroextracted with PEG and the final concentrate was plaque assayed in either BS-C-1 (entero- and reovirus) or MA-104 (SA-11 virus) cell monolayers. Results represent the mean values for four experiments of each virus and sample volume tested.

Effect of Adsorption-Interfering Substances on the Virus Recovering Efficiency of the Layers

Organic impurities in certain types of samples have been shown to interfere with virus adsorption to the filtration matrix (Wallis and Melnick, 1967a). In order to study the effect of such interfering substances, 1,000-1,200 L of conditioned tap water were first passed through the layers. They were then used for the concentration of poliovirus 1 (Sabin) from 20 L volumes of experimentally-contaminated tap water samples. Plaque assay of the filtrates revealed the presence of only 10.0% of the added virus. This indicated that there was no noticeable reduction in the virus adsorbing efficiency of the layers in spite of the prior passage of large volumes of conditioned water through them.

D. Talc-Celite Layers in Virus Recovery from Tap Waters Experimentally-Contaminated with Field Isolates and Sewage

Recovery of Field Isolates Added to Tap Water Samples

So far the talc-Celite concentration technique had been tested with laboratory-adapted strains. However, basic differences are known to exist between the laboratory-adapted virus strains and those that are freshly recovered from clinical specimens (Seto et al., 1965; Takemoto, 1966). Because these differences are known to influence the adsorption-elution characteristics of virions on various chromatographic columns (Ozaki et al., 1965), they could conceivably affect the behavior of enteric viruses on talc-Celite layers as well. The basic aim of the following experiments was, therefore, to test the efficiency of the talc-Celite technique in the recovery of field isolates of enteric viruses added to tap water samples

To a 1-L volume of conditioned potable water, a known amount of the virus under test was added and after thorough mixing, a 20 ml volume was removed to act as control. The remaining sample was then passed through a small layer of talc-Celite (47 mm diameter). A 10 ml volume of 10% FCS in saline (pH 9.0) was used to elute the layer-adsorbed virus. The control and eluate were plaque assayed to determine the amount of input virus recovered. Results summarized in Table 37 show that when working with 1-L volumes it was possible to recover between 82 to 93% of the input PFU of the viruses tested.

TABLE 37

TALC-CELITE LAYERS IN THE RECOVERY OF FIELD STRAINS OF ENTERIC VIRUSES FROM EXPERIMENTALLY-CONTAMINATED SAMPLES OF TAP WATERS

Virus	Input PFU/L	PFU recovered	% recovery	Standard deviation
polio 1 (vaccine strain)	7.2×10^4	5.8×10^4	82.0	±9.0
polio 1 (non-vaccine strain)	1.5×10^5	1.4×10^5	93.0	±10.0
echo 1	2.4×10^4	2.0×10^4	88.0	±4.0
coxsackie	1.8×10^4	1.6×10^4	90.0	±4.0
reo	2.2×10^5	1.9×10^5	89.0	±9.0

The pH of a 1-L volume of potable water was adjusted to 6.0 and EBSS was added to a final concentration of 1:100. After adding a known number of PFU to the sample it was passed through a layer containing a mixture of 300 mg talc and 100 mg Celite 503. The layer-adsorbed virus was eluted with 10 ml 10% fetal calf serum in saline (pH 9.0). Plaque assays were performed in monolayers of BS-C-1 cells. The results represent the mean of five experiments with each virus strain.

Recovery of Indigenous Viruses from Samples of Tap Waters Experimentally-Contaminated with Raw Sewage

Contamination of potable waters with sewage leads to the addition of viruses as well as certain organic and inorganic impurities. It has been reported that the presence of such impurities in potable waters could adversely affect the virus recovering efficiency of certain techniques which otherwise gave excellent virus recoveries (Fattal et al., 1974; 1977). The following experiments were designed to study how the virus recovering efficiency of the talc-Celite techniques was affected when tap water samples were contaminated with raw sewage.

