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ELUTION OF ENTERIC VIRUSES FROM MISSISSIPPI SEDIMENTS USING LECITHIN-SUPPLEMENTED ELUENTS

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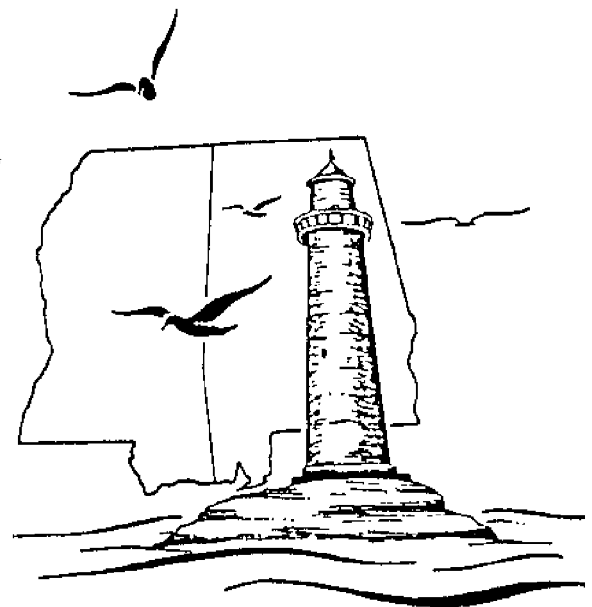

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CHAPTER I

INTRODUCTION

The release of raw and partially treated sewage into the ocean environment is a common event in our society. This may occur due to leaching of wastewater from septic tanks along coastal regions or may be a designed disposal process for the community. This method is very economical and in many cases the sewage is not treated. If treatment is not employed, no money or property is required for the facility. The process is deemed acceptable by many people because of the large dilution factor provided by the ocean. However, environmental changes, both short and long term, may lead to the contamination of recreational and seafood harvesting areas.

Many types of viruses are shed in human feces. Foremost among these are the enteroviruses, consisting of polioviruses, coxsackieviruses A and B and echoviruses. Reoviruses, adenoviruses, hepatitis viruses, the Norwalk agent and rotaviruses are also spread by the fecal route. These viruses may cause diseases ranging from a mild rash to encephalitis to paralysis. Rotaviruses have recently been found in the feces of patients with severe gastroenteritis and are now thought to be the primary cause of non-bacterial gastroenteritis.

Enteric viruses shed in fecal wastes will adsorb to estuarine sediment particles and these particles infer an extended viability to the virus. This increased viability and the tendency of sediment particles to settle can contribute to a higher level of viral contamination in a defined coastal area. It has been proposed that environmental factors, few of which are understood, can lead to the release of these particles and produce an abrupt rise in the level of virus in the overlying water. This rise may not be consistent with bacterial indicator levels. It is known that virus particles remain infective when bound to sediment, therefore; virus need not be released from the sediment to present a public health hazard. This could be a factor when wave action, normal or storm induced, rainfall run-off or recreational boating resuspend the sediment particles. Resuspension of the particles is thought to increase the recreational water hazard by bringing virus into contact with filter-feeding shellfish such as oysters, clams and crabs or by primary human contact (skiing and swimming). The consumption of raw shellfish is a documented cause of many virus-associated disease outbreaks; therefore, the ability to test an area for sediment-bound virus would greatly aid the shellfish industry as well as the environmental epidemiologist.

Sediment, as one of several factors crucial to the prolonged survival of viruses in the marine environment, is important to the overall understanding of marine sewage pollution. The ability to elute virus from estuarine sediment has been found to vary widely with sediment composition and virus type and the initial elution process is the critical step in developing a workable procedure. This study was designed to develop a suitable mixture for the initial elution of virus from local, estuarine sediments and, if possible, to discover what factors contribute to the binding of virus to sediment. Four sediments of different compositions were used to simulate the constantly changing make-up of Mississippi estuarine sediments. Three viruses (poliovirus type 1, coxsackievirus B1 and echovirus 11) were used to determine each eluent's ability to desorb a variety of viruses.

CHAPTER II

LITERATURE REVIEW

The discharge of raw or partially treated sewage into the ocean environment has been ignored and even condoned. Near Miami, raw sewage is released from a deep marine outfall and allowed to be carried out to sea by the Gulf Stream. However, these effluents can reach the nearby coastline by the action of errant currents and tidal action (39). In some areas, leaching of raw sewage from underground septic tanks occurs during periods of heavy rainfall or from a high water table (24). The problem is complicated in many of these areas because of their tendency to be sheltered from heavy tidal action and a significant flushing effect.

Enteroviruses are known to survive sewage treatment. Berg et al. (4) reported that as much as 15% of the viruses present in primary sewage effluents may survive accepted chlorination processes. In the same study, greater than 99.9% of the total coliforms, fecal coliforms and fecal streptococci were destroyed. Wellings et al. (48) demonstrated free and solid associated viruses in chlorinated effluent samples taken from sewage treatment plants in Florida.

Sattar and Westwood (38) found virus in up to 58% of samples collected after chlorination in a sewage treatment plant in Ottawa. Stagg et al. (43) have shown that the attachment of bacteriophage MS-2 to bentonite significantly decreased the virus inactiviation rate by hypochlorous acid. Thus, sewage treatment, even with effluent chlorination, may lead to a false sense of security.

Many viruses readily adsorb to suspended solids and particulate matter in the environment (5, 6, 17, 20, 21, 23, 27, 35, 40). This adsorption does not necessarily inactivate the virus. Shaub and Sagik (40) have shown that the infectivity (or plating efficiency) of encephalomyocarditis (EMC) virus was increased by binding to montmorillonite clay as compared to freely suspended virus. In animal infectivity studies, the LD_{50} for suckling mice innoculated by ingestion was increased by approximately one log when the virus was bound to clay. There was no significant difference between the LD_{50}'s of control and clay-bound virus when an intracerebral infection route was used. Moore et al. (35) demonstrated that poliovirus type 1 and bacteriophages T7, T2 and f2 continued to be infective when adsorbed to bentonite clay.

The problem of particulate binding is compounded by the tendency of adsorbed particulate matter to protect the virus from inactivation. Liew and Gerba (32) reported the

thermostabilization of poliovirus and echovirus by sediments at temperature up to 50°C. LaBelle and Gerba (28) demonstrated that, under natural conditions, the time required to inactivate 99% of poliovirus type 1 in seawater was increased from one hour in seawater alone to 4.25 days after adsorption to natural sediment. In a separate study, LaBelle and Gerba (29) showed that adsorption to sediment significantly prolonged virus survival.

It has been suggested that heavy metal ions contribute to the rapid inactivation rates for virus in natural seawater. Murray and LaBand (36) demonstrated a loss of infectivity when poliovirus was adsorbed to MnO_2, Al_2O_3 and CuO. Protein interaction with the metals through strong Van der Waals forces is thought to cause capsid disruption or rearrangement leading to inactivation. Fugioka et al. (16) have presented strong evidence for the presence of a microorganism(s) which confer antiviral activity to the coastal waters of Hawaii. Viral inactiviation was shown to decrease when natural seawater was sterilized by autoclaving or filtration and when unsterile natural seawater was amended by antibiotics. Adsorption to sediment could provide protection from these factors by reducing their contact with viruses.

Ultraviolet light in the form of sunlight has been suggested as a contributing factor in the natural dieoff of virus in seawater. Hill et al. (25) showed that

enteroviruses are rapidly inactivated by high intensity UV light. The same mechanism may be responsible for some natural virus inactivation. Because of the large attenuation of ultraviolet light by seawater the mechanism would only apply to viruses present at the water's surface. This illustrates the importance of particulate binding because of the tendency of bound virus to concentrate in the aerobic, bottom sediments.