Raw sewage was added to a 20-L volume of conditioned tap water to give a final concentration of either 0.05% or 0.1%. The sample was then passed through a layer of talc-Celite (142 mm diameter). The layer-adsorbed virus was eluted with 100 ml of 10% FCS in saline (pH 9.0). The eluate was concentrated 10-fold by overnight hydroextraction with PEG (6,000) at 4°C, and then plaque assayed. The number of enteric virus PFU in the samples of raw sewage used for experimental contamination was determined by concentrating 200 ml of it by the talc-Celite technique as described previously (Sattar and Westwood, 1976b). Results presented in Table 38 show that between 86 to 87% of the indigenous enteric virus PFU contained in raw sewage added to the sample could be recovered by this technique. This clearly indicated that the presence of sewage, at least in the concentrations tested in these experiments, in tap water samples does not adversely affect the enteric virus recovering efficiency of the talc-Celite layers.

TABLE 38

TALC-CELITE LAYERS IN THE RECOVERY OF INDIGENOUS ENTERIC VIRUSES OF TAP WATER SAMPLES EXPERIMENTALLY-CONTAMINATED WITH RAW SEWAGE

Final conc. of raw sewage in sample	Calculated input enteric virus PFU in sample	PFU recovered in sample conc.	% recovery	Standard deviation
0.05%	13.0	11.0	87.0	±4.0
0.10%	22.5	19.0	86.0	±4.0

The pH of a 20-L volume of tap water was adjusted to 6.0 and EBSS was added to give a final concentration of 1:100. Raw sewage was then added to the desired concentration and the sample was passed through 142 mm diameter layers of talc (1.2 gm)-Celite (0.4 gm). The layer-adsorbed virus was eluted with 100 ml of 10% fetal calf serum in saline (pH 9.0). The eluate was subjected to overnight hydroextraction with PEG at 4°C. Final concentrate was subjected to plaque assay in BS-C-1 cells. The results represent an average of four experiments at each sewage conc. tested.

The concentration of enteric virus plaque forming units (PFU) in the raw sewage used for experimental contamination was determined by processing 200 ml of it through talc-Celite layers (Sattar and Westwood, 1976b).

DISCUSSION

Talc-Celite Layers

From the data presented in this section of the study, it can be seen that the talc-Celite technique, in combination with second-step concentration with PEG, represent a relatively simple and efficient means of recovering entero-, reo- and rotaviruses from large volumes of tap waters. In this procedure virus adsorption to the layers is carried out at pH 6.0 and the recovery of the layer-adsorbed virus is achieved by using an eluent of pH 9.0. This is in contrast to a number of other techniques reported for the concentration of enteroviruses from the water environment (Hill et al., 1971; Sobsey, 1976; Gerba et al., 1978; Wallis et al., 1979) where pH extremes of 3.5 and 11.5 are necessary for virus adsorption to and elution from the filtration matrix. Because of their relatively pH labile nature, such techniques become potentially unsuitable for the recovery of parvoviruses (Sobsey and Jones, 1979) as well as reo- (Estes et al., 1979b) rota- (Farrah et al., 1978b; Estes et al., 1979b) and adenoviruses (Fields and Metcalf, 1975). It has also been shown that certain strains of enteroviruses are unable to withstand such pH extremes (Shaffer et al., 1977; Gerba et al., 1977).

In addition to possible virus inactivation at pH levels of 3.5 and 11.5, techniques using these pH extremes demand extra care and equipment in the adjustment and maintenance of the desired pH values (Farrah et al., 1976c). Moreover, the pH of the concentrates obtained through these procedures needs to be readjusted to the physiological level before their inoculation into cell cultures. When working with Zeta filters (Sobsey and Jones, 1979) a drastic reduction in the sample pH is not needed. In our experience, however, the flow rates through the presently available Zeta filters was considerably less when compared to talc-Celite layers of the same diameter.