The protection which particulate matter confers to many viruses can have a tremendous effect on the actual (as opposed to perceived) water quality of a given area. Some researchers have suggested that water quality analysis should include a virological examination of the sediment (21). For a method to be useful in the routine virological evaluation of sediments, it must meet several requirements: (1) The method must be able to handle large amounts of sediments. Since the population of viruses in a given sample is likely to be low, sample sizes will need to be large (100 to 500 grams). (2) The time required to perform the procedure should be as short as possible and similar to the time required for bacterial analysis. (3) The method should be easy to teach and use and applicable to semi-skilled personnel. (4) The method should not require costly equipment or materials. (5) The method must work equally well with all types of viruses and sediments. Sediment composition at a given site may change drastically

over a period of time and different locations allow for even more diversity, (6) The method must yield a high and consistent percentage of recovery, (7) The method and materials used must be compatible with the method of quantitation. If a plaque assay is used, the eluent must not be toxic to mammalian cell culture or interfere with plaque formation. If a serological assay is used, the medium must also be free of substances which would interfere with binding.

Methods used in previous studies to elute virus from sediments range from the simple to the very complex. All of these procedures employ an initial elution from the sediment followed by a means of concentrating the virus present in the eluent. Deflora et al. (12) used sterile seawater to elute naturally occuring viruses from sandy sediments and slime collected near a sewage outfall. The eluate was further concentrated by adsorption to a polyelectrolyte at pH 5.0 and collected on a fiberglass filter. The virus was eluted from the polyelectrolyte with 4 ml of borate buffer at pH 9.0.

Many methods use a glycine buffer at an alkaline pH either with or without the addition of EDTA. Gerba et al. (21) tested various glycine-EDTA eluents for their ability to elute virus from 10 grams of dried sediments collected along the Texas coast. They used 30 ml (1:3 w/v) of a solution consisting of 0.05M EDTA prepared in 0.25M glycine

buffer with a final pH of 11.0 (after the addition of sediment) for the initial elution. $AlCl_3$(1M) was added to a final concentration of 0.06M and the resulting mixture passed through a Millipore filter. Virus was eluted from the filter with two 15ml portions of 0.25M glycine buffer at pH 11.5. The eluate was neutralized to prevent inactiviation by the high pH. This method produced an average recovery of 83%. A solution of ten per cent fetal calf serum and 0.01M EDTA in 0.25M glycine buffer at pH 10.5 also yielded good recoveries (60%) but this eluent could not be reconcentrated. An average recovery of 50% was reported when the glycine-EDTA method was used to recover virus from 500 grams of wet, virus-seeded sediment.

Gerba et al. (19) used the method to elute viruses from 400ml of wet sediment collected at several sites within a polluted community canal system. Large numbers of viruses (177-332 PFU) were found at the various sites. LaBelle et al. (30) also used the method of Gerba (21) to study the level of virus at sites in the Galveston Bay area. The procedure was modified by using tryptose phosphate broth with 10% fetal calf serum at pH 11.5 to elute virus from the membranes. Per cent recovery was quoted as the 50% figure given by Gerba (21) but the efficiency of the procedure with the new membrane eluent was not reported.

Shaiberger et al. (39) used the method of Gerba (21) to elute virus from 400 gram sediment samples collected near Miami, Florida. The procedure was modified to use a series of Filterite filters instead of Cox filters. The composition of the sediment varied from muddy sand to coarse sand and the efficiency of recovery was determined to be 50%.

Beef extract has been used by several investigators (3, 47, 48) to elute filter-adsorbed viruses. Solutions of beef extract have also been used in the elution of viruses from particulate matter. Wellings et al. (48) used 3% beef extract to elute virus from sludge samples taken from activated sludge treatment plants in Florida. In this study, a 30% beef extract solution was added to 500ml of sludge to give a final concentration of 3% and the pH adjusted to 9.0. The samples were sonicated at 100 watts for 15 minutes in a cooling cell. After centrifugation, the supernatants were placed in dialysis bags with an average pore size of 24nm and concentrated with Carbowax 20,000 overnight. Sufficient phosphate buffered saline (pH 7.4), with 2000 units of penicillin and 2000 ug of streptomycin, was added to bring the total volume to 5ml. The interior of the dialysis bag was washed with this solution and the samples stored at -70°C. Although no attempt was made to determine the efficiency of this method, virus was isolated from 60% of the sludge samples

tested. When this method was used with various other samples (mud, soil and sludge collected in or around the treatment plants and near a sewer pipe leak), virus was found in 100% of the samples tested.

Berg and Dahling (2) used beef extract mixtures to elute virus from river water solids. It was found that a 10% solution of beef extract in McIlvaine buffer (Na_2HPO_4 and citric acid) at pH 7.0 eluted from 39% to 63% of the adsorbed virus (poliovirus 1). The composition of the particulate matter was not determined.

Hurst et al. (26) evaluated both glycine and beef extract-based eluents for their ability to elute virus from activated sludge. In conjunction with sonication, a 3% beef extract solution (pH 9.5) yielded better recoveries (21% to 66%) than 0.05M glycine at pH 11.5 (4% to 8%). However, when mechanical mixing was used instead of sonication, the glycine mixture gave better results (30% to 89%) than beef extract (21% to 46%).

Bitton et al. (7) compared the abilities of glycine-EDTA, beef extract, isoelectric casein and non-fat dry milk to elute virus from 10 grams of soil. Isoelectric casein (0.5%, pH 9.0) was found to give the best recovery (125.5%) of poliovirus type 1 from a soil composed primarily (94%) of sand. Non-fat dry milk (0.5%, pH 9.0) also yielded a good recovery (95.0%) followed by 0.25M glycine + 0.05M EDTA at pH 11.0 (62.5%) and 3% beef extract

at pH 9.0 (48.3%). Isoelectric casein was also tested for its ability to elute poliovirus type 1 from 100 grams of four soil types. These soil types ranged in sand content from 74% to 96% and clay content from 0.1% to 13%. No significant difference was found between the mean recoveries which ranged from 45.0% to 52.0%. The ability of isoelectric casein to elute viruses other than poliovirus type 1 was determined. Compared to the exceptional recovery of poliovirus type 1 (125.5%), the elution of coxsackievirus B3 was only slightly less (118.0%), followed by echovirus type 4 (74.5%).

Earlier work done in this laboratory, using various methods and eluents described in the literature in conjunction with Mississippi estuarine sediments, did not yield satisfactory results (46). Glycine eluents described by Gerba (21) were used at pH values from 10.0 to 11.5 to elute poliovirus type 1 from a Mississippi estuarine sediment with an average sand, silt and clay content of 79.2%, 11.7% and 9.1%, respectively. All recoveries were found to be 1.0% or lower. When EDTA (0.05M) and fetal calf serum (10%) were added, recoveries increased slightly (less than 2.0% and 4.5%, respectively).

The use of a 0.5% skim milk solution at pH 9.0 also gave poor recoveries (0.2%). Addition of 10% newborn calf serum increased the recovery to 11.0%. Mixtures of 10% beef extract paste (Difco) at pH 9.0, alone and with 10%

newborn calf serum, yielded recoveries of less than 3.0%. However, when powdered beef extract (Inolex) was used at a concentration of 3% and a pH of 9.0, recoveries increased to 42.0%. Concentrations of powdered beef extract between 3% and 15% did not yield significantly different results (42.0% to 48.0%); however, at concentrations lower than 3% and higher than 15%, recoveries decreased. A nutrient broth solution was also tested as an eluent. Nutrient broth (Difco) is approximately 40% beef extract and 60% peptone. Its use in a 4% solution at pH 7.5, yielded an average recovery of 50.0%.

Experiments were also done to a limited extent with another Mississippi costal sediment (46). The sand, silt and clay content of this sediment averaged 10.1%, 48.2% and 41.7%, respectively. When eluted with glycine, skim milk and (Difco) beef extract-based eluents the recoveries again were low (less than 5.0%). Three per cent Inolex beef extract (pH 9.0) and 4% nutrient broth (pH 7.5) eluted an average of 32.0% and 40.0%, respectively.