Although studies with samples experimentally-contaminated with adenoviruses have not yet been carried out, the isolation of adenoviruses from field samples of waste and surface waters (Sattar, 1978a) shows that these viruses, when present, can also be concentrated by the talc-Celite technique.

It has been shown that basic differences exist in the adsorptive behavior of entero- and rotaviruses to aluminum hydroxide and activated sludge flocs (Farrah et al., 1978a). However, under the experimental conditions used in this technique, the adsorption of rotaviruses to talc-Celite layers was as efficient as that of other enteric viruses.

Organic impurities in certain types of samples have been shown to interfere with virus adsorption to the filtration matrix (Wallis and Melnick, 1967b). In the experiments done in this study such interference was not noted. Layers, after having had 1200 L of conditioned tap water passed through them, were still able to adsorb virus particles subsequently filtered through them.

Presence of EBSS in the sample aids in virus adsorption to the layers. Whereas this is assumed to be due to the types and amounts of cations present in it, conclusive experimental evidence to this effect is yet to be generated. Unlike the use of aluminum chloride (Farrah et al., 1978c), addition of EBSS to tap water samples was not found to result in the production of floccules. EBSS is relatively inexpensive and commercially and readily available in the powder form.

For the recovery of layer-adsorbed entero- and reoviruses, 10% FCS in saline (pH 9.0) was found to be the best eluent. However, we noted it to be inhibitory to rotaviruses. Such rotavirus inhibitory activity in commercially available sera from fetal calves has recently been reported by other investigators (Clark et al., 1979; Estes et al., 1979a; Schlafer et al., 1979). Testing of a number of other eluents showed 3% BE and TPB, both at pH 9.0, to be highly efficient in the recovery of layer-adsorbed rotaviruses. Subsequently, it was shown that the use of these two eluents could be readily extended to working with entero- and reoviruses as well. Although other studies have also shown BE to be a good virus eluent (Rao and Labzoffsky, 1969; Katzenelson et al., 1976b; Fattal et al., 1977; Landry et al., 1978), the use of TPB offers certain advantages over it. Apart from being less expensive, the final concentrates obtained with TPB are relatively easy to pass through sterilizing membranes. Therefore, TPB represents a good general purpose eluent for the talc-Celite technique.

Solutions of individual basic amino acids such as arginine and glycine could also elute polio- and rotaviruses from the layers, but their eluting efficiency was slightly lower in comparison with that of BE or TPB. Because the use of single amino acid solutions could provide an inhibitor-free and readily standardizable virus eluent, further work on the improvement of their eluting efficiency would be of great value.

Presence of virus aggregates in experimentally-contaminated samples and their subsequent disaggregation during sample processing could give an erroneous impression of the virus-recovering efficiency of a given technique (Fattal et al., 1977). Every effort has been made in the study to minimize the presence of virus clumps in the samples. In this context, the following major points need to be stated: (a) all virus pools were stored at -80°C; such storage has been shown to minimize virus aggregation (Katzenelson et al., 1974); (b) whenever possible virus pools were carefully examined under the electron microscope to rule out the presence of detectable virus clumps; (c) suspension of virus aggregates in balanced salt solutions has been shown to result in their disaggregation and also in the avoidance of aggregation of already dissociated particles (Young and Sharp, 1977); (d) the plaque titre of rotavirus pools was found to be the same before and after their passage through 80 nm pore diameter membrane filters; (e) all fractions tested by plaque assay contained either FCS or TPB and presence of such protein-rich materials has also been shown to contribute to virus disaggregation (Hamblet et al., 1967); all dilutions for plaque assay were made using EBSS and since all fractions, including virus controls to determine the amount of input PFU, were similarly treated, plaques detected most likely resulted from dissociated infectious virus particles rather than virus clumps.

The time required for the passage of 1,000 L of potable water through the layers ranged between 4-5 hours. This rate of sample filtration compares favorably with that of other techniques reported in the literature (Hill et al., 1976; Wallis et al., 1979).