From the data given above, it is obvious that glycine-EDTA elution methods are not compatable with Mississippi sediments. The comparative success of such eluents as powdered beef extract and nutrient broth is also shown. A constituent of any crude tissue-based infusion, such as beef extract, is a compound known as phosphatidyl choline or lecithin. Phosphatidyl choline is found in most

mammalian cell membranes where it forms lipid bilayers and plays an important role in a membrane's selective permeability. Phosphatidyl cholines may also act as natural emulsifiers and surfactants and are used in many commerical food preparations. It is thought that these properties might explain the ability of beef extract and nutrient broth-based mixtures to elute virus from sediments. This study was designed to test that theory using several different types and concentrations of lecithin.

CHAPTER III

MATERIALS AND METHODS

Laboratory Practice and Equipment

No attempt was made to perform extractions under aseptic conditions. All procedures requiring sterile conditions were performed under a laminar flow hood using standard aseptic techniques. Virus contaminated glassware was placed in 3% Lysol and autoclaved before disposal or washing. Glassware was soaked in a 2% solution of MICRO (International Products Corporation) for 24 hours, rinsed in tap water and again in distilled water. The glassware was allowed to dry, wrapped in aluminum foil and sterilized in either a hot-air oven (Blue M Company) for three hours at 350°F or autoclaved for 20 minutes at 15 PSI, 121°C.

Media and reagents were prepared using reverse osmosis, Type 1 water (Millipore Corporation). Tissue culture media were filter sterilized using positive pressure filtration (90 mm filter holder, Millipore Company; 0.45 um pore size filters, Cox Instrument Company). Reagents were either filter sterilized or autoclaved at 15 PSI, 121°C for 20 minutes. Vertical-flow laminar-air hoods (The Baker Company Incorporated) were used for tissue culture, media filtration and other procedures requiring sterile technique.

Tissue Culture

The Buffalo Green Monkey Kidney (BGM) cell line was used for all viral assays. This cell line has been shown to be more sensitive to enteric viruses than either primary rhesus or African green monkey kidney cells (11). Cell passages between 100 and 160 were used for all assays.

Stock cultures of BGM cells were grown in L15 (Appendix) medium in 150 cm^2 disposable Corning tissue culture flasks (Corning Glass Works). The cells were incubated in a 37°C circulating incubator (Percival Manufacturing Company) until confluent. For subcultivation, the flasks were washed with a trypsin-versine, (ATV, Appendix) solution three times, leaving a residue (approximately 1 ml) after the third wash. The flasks were then incubated at 37°C for approximately five minutes or until the cells detached from the surface. The cells were resuspended in L15 growth media and dispersed into sterile 150 cm^2 flasks. For plaque assay, the same procedures were performed and the cells dispersed into ten to twenty 60 mm tissue culture dishes (Corning Glass Works). The dishes were then stacked and placed in a plastic chamber containing several beakers of water. The chamber was covered with aluminum foil to prevent excess evaporation from the dishes and the cells allowed to grow to confluency at 37°C.

Viruses

Poliovirus type 1 (American Type Culture Collection (ATCC), VR-192, Sabin), Coxsackievirus B1 (ATCC, VR-1032, Conn-5) and Echovirus type 11 (ATCC, VR-1052, Gregory) were used in all experiments. Virus pools were prepared by pipetting 1.5 ml of a 1/100 dilution of virus onto confluent monolayers of BGM cells in 150 cm^2 flasks. The flasks were placed on a rocker apparatus (Bellco Biological Company) and the virus was allowed to adsorb to the cells for one hour. Following adsorption, 30 ml of maintenance media was added to each flask. The flasks were incubated at 37°C until greater than 98% of the cells showed cytopathic effect (CPE). The flasks were frozen at -70°C; cells were lysed by rapid thawing and refreezing three times. Cell debris were cleared from the suspension by centrifuging at 1000 x g for 10 minutes. The virus suspension was filtered through a 0.45 filter (Nalgene Company) to remove aggregates and dispensed in 1.5 ml aliquots into 2 ml sterile, low-temperature plastic ampules (Vanguard) and stored at -70°C. Virus pools were titered by plaque assay. The vials were rapidly thawed at 37°C before use.

Plaque Assay

A single agar overlay plaque assay method was used for the titration of all samples and virus pools. The samples to be titered were diluted in a L15 medium using

log 10 dilutions. Growth media was removed from the confluent monolayers of BGM cells in 60 mm plastic tissue culture dishes. Three replicates were inoculated with 0.5 ml of virus suspension at each dilution. The virus was allowed to adsorb for one hour with redistribution of the virus inoculum at five minute intervals. Following adsorption, 5 ml of agar overlay medium (Appendix) was added to each plate. After cooling, the plates were inverted, placed in a plastic moisture chamber and incubated in the dark at 37°C for three days. Plaques were counted, beginning on the third day, for three days or until no new plaques appeared.

Sediment Collection

Four sediment collection sites were chosen based on the percent composition of sand, silt and clay. An attempt was made to cover the range of these values found in the Mississippi Sound. Eight to ten kilograms of sediment was collected at each site using a hand-held Ekman standard dredge (Wildco, Catalog Number 196). Only the top 2 to 3 cm of sediment were retained from each dredging. The sediment was homogenized, analyzed for sand, silt and clay content (Table 1) and stored in 500 gram lots at -20°C.

Artifical Seawater

Artifical seawater was prepared using Instant Ocean (R) (Eastlake, Ohio) artificial sea salts. The salinity and conductivity of the seawater was measured with a Model

Table 1

SEDIMENT PROFILES

		%			
SITE	GRVL	SAND	SILT	CLAY	SIZE TERM
1	0.0	99.0	1.0	0.0	MEDIUM SAND
2	0.0	52.3	30.3	17.4	MEDIUM SILTY, FINE SAND
3	0.1	89.3	6.0	4.6	SLIGHTLY GRANULAR MUDDY FINE SAND
4	0.0	37.3	39.2	23.5	VERY FINE, SANDY MUD

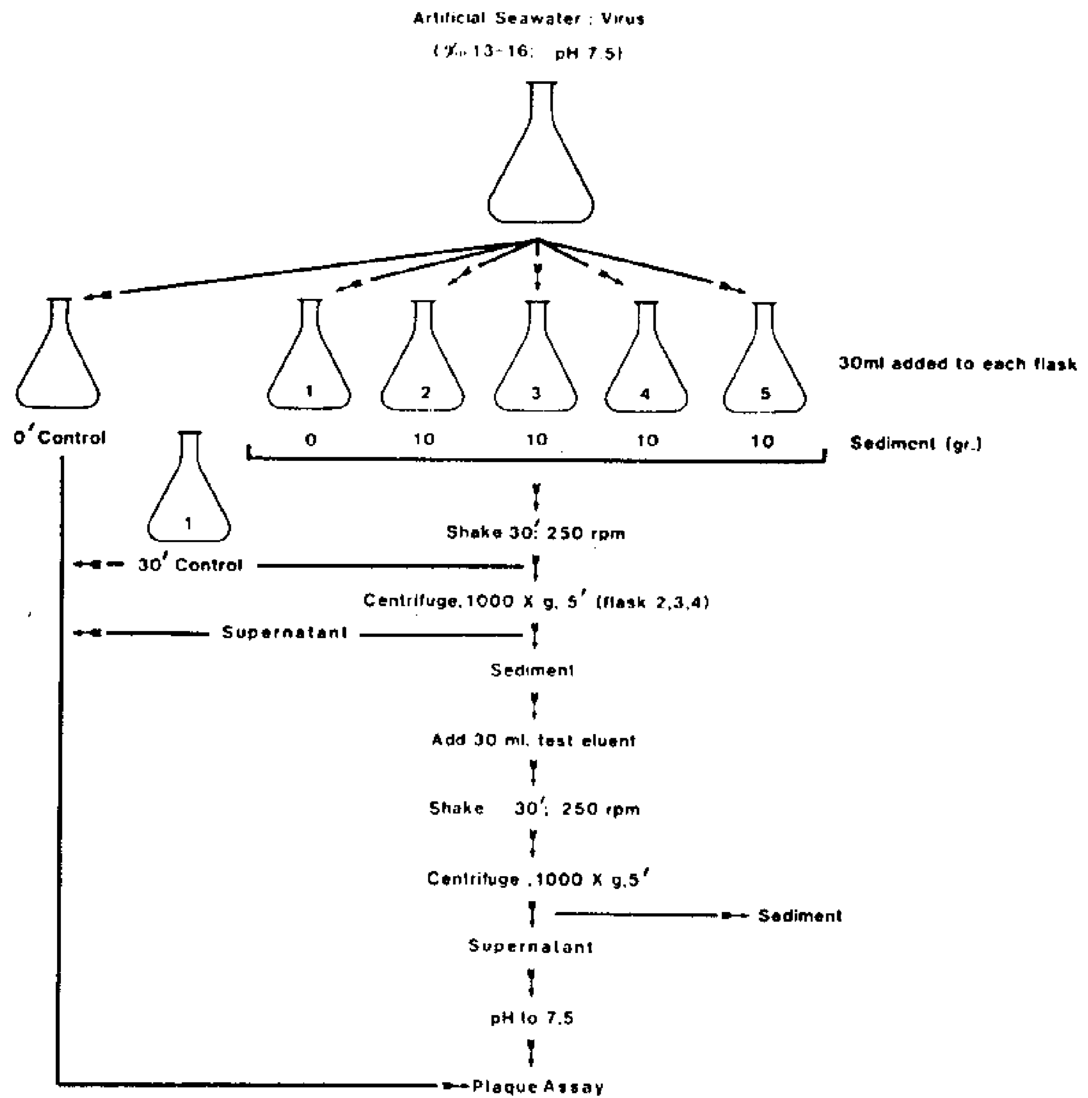
YSI 33 S-C-T meter and adjusted using Type I reverse osmosis water. Salinity and conductivity were adjusted to 15 ppt and 20,000 uMHO. A Sargent-Welch Model LSX pH meter was used to measure the pH which averaged 7.5. The seawater was filtered through a 0.45 um filter and stored at room temperature.

Experimental Procedure

Figure 1 shows the procedure used for all experiments. Virus was added to the filtered seawater in a ratio calculated to produce approximately the same titer (10^3 to 10^5 PFU/ml) for all experiments. Sediments were autoclaved and a known amount of each sediment type was dried overnight in a drying oven at 80°C. The weight of the dried sediment was measured and the amount of wet sediment equal to 10 grams of dry sediment was calculated. The sediments were stored in sealed containers at 4°C and remixed before each experiment.

Ten grams (dry weight) of each sediment type was added to one of four 500 ml centrifuge bottles. Thirty milliliters of the virus-seawater mixture was added to each bottle and to a fifth bottle which contained no sediment. This bottle was used as the input control. The bottles were shaken at 250 RPM on a rotary shaker (New Brunswick Scientific Company) at room temperature for 30 minutes to allow virus adsorbtion to sediment. The four sediment-containing bottles were centrifuged at 1000 x g

Figure 1. Sediment Extraction Procedure



for five minutes. A sample of the supernatant was taken from each flask and used to determine the per cent adsorption of virus to each sediment. The titer in the flask containing no sediment was used as the original titer. The remainder of the supernatant was discarded. Thirty milliliters of a test eluent was added to each bottle containing sediment; the bottles were shaken for 30 minutes at 250 RPM and centrifuged for five minutes at 1000 x g. A sample of each supernatant was taken to determine the amount of virus eluted. All samples were frozen at -70°C until titered.

CHAPTER IV

RESULTS AND DISCUSSION

Introduction

Many of the published studies dealing with the elution of enteric viruses from sediments employ either the glycine method of Gerba (21) or a variation of this method. Previous work performed in this laboratory indicated that these methods were not suitable for use with Mississippi estuarine sediments (46). Therefore, other methods were developed and tested with greater success. However, samples of sediment collected at different times of the year varied in sand, silt and clay content. More control was needed over the composition of the sediments in order to make definitive statements concerning the efficiency of virus elution.

The use of autoclaved sediments has been criticized due to the possible alteration and/or destruction of sediment components. Previous studies using natural, untreated sediments collected along the Mississippi Gulf Coast encountered problems of cytotoxicity. Autoclaving the sediments removes or destroys the cytotoxic component of the sediment and also eliminates any gross bacterial contamination and/or background viruses. The use of dried sediment also introduces problems not associated with the

autoclave process. Drying the sediment does destroy cytotoxicity and reduce contamination; however, clay particles in a sample form a modified matrix after which no amount of grinding can produce the range or distribution of particle sizes found in the original sample (Lytle, T. and Lytle, J., Gulf Coast Research Laboratory, Ocean Springs, Mississippi, personal communication). Since clay is considered a major controlling factor in the adsorption and elution of virus from sediment, drying seems inappropriate. Autoclaving does not alter the clay because hydration of the clay is retained during the process.

Adsorption of Virus to Sediment

Table 2 summarizies the adsorption of each virus to each sediment type. For the sediments which contain clay (Numbers 2 through 4) the average adsorption did not drop below 98%. Adsorption to sediment 1 which contained no clay and very little silt was considerably less than the other sediments for coxsackievirus and echovirus but only slightly less for poliovirus. The fact that poliovirus is strongly adsorbed regardless of clay content of the sediment indicates a structural and/or charge-related difference from coxsackievirus and echovirus. When the average adsorption for each virus is plotted against the per cent clay in the sediment, the resultant line resembles that of a power curve described by the equation $y=ax^b$, $(a \neq o)$. Figure 2 shows the resultant theoretical curves