Talc-Celite layers have been shown to be as efficient as PE 60 in the recovery of viruses from surface and waste waters (Sattar and Westwood, 1976b). The layers also have been used in the recovery of animal viruses from water contaminated with farm wastes (Derbyshire and Brown, 1978). Talc and Celite both are not only more readily available but also considerably cheaper than PE 60. The use of these layers is also more economical than that of a variety of membrane or cartridge filters. With $15.00 to $25.00 required for the processing of each 1,000-L tap water sample, such techniques could be at least ten times more expensive than the use of talc-Celite layers.

It has already been shown (Sattar and Westwood, 1976) that two to three days of storage of preformed talc-Celite layers in a moist state at 4°C does not affect their virus-recovering efficiency. It has also been found (Sattar and Westwood, 1976a) that virus particles adsorbed to the layers could retain their infectivity for at least 24 hours. In other words, after passing a virus-containing sample through the layer, the layer-adsorbed virus can be eluted 24 hours later without any significant loss in its infectivity. In actual practice this makes it possible to carry preformed layers to the sample collection site instead of having to transport large-volume samples to the laboratory for processing. Passage of the sample through the layer can be accomplished at or near the site of sample collection and the layers brought back to the laboratory for virus elution and further processing of the eluate.

PEG Hydroextraction

Second-step concentration of eluates forms an integral part of the techniques designed to recover viruses from large volumes of water samples. The simplicity and efficiency of the second-step concentration process in virus recovery are, therefore, crucial to the overall performance of the sample concentration procedure.

A number of techniques (Wallis et al., 1972b; 1972c; Katzenelson et al., 1976b; Farrah et al., 1977b) for second-step virus concentration call for adjustment of sample pH to highly acidic (pH 3.5) or highly alkaline (pH 11.5) levels. Such pH extremes were undesirable for the reason previously stated. Moreover, lowering of sample pH to 3.5 also results in the flocculation of a number of organic chemicals present in the eluate. The presence of such floccules makes it difficult to use small diameter membrane filters for second-step concentration (Farrah et al., 1976a). Centrifugation of the eluate to remove the flocculated material results in the loss of floccule-associated virus.

Although the organic flocculation technique (Katzenelson et al., 1976b) also involves the lowering of sample pH to 3.5, its relative simplicity and the reported efficiency in the recovery of enteroviruses prompted its inclusion in this study. Using this technique, Katzenelson et al. (1976b) were able to recover nearly all the poliovirus from experimentally-contaminated samples of 3% beef extract (eluent); but when the technique was applied to experimentally-contaminated samples of eluates, virus recovery was about 75%. However, using this method, we could recover only 66% of added poliovirus 1 (Sabin) from samples of 3% beef extract. Of the remaining virus, 27% was detectable in the supernatant obtained after organic flocculation. Prior to these experiments, the pH meter (Fisher Scientific, Model No. 220) was thoroughly checked for any possible malfunctioning. Moreover, beef extract from two different manufacturers (Difco and Oxoid) was used to rule out the possibility of any inherent variations in its virus-recovering efficiency.

During preliminary studies in our laboratory with the two-phase separation technique (Shuval et al., 1969) no more than 16% of the added poliovirus 1 (Sabin) could be recovered from 1-L samples of surface waters. The technique was also found to be cumbersome and time-consuming.

The use of PEG hydroextraction for virus concentration was initially reported by Gibbs and Cliver (1965). The results of subsequent applications (Cliver 1967a;Shuval et al., 1967) of this technique to virus recovery from water samples were not encouraging. But more recent studies by Wellings et al. (1975) have shown PEG hydroextraction to be relatively simple and efficient in virus concentration from samples of sewage and effluent. The results reported in this study confirm and further extend these observations.