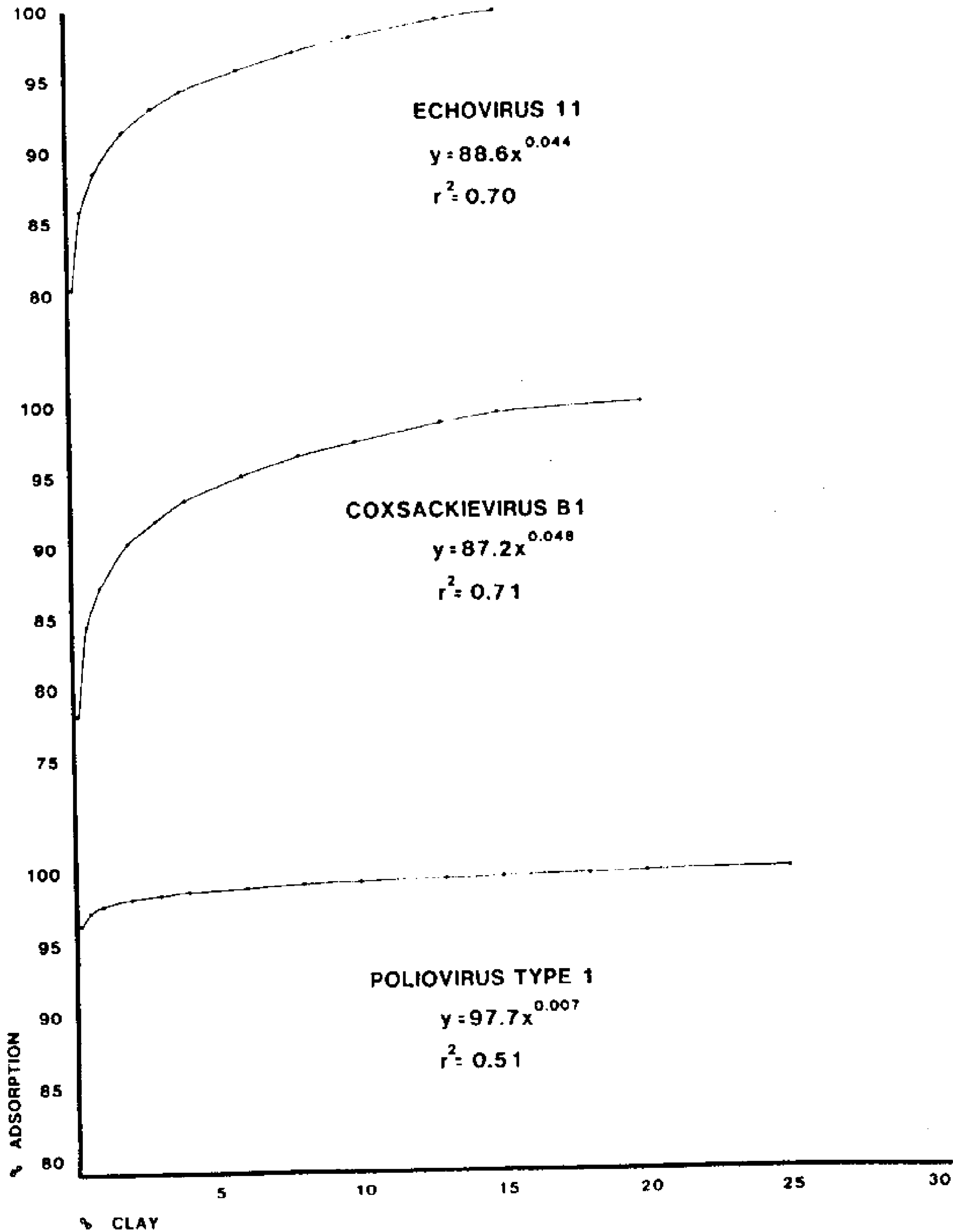
Table 2

ADSORPTION OF VIRUS
TO SEDIMENT

VIRUS	NUMBER OF EXPERIMENTS	SEDIMENT NUMBER 1	2	3	4
POLIOVIRUS 1	27	93.4 ± 4.3[1]	99.8 ± 0.3	98.3 ± 2.7	99.9 ± 0.1
COXSACKIEVIRUS B1	16	64.6 ± 13.6	98.4 ± 0.9	98.6 ± 0.9	99.0 ± 0.7
ECHOVIRUS 11	15	66.6 ± 12.6	98.9 ± 0.6	98.9 ± 0.7	99.5 ± 0.4

[1]Average per cent adsorption ± standard deviation

Figure 2: Theoretical Equations and Their Plots Resulting From Power Curve Regression Analysis for Each Virus' Adsorption versus the Per Cent Clay In the Sample



when individual adsorptions (each experiment) for the three viruses are subjected to a power curve regression analysis. Note that since the regression analysis formula does not allow either variable to equal zero, a value approaching zero (0.001%) was substituted for the per cent clay in sediment 1. The correlation coefficients for the three equations indicate that poliovirus type 1 (r^2=0.51) is not described well by the equation. However, coxsackievirus B1 and echovirus 11 (r^2=0.71 and 0.70, respectively) give a considerably better, albeit imperfect, fit. Adsorption of some virus to sand, silt and organic components of the sediments probably lowers the correlation between adsorption and per cent clay. The fact that poliovirus does not follow the power curve model indicates a non-preference for which type of material it binds to.

Elution of Virus from Sediment

Previous studies in this lab indicated that nutrient broth (Difco), Inolex beef extract and isoelectric casein (Difco) all had potential for the elution of virus from Mississippi estuarine sediments (46). Table 3 summarizies initial experiments to determine the effectiveness of these mixtures to elute poliovirus type 1 from the four sediments studied. With the exception of 4% nutrient broth (pH 7.5) the results were extremely variable. The lowest recoveries were almost always obtained from the sediments containing the highest amount

Table 3

PER CENT ELUTION OF POLIOVIRUS FROM SEDIMENTS USING NUTRIENT BROTH, ISOELECTRIC CASEIN AND BEEF EXTRACT SOLUTIONS

		SEDIMENT NO.[2]				
ELUENT[1]	pH	1	2	3	4	AVERAGE FOR ALL SEDIMENTS
4% NUTRIENT BROTH	9.0	32.5	2.0	3.3	2.0	10.0
4% NUTRIENT BROTH	7.5	6.2	10.4	7.3	5.3	7.3
5% INOLEX BEEF EXTRACT	9.0	48.9	3.1	0.6	1.8	13.6
0.5% ISOELECTRIC CASEIN	9.0	65.3	0.3	29.4	1.2	24.0

[1]Prepared in 0.2M TRIS buffer containing 0.85% NaCl

[2]Each value represents an average of two replicates

of clay (Numbers 2 and 4). Isoelectric casein (0.5%, pH 9.0) produced the highest overall recovery (24.0%) and also yielded satisfactory recoveries from sediments 1 and 3 (65.3% and 29.4%, respectively); however, elution from the remaining two sediments was poor (less than 2%). Nutrient broth (4%, pH 7.5) produced the most consistent recovery of the four sediments tested but the overall recovery was less than the other three eluents. The overall recovery of virus with Inolex beef extract was slightly higher than with either of the nutrient broth eluents. The higher concentration of Inolex beef extract than nutrient broth (5% vs. 4%) is the most likely reason for this difference. However, Difco nutrient broth contains only 38% beef extract and this may also have contributed to lower recoveries. It is possible that the peptones present in the nutrient broth may have in some way hindered the elution of virus but this is not considered likely.

Table 4 shows the elution profile of poliovirus type 1 from the four sediments using various lecithin solutions. Again, recovery of virus is usually higher from sediments containing little or no clay. The recoveries for the first three elutents are quite similar for each sediment and this is reflected by a similarity in overall recovery. Elution of the four sediments using 0.2% purified lecithin is extremely poor (less than 1%) with an overall recovery of only 0.5%. Adsorption of viruses to a

Table 4

PER CENT ELUTION OF POLIOVIRUS
FROM SEDIMENTS USING LECITHIN SOLUTIONS

ELUENT[1]	SEDIMENT NO.[2] 1	2	3	4	AVERAGE FOR ALL SEDIMENTS
1% CRUDE SOYBEAN LECITHIN	38.8	1.0	0.7	0.5	10.3
1% SEMI-PURIFIED SOYBEAN LECITHIN	33.5	0.03	3.8	0.0	9.3
1% EGG LECITHIN	40.2	0.02	3.7	0.02	11.0
0.2% SYNTHETIC LECITHIN	0.0	0.7	0.9	0.3	0.5

[1]Prepared in 0.2M TRIS buffer at pH 9.0 containing 0.85% NaCl

[2]Each value represents an average of two replicates

surface is thought to be primarily due to electrostatic bonds (5, 6). These bonds are in a constant state of flux, breaking and re-forming. Due to its ability to act as a surfactant, lecithin might be able to modify the bond kinetics and allow the electrostatic bonds to be broken. If proteins in a solution are able to attach to a site previously bound to a virus, the virus would be prevented from re-attaching to that site. After enough sites are covered, the virus might be able to break free of the sediment. This could explain the results seen in Table 4. The crude extract would be expected to contain the greatest amount of extraneous protein. However, the semi-purified and egg preparations also contain a significant amount of protein. Only the synthetic purified form contains little or no protein. If no proteins are present to cover the binding sites as the bonds are broken, then the virus is allowed to re-attach to the sediment, hence the low recovery with purified lecithin alone. Based upon this assumption a two-component eluent appeared necessary for efficient virus desorption. Due to the satisfactory recovery from two of the sediments using 0.5% isoelectric casein, it was used as the basic solution to which various types and concentrations of lecithin were added for further testing. Preliminary experiments were performed to ensure that lecithin did not harm BGM cells in tissue culture. Lecithin mixtures (prepared in the aforementioned basal

eluent) of 1%, 3%, 5%, 8% and 10% were tested for their effect on tissue culture cells for one hour (the adsorption time used in the plaque assays). Concentrations of 1% and 3% had no effect on the cells. However, concentrations higher than 3% caused cell detachment after 30 minutes possibly due to the high viscosity of these solutions.

Table 5 summarizes the results of experiments which incorporated a crude extract of soybeans as the lecithin source. When 1% lecithin was used in the eluent, the recovery of poliovirus from sediment 1 was reduced from 65.3% for 0.5% IC (Isoelectric casein) alone and 38.8% for 1% crude soybean lecithin alone to 25.8% using the combined mixture. However, the overall recovery of virus from the other three sediments was increased. Recovery of coxsackievirus from each sediment was similar to echovirus with the overall recovery of coxsackievirus higher than that of echovirus and nearly equal that of poliovirus. The greatest average recovery for each sediment was with sediment 2 (31.4%). Average recoveries for sediments 1 and 3 were similar (22.3% and 20.9%, respectively) followed by sediment 4 (9.2%). If the mechanism for desorption described above is appropriate, the increase in recovery from sediments 2 and 4, which contain the greatest amount of clay and theoretically the largest number of binding sites, could be explained by the larger number of proteins available to cover these sites. When the concentration of

Table 5

PER CENT RECOVERY OF ENTEROVIRUSES USING SOYBEAN LECITHIN SUPPLEMENTED ELUENTS

ELUENT[1]	VIRUS[2]	SEDIMENT NO.[3] 1	2	3	4	AVERAGE FOR ALL SEDIMENTS
1% CRUDE SOYBEAN LECITHIN	P	25.8	17.8	38.6	7.0	22.4
	C	22.5	37.8	12.6	13.9	21.7
	E	17.0	42.4	6.7	5.6	17.9
AVERAGE FOR ALL VIRUSES		22.3	31.4	20.9	9.2	
3% CRUDE SOYBEAN LECITHIN	P	14.3	6.8	25.4	1.0	11.8
	C	45.6	80.9	40.2	21.8	47.1
	E	46.7	94.7	43.5	33.2	54.5
AVERAGE FOR ALL VIRUSES		35.5	60.8	36.4	18.7	

[1] In 0.2M TRIS buffer (pH 9.0) with 0.5% Isoelectric Casein and 0.85% NaCl

[2] P, Poliovirus Type 1, Sabin; C, Coxsackievirus B1; E, Echovirus Type 11

[3] Each value is the average of at least two replicates

lecithin in the eluent was increased to 3%, the recoveries for coxsackievirus and echovirus increased dramatically; however, the recovery for poliovirus dropped by approximately half. Echovirus exhibited the highest elution (54.5%) followed closely by coxsackievirus (47.1%). Poliovirus recovery dropped to 11.8%. An increase in the amount of protein would be expected to produce higher recoveries and does in the cases of coxsackievirus and echovirus. The lower recovery of poliovirus may indicate the presence of some interfering substance in the crude extract. Average elution of sediment 2 was again the greatest (60.8%). Averages for sediments 1 and 3 were within 1% (35.5% and 36.4%, respectively). The lower recoveries (compared to sediment 2) from sediment Numbers 1 and 3, seen here and with the 1% lecithin eluent above, may result from the desorption of proteins from the sand and/or silt components allowing virus to re-attach to those particles. The overall recovery for sediment 4 was lowest (18.7%) but more than twice as high than with the 1% crude soybean lecithin-containing eluent.

Results of experiments using two concentrations of semi-purified soybean lecithin are shown in Table 6. With the addition of 1% semi-purified soybean lecithin to the basal eluent the recovery of poliovirus from sediment 1 is 47.9%. Again, this is lower than that for 0.5% IC alone but higher than for 1% semi-purified lecithin alone.

Table 6

PER CENT RECOVERY OF ENTEROVIRUSES USING
SEMI-PURIFIED SOYBEAN LECITHIN SUPPLEMENTED ELUENTS

ELUENT[1]	VIRUS[2]	SEDIMENT NO.[3] 1	2	3	4	AVERAGE FOR ALL SEDIMENTS
1% SEMI-PURIFIED SOYBEAN LECITHIN	P	47.9	0.5	0.3	0.3	19.5
	C	55.6	55.1	61.6	48.4	55.1
	E	81.9	66.3	68.6	31.6	62.1
AVERAGE FOR ALL VIRUSES		61.8	40.6	53.1	26.7	
3% SEMI-PURIFIED SOYBEAN LECITHIN	P	75.1	9.7	61.4	2.6	37.2
	C	54.4	91.5	78.4	55.7	70.0
	E	58.9	78.5	69.0	56.8	65.8
AVERAGE FOR ALL VIRUSES		62.8	59.9	69.6	38.3	

[1, 2, 3] See Table 5 for explanation of notes

Recovery was less than 1% for the other three sediments and may be due to the lower concentration of protein present in the semi-purified product. Overall recovery of poliovirus was 19.5%. Elution of coxsackievirus was satisfactory and consistent. Recovery ranged from 48.4% for sediment 4 to 61.6% for sediment 3 with an overall recovery of 55.1%. Elution of echovirus was higher than that for coxsackievirus but less consistent. Recovery was highest for sediment 1 (81.9%) and lowest for sediment 4 (31.6%) with an overall recovery of 62.1%. Increasing the concentration of lecithin to 3% resulted in an increase in recovery for all but two of the virus-sediment combinations. Elution of poliovirus, although higher than with the 1% solution, was variable allowing good recoveries with sediments 1 and 3 and poor recoveries with the remaining two. Overall recoveries of coxsackievirus and echovirus rose with the concentration of lecithin in the eluent even though the recoveries of each decreased for sediment 1. In general, recoveries of all three viruses increased and became less consistent for coxsackievirus and more consistent for echovirus while the consistency of poliovirus recovery remained approximately the same. The increase in recovery over the less pure form is attributable to the greater overall concentration of lecithin in the eluents.

Table 7 summarizes the experiments performed using lecithin derived from egg yolk. A 1% solution of this eluent produced much the same elution profile for poliovirus that was seen using 0.5% IC alone. Elution of coxsackievirus was not as consistent as with 1% semi-purified soybean lecithin but produced approximately the same overall recovery. Elution of echovirus was greatly increased with elution from sediment 1 being 138.4%. The recovery of more than 100% may be due to the disaggregation of virus clumps by the eluent. With an increase in the lecithin concentration to 3% the recovery of poliovirus was increased although recovery from sediments 2 and 4 remained low. The increase in poliovirus recovery after an increase in the concentration of semi-purified (and egg) lecithin indicates that there is an interfering or aggregating substance in the crude extract which is not present or has no effect in the two purer forms. Elution of coxsackievirus from the four sediments increased slightly from 56.7% to 64.0%. Elution of echovirus dropped dramatically from 93.9% to 46.8% and became very consistent (range, 43.4% for sediment 4 to 50.4% for sediment 2). The variations seen between recoveries using semi-purified soybean lecithin and those with egg lecithin could be caused by differences in the types and effectiveness of the proteins and lecithins found in the two preparations.

Table 7

PER CENT RECOVERY OF ENTEROVIRUSES USING EGG LECITHIN SUPPLEMENTED ELUENTS

ELUENT[1]	VIRUS[2]	SEDIMENT NO.[3]				AVERAGE FOR ALL SEDIMENTS
		1	2	3	4	
1% EGG LECITHIN	P	56.4	0.8	36.3	0.3	23.4
	C	64.9	54.5	69.0	38.6	56.7
	E	138.4	90.1	80.6	66.7	93.9
AVERAGE FOR ALL VIRUSES		86.6	48.4	62.0	35.2	
3% EGG LECITHIN	P	102.6	1.1	65.5	0.6	42.5
	C	41.9	67.6	73.4	73.1	64.0
	E	44.6	50.4	48.8	43.3	46.8
AVERAGE FOR ALL VIRUSES		63.0	39.7	62.5	38.7	

1, 2, 3 See Table 5 for Explanation of notes

Table 8 summarizes experiments using a purified preparation of a synthetic lecithin. The low concentration used is a reflection of the cost of the product (approximately $60.00 per gram) and the purity (99.0%). Elution of poliovirus is, again, very inconsistent. Recovery from sediment 1 was 109.2% while less than 0.1% was recovered from sediments 2 and 4. The recoveries for sediments 1 and 3 were approximately twice those of 0.5% IC alone. This may reflect an enchancement of the ability of isoelectric casein to elute poliovirus from sand since elution with 0.2% synthetic lecithin alone recovered less than 1% of the adsorbed virus (Table 4). The possible need for a higher concentration (or different type) of protein for the elution of sediments 2 and 4 is therefore understandable. With no proteins added by the synthetic lecithin preparation, the recoveries are even lower than with 0.5% isoelectric casein alone. Elution of coxsackievirus and echovirus was more consistent than that of poliovirus but less so than with some other eluents. The low recovery of coxsackievirus from sediment 1 and high recoveries from the other three sediments is a pattern not seen with any other eluent. These may also be attributable to the lack of additional proteins. A different protein than isoelectric casein may be required for better coxsackievirus recovery from sediments which are mainly sand.