Poor virus recoveries obtained by Cliver (1967a)and Shuval et al. (1967) with PEG hydroextraction were considered to be due to adsorption or entrapment of virus on the inside surface of the dialysis sac. The recovery of nearly 90% of the input enteric virus PFU in our experiments shows that only small amounts of virus, if any, were being lost by retention or inactivation in the dialysis sac.

It has been reported that either PEG molecules or other low molecular weight impurities could enter the concentrate as a result of counter dialysis (Howe et al., 1964; Gibbs and Cliver, 1965; Shuval et al., 1967). This has been shown to make the concentrate cytotoxic to certain types of cells (Gibbs and Cliver, 1965). In the experiments reported in this study, the concentrates never appeared to be toxic to the cell cultures. From the results, one can also argue strongly against any noticeable anti-viral effect in the concentrates. Shuval et al. (1967) also believe that PEG may not be anti-viral.

The data presented in this study show that the PEG hydroextraction technique works equally efficiently in the concentration of laboratory-adapted as well as field strains of at least three major groups of enteric viruses. Although tests with experimentally-contaminated samples of adenoviruses were not done, the isolation of adenoviruses during field studies in our laboratory (Sattar, 1978a) shows that these viruses were not being adversely affected by this procedure.

PEG hydroextraction, therefore, represents a simple and efficient means of second-step concentration of eluates. The promise shown by this technique has prompted its inclusion in the revised tentative procedure for virus concentration from finished waters in the forthcoming edition (15th) of the Standard Methods for the Examination of Water and Wastewaters (M. Sobsey, personal communication).

GENERAL DISCUSSION

Rotaviruses have now come to be recognized as important pathogens of man and animals (McNulty, 1978; Holmes, 1979). The ability of a variety of animal rotaviruses to grow in cell cultures has been very helpful in the investigation of the basic properties of this virus group as a whole. Although certain human rotavirus strains have recently been adapted to grow in cell culture (Wyatt *et al.*, 1980), easy and reliable means of *in vitro* cultivation and quantitation of human rotaviruses in general have not yet become available. Close similarities between SA-11 and human rotaviruses, therefore, continue to make it a suitable substitute for them (Kapikian *et al.*, 1976; Schoub *et al.*, 1977). An understanding of the role of proteolytic enzymes in the replication of SA-11 should be helpful in developing better ways for the *in vitro* cultivation of human rotaviruses. It could also assist in determining the part such enzymes may play in the pathogenesis of rotaviral infections.

Rotaviruses have recently been implicated as etiological agents in waterborne outbreaks (Freij *et al.*, 1978; Lycke *et al.*, 1978). In spite of the mounting evidence for the potential of rotaviruses to be transmitted through sewage-polluted waters, techniques for their concentration and recovery from incriminated water samples have been generally unavailable. The SA-11 plaque assay system reported in this study made it possible to develop methods for their recovery from the water environment.

The enteric virus-recovering efficiency of the talc-Celite layers compares favorably with other techniques reported in the literature (Hill *et al.*, 1976; Fattal *et al.*, 1977; Gerba *et al.*, 1978; Wallis *et al.*, 1979). Using this technique in combination with PEG hydro-extraction, samples of tap water could be concentrated as much as 100,000-fold. In spite of this high degree of concentration, sample concentrates were found to be free from cytotoxicity.

Experiments using relatively pure water samples contaminated with laboratory-adapted strains of enteric viruses give only a preliminary indication of the virus recovering efficiency of a given technique. The potential of such a technique can only be determined by testing it under experimental conditions which resemble as closely as possible the situation it may encounter in the field. As can be seen from the results of the experiments done with field isolates and sewage-contaminated potable water samples, the performance of the talc-Celite technique in the recovery of enteric viruses was highly satisfactory under these simulated field conditions.

In addition to the studies reported here, the following aspects of the development of the talc-Celite technique require further investigation:

(1) Presence of EBSS in the sample is needed for virus adsorption to the layers. Which component(s) of EBSS plays an active role in this regard needs to be determined.

(2) What is the average pore diameter of the talc-Celite layer?

(3) At the present time, 1,000 L of tap water samples can be filtered through the layers in 4 to 5 hours. If the rate of filtration of the sample could be further speeded up without adversely affecting the virus recovering efficiency of the layers, it would make the technique more widely acceptable.

(4) The Norwalk agent (Ouiverkerk et al., 1978; Morens et al., 1979), hepatitis A virus (Goldfield, 1976) and adenoviruses (D'Angelo et al., 1979) are among the important infectious agents transmitted by the water route. Because of the present difficulties in either the cultivation or quantitation of these viruses in cell cultures, they could not be included in this study. However, if and when suitable model systems for these viruses become available, attempts should be made to extend the use of the talc-Celite technique to include them.

(5) The possible use of solutions of single basic amino acids as virus eluents in the talc-Celite technique deserves further study. Even if a combination of two or more such amino acids proves to be necessary in this regard, it would still represent a chemically-defined and virus inhibitor-free eluent.

The testing of the talc-Celite technique under field conditions did not constitute a part of the present study. However, the technique has been used extensively in field surveys for the recovery of viruses from samples of sewage, effluents and raw and finished waters (Sattar, 1978a).

APPENDIX I

Statistical Analysis of the Data from Plaque Titrations of Rotavirus SA-11 in MA-104 Cells

The following analysis is for the data presented in Table 12.

Analysis of variance			
Source of Variation	Degrees of freedom	Sum of squares	Mean square
Time	9	108.08	12.01
Error	40	1007.20	25.18
Total	49	1115.28	

The following model was used for the statistical analysis

$$Y_{ij} = \mu + \tau_i + \epsilon_{ij} \qquad i = 1,\ldots\ldots, 10 \quad j = 1,\ldots\ldots, 5$$

where Y_{ij} is the observed number of plaque counts on the i^{th} time period on the j^{th} replication,

μ is the mean effect,

τ is the time effect

and ϵ_{ij} are random variables assumed normally distributed with mean zero and same variance σ^2.

The effect of time on the reproducibility of the plaque assay system was tested. By analysis of variance the mean square of time was found to be smaller than the mean square of error. Hence it was concluded that no significant time effect is present.

APPENDIX II

<u>The Following Publications are Based on the Research Carried Out for this Thesis.</u>

1. Ramia, S. and S.A. Sattar. 1979. Second-step concentration of viruses in drinking and surface waters using polyethylene glycol hydroextraction. Can. J. Microbiol. <u>25</u>: 587-592.

2. Ramia, S. and S.A. Sattar. 1979. Simian rotavirus SA-11 plaque formation in the presence of trypsin. J. Clin. Microbiol. <u>10</u>: 609-614.

3. Sattar, S.A. and S. Ramia. 1979. Use of talc-Celite layers in the concentration of enteroviruses from large volumes of potable waters. Water Res. <u>13</u>: 637-643.

4. Sattar, S.A. and S. Ramia. 1979. Talc-Celite layers in virus recovery from potable waters experimentally-contaminated with field isolates and sewage. Water Res. <u>13</u>: 1351-1353.

5. Sattar, S.A., V.S. Springthorpe and S. Ramia. 1979. Sewage disposal and viral pollution of the Ottawa River. Water Pollut. Res. in Canada. <u>14</u>: 45-62.

6. Ramia, S. and S.A. Sattar. 1980. Concentration of seeded simian rotavirus SA-11 from potable waters using talc-Celite layers and hydroextraction. Appl. Environ. Microbiol. <u>39</u>: 493-499.

7. Ramia, S. and S.A. Sattar. 1980. Proteolytic enzymes and rotavirus SA-11 plaque formation. Can. J. Comp. Med. <u>44</u>: 232-236.

8. Ramia, S. and S.A. Sattar. 1980. Role of trypsin in plaque formation by simian rotavirus SA-11. Can. J. Comp. Med. In press.

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