Table 8

PER CENT RECOVERY OF ENTEROVIRUSES USING SYNTHETIC LECITHIN SUPPLEMENTED ELUENTS

ELUENT[1]	VIRUS[2]	SEDIMENT NO.[3] 1	2	3	4	AVERAGE FOR ALL SEDIMENTS
0.2% SYNTHETIC LECITHIN	P	109.2	0.08	52.0	0.02	40.3
	C	29.7	80.3	95.0	73.0	69.5
	E	53.1	46.8	57.6	31.3	47.2
AVERAGE FOR ALL VIRUSES		64.0	42.4	68.2	34.7	

[1, 2, 3] See Table 5 for explanation of notes

Table 9 contains the average recoveries of each virus from each sediment for all lecithin-IC eluents tested. The recovery pattern from all four sediments for each of the viruses tested is echovirus > coxsackievirus > poliovirus. This pattern corresponds to that seen with 3% soybean lecithin, 1% semi-purified lecithin and 1% egg lecithin. The elution pattern coxsackievirus > echovirus > poliovirus was produced by 3% semi-purified lecithin, 3% egg lecithin and 0.2% synthetic lecithin. The other eluent (1% soybean lecithin) produced the pattern poliovirus > coxsackievirus > echovirus. These data combined with the similarity of the coxsackievirus and echovirus adsorption curves and their dissimilarity to the poliovirus curve (Figure 2) may point to differences in the surface makeup of the viruses and/or the manner in which they interact with the sediment. Coxsackievirus and echovirus appear to be more similar to each other than to poliovirus. The recovery pattern of each sediment with all three viruses for all seven lecithin-IC eluents is 1 > 3 > 2 > 4 (Table 9). This pattern is the most common and is seen for 1% semi-purified lecithin, 1% egg lecithin and 3% egg lecithin. The pattern 3 > 1 > 2 > 4 is seen for 3% semi-purified lecithin and 0.2% synthetic lecithin. Two other patterns also observed are 2 > 1 > 3 > 4 (1% lecithin) and 2 > 3 > 1 > 4 (3% lecithin). The most common of the patterns corresponds to the order of increasing clay

Table 9

AVERAGE PER CENT RECOVERIES
FOR EACH VIRUS USING ALL ELUENTS

	SEDIMENT NO.				
VIRUS[1]	1[2]	2	3	4	AVERAGE (ALL VIRUSES)
P	59.2	6.1	43.7	2.0	27.7
C	43.4	64.9	58.2	44.2	52.7
E	62.9	67.0	53.5	38.3	55.4
AVERAGE (ALL SED.)	55.0	45.5	51.8	27.9	

[1]P,Poliovirus type 1; C,Coxsackievirus B1; E,Echovirus 11

[2]Each value is an average of at least 56 experiments

concentration in the sediments. This indicates that the amount of clay in the sample may be the determining factor for the elution of virus from a sediment. The high charge concentration and large surface area of clay particles makes this possibility even more attractive.

Table 10 summarizes the ability of each eluent tested to elute all viruses tested from all sediments tested. This is an important consideration since neither the composition of the sediment nor the types of viruses are likely to be known for a given natural sample. Four of the seven eluents tested produced average recoveries above 50%. One per cent egg lecithin and 3% semi-purified soybean lecithin gave the best recoveries and differed by less than 0.5%. However, 0.2% synthetic lecithin, 3% egg lecithin and 1% semi-purified lecithin all have recoveries within 8% of each other and within 13% of the two best eluents. The positive effect which the lecithin preparations exert could be attributed to the additional proteins present in the product if not for the good recoveries with 0.2% synthetic lecithin which contains very little if any extraneous protein. The purity of the egg yolk and semi-purified soybean preparations is given as approximately 60% and 50%, respectively, based on choline determinations done by the manufactoring company. This translates to approximately a 0.6% and 1.8% pure lecithin concentration for 1% and 3% solutions of the egg

Table 10

OVERALL RECOVERIES (ALL VIRUSES AND ALL SEDIMENTS FOR EACH LECITHIN ELUENT)

LECITHIN TYPE		PER CENT RECOVERY[1]
1%	EGG	58.0 ± 52.9[2]
3%	SEMI-PURIFIED SOYBEAN	57.6 ± 31.0
0.2%	SYNTHETIC	52.3 ± 37.3
3%	EGG	51.1 ± 35.2
1%	SEMI-PURIFIED SOYBEAN	45.6 ± 26.5
3%	CRUDE SOYBEAN	37.8 ± 30.1
1%	CRUDE SOYBEAN	21.0 ± 15.3

[1]Each value is an average of at least 24 experiments

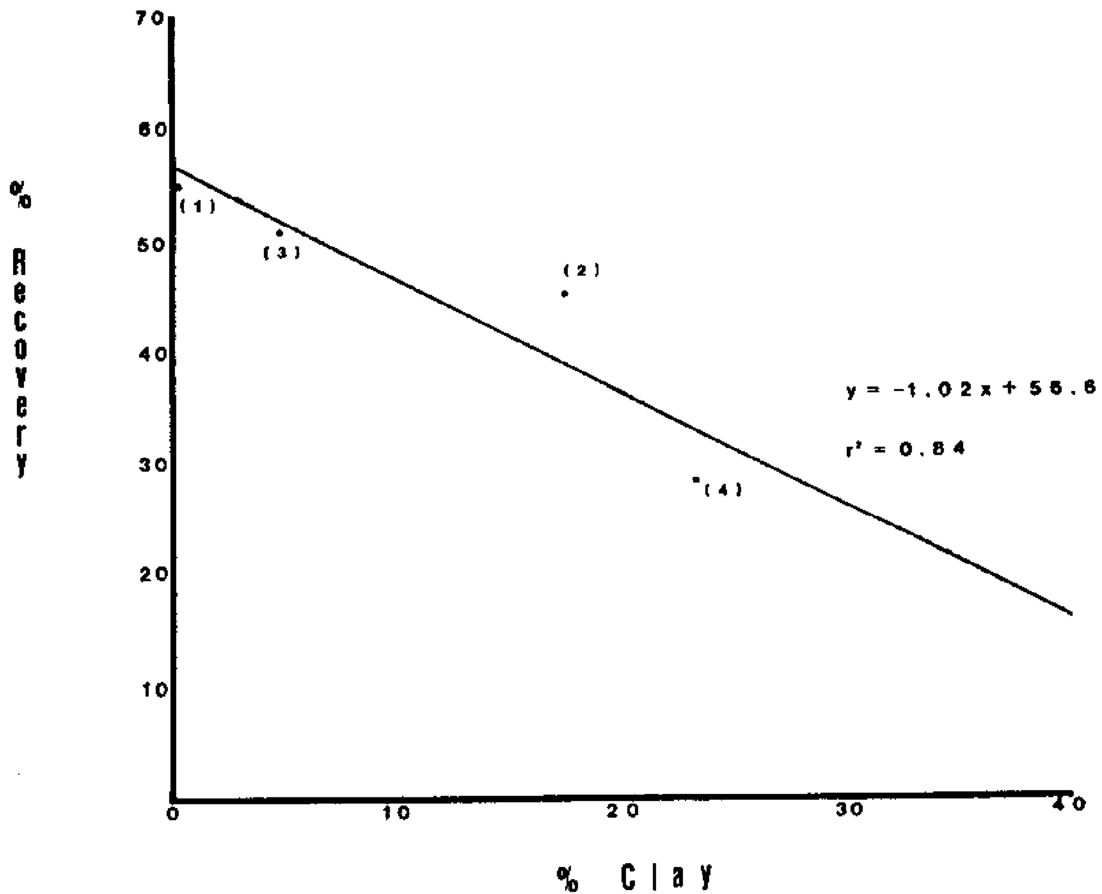
[2]Per cent recovery ± standard deviation

yolk-derived compound and a 0.5% and 1.5% pure lecithin concentration for the soybean derived compound. Experiments using a higher concentration of the synthetic preparation might demonstrate the positive effect of lecithin on the elution of virus although the cost might be prohibitive.

In an environment where electrostatic forces determine the binding of one surface to another, bonds are constantly breaking and re-forming. The ratio of the number of bonds formed to the number of bonds broken is a binding constant describing how well the surfaces are held together. It is possible that lecithin, which can act as a surfactant, might lower the binding constant by breaking bonds or preventing them from forming. The protein present in the eluent could then bind to the charges on the sediment preventing bonds between the virus and particle from re-forming. This model would explain the success of the two-fold eluent composed of the lecithin source and a protein source (isoelectric casein).

As discussed previously, the amount of clay in a sediment is thought to influence the interaction of viruses with the sediment particles. Figure 2, mentioned earlier, illustrates the possible effects of clay on adsorption. It is logical to assume that clay also affects the viruses' subsequent elution from sediment. Figure 3 shows the average recoveries for all eluents and all viruses from

Figure 3: Theoretical Equation and Its Plot Resulting From Linear Regression Analysis of Average Overall Recovery Using Each Sediment versus the Per Cent Clay in Each Sediment



each sediment plotted against the per cent clay in that sediment. The data were subjected to a linear regression analysis and the line corresponding to the resulting equation is shown. The correlation coefficient for this equation is 0.84 and the resulting line has a negative slope indicating a trend toward lower recoveries as the clay concentration is increased. It is generally believed that the large number of surface charges, the large surface area and the complex structure of many clays make it difficult to elute virus. However, caution must be used when interpreting the regression analysis due to the small number(4) of clay concentrations used.

CHAPTER V

CONCLUSIONS

Poliovirus, coxsackievirus and echovirus adsorb well to sediments which contain even small amounts of clay. Poliovirus also showed a high affinity for sediment 1 which contained no clay. Coxsackievirus and echovirus were not able to adsorb as well to this sediment. The ability to elute virus from the sediments seems to be directly dependent on the amount of clay in the sample. Differences in the elution profile of each virus were seen, indicating a difference in surface structure between the viruses. Coxsackievirus and echovirus appear to be more closely related to each other than to poliovirus. The best eluents for enterovirus recovery were 1% egg lecithin and 3% semi-purified soybean lecithin although all but the two crude lecithin eluents produced comparable results.

Future studies in this area should include more sediments of known composition. More complete characterization of the sediments is needed. This might include organic matter content, average and range of particle sizes, types and amounts of heavy metals present and most importantly, the types and amounts of clays in the sample. Other experiments could be conducted using

sediment which had been separated into its component fractions. A more complete understanding of virus adsorption is also vital. An experiment wherein a sediment is pre-treated with a protein solution (possibly the eluent) before attempting to adsorb the virus would provide information on the role of proteins in virus elution. If less virus adsorbs after pre-treatment, then the protein is probably covering many of the binding sites thus preventing the virus from attaching. The use of many sediments with different amounts of sand, silt and clay would allow for better statistical testing than can be done for four values.

APPENDIX A

MEDIA AND REAGENTS

<u>BGM Growth Media</u>

L15	440.0 ml
Newborn Calf Serum	50.0 ml
Antibiotic-Antimycotic Mixture (100X)	5.0 ml
L-Glutamine (29.2 mg/ml) Solution (100X)	5.0 ml
Gentamicin (10 mg/ml)	1.0 ml

L15: Lebovitz's L15 medium. Powdered medium was dissolved in reverse osmosis, type 1 water and sterilized by membrane filtration.

<u>ATV(10X) Versene Dispersant</u>

NaCl	80.0 g
KCl	4.0 g
Dextrose	10.0 g
Trypsin (Difco 1:250)	5.0 g
Na_2HCO_3	5.8 g
EDTA	2.0 g
Type 1 Water	QS to 1 liter

Overlay Medium

Nutrient Solution:

L15 (2X)	43.0 ml
Newborn Calf Serum	2.0 ml
Antibiotic-Antimycotic Solution (100X)	1.0 ml
L-Glutamine (29.2 mg/ml) Solution (100X)	1.0 ml
Non-Essential Amino Acids (100X)	1.0 ml
1% $MgCl_2$	1.0 ml
Gentamicin	0.2 ml
1:300 Neutral Red Solution	0.6 ml

Agar Solution

Nobel Agar	1.5 g
Type 1 Water	50.0 ml

After autoclaving the agar solution both solutions were adjusted to 45°C. The solutions were combined just before used. $MgCl_2$ and neutral red should only be added after the two solutions are mixed to prevent precipitation of other components.

REAGENTS

Antibiotic-Antimycotic Mixture

Penicillin, Streptomycin and Fungizone (PSF,100X) purchased from Grand Island Biological Company, Cat. No. 600-5240.

Newborn Calf Serum

Purchased from Sterile Systems, Inc.

L-Glutamine

L-Glutamine (200 mM, 29.2 mg/ml) purchased from Grand Island Biological Company, Cat. No. 320-5030.

MEM Non-Essential Amino Acids

MEM Non-Essential Amino Acids (100X) purchased from Grand Island Biological Company, Cat. No. 320-1140.

Neutral Red

Neutral Red Solution (3333.0 mg/L, Sodium Salt) purchased from Grand Island Biological Company, Cat. No. 630-5330.

Tris (hydroxymethyl) aminomethane Buffer

24.2 g Tris (hydroxymethyl) aminomethane (enzyme grade) was dissolved in 1000 ml type 1 water (0.2 M) was used to adjust the pH to 9.0.

APPENDIX B

SUMMARY OF LECITHIN
TYPES USED

Designation	Supplier	Cat. No.	Approx. % Lecithin
Crude Lecithin Soybean Extract Technical Grade	Matheson, Coleman and Bell	LX210	10 - 20
Semi-Purified Lecithin, Type IV-S From Soybean	Sigma	P3644	40 - 50
Semi-Purified Lecithin, Type X-E From Dried Egg Yolk	Sigma	P5394	60
DL-α-Phosphatidyl-choline, Dipalmitoyl Grade I; Crystalline, Synthetic	Sigma	P6138	99

APPENDIX C

EXPERIMENTAL MATERIALS

ITEM	MANUFACTURER
Centrifuge	Ivan Sorrall Inc.
Filter holder	Millipore Co.
Forma bio-freezer	Forma Scientific
Incubator	Percival Mfg. Co.
Incubator shaker	New Brunswick Scientific
Inverted microscope	Olympus
Magnetic stirrer	E. H. Sargent & Co.
Media Filter (90 mm, 0.45 um)	Millipore Co.
Media holding tank for sterilization	Gibco Co.
Nalgene filter unit	Nalge Co.
pH meter	Sargent-Welch Scientific
Salinity (S‰), temperature (°C), conductivity (uMHO) and pH meter	Simpson Electric Co.
Sterile plastic ampule	Vanguard International
Tissue culture flask	Corning Glass Works
Tissue culture plates	Corning Glass Works
Vertical-flow laminar air hood	Baker Co., Inc.

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