VIRUS REJECTION BY THE REVERSE OSMOSIS - ULTRAFILTRATION PROCESSES

Technical Report
to the

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and Development Center

by

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ABSTRACT

Rejection of viruses by commercial grade asymmetrical cellulose acetate membranes commonly used in the ultrafiltration and reverse osmosis processes was evaluated. Various concentrations of coliphage T2 and poliovirus were inocculated in the feed water of a bench scale ultrafiltration-reverse osmosis unit and virus concentrations in the product were determined.

The insoluble polyelectrolyte technique for concentrating extremely low levels of virus was modified and standardized for use in this study. The effectiveness of this technique to quantitatively detect low virus concentrations was found to be directly dependant upon the pH of the suspending medium and the type virus employed.

Membrane penetration studies indicated that limited numbers of virus passed the membranes. The penetration of viruses may be attributable to the presence of random areas of imperfect crosslinkage of the cellulose acetate in the dense layer of the membrane. These random areas of imperfection appear to be minimized

for the tightest membrane used in this study. Despite limited virus penetration, all of the cellulose acetate membranes used in this study rejected an extremely high percentage of the viruses and provided a product water of excellent quality.

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SYMBOLS AND ABBREVIATIONS

pfu	Plaque Forming Unit
gpd/ft^2	Water Flux - gallons per day/square foot of membrane surface area
PBS	Phosphate Buffered Saline
HBS	Hank's Balanced Saline

CHAPTER I

Introduction

Major epidemics have been attributed to water-borne viruses. Horsfall (1957) indicates that the average person in the United States is afflicted by viral diseases during ten percent of his life.

About one hundred identifiable enteric viruses have been found in human feces. These viruses are classified in six main groups and are presented in Table 1. More than seventy strains of viruses have been recovered from water and municipal wastewater during the past thirty years. Clark, et al. (1964) reported that 39 percent of all the samples of chlorinated effluents of conventional treatment analyzed contained viruses.

The effectiveness of virus removal by water and wastewater unit processes has been under investigation for many years. This type of research has been necessitated by the epidemiological significance of viruses such as the obvious case of the New Delhi infectious hepatitis outbreak in 1955-56 (Dennis, 1959) as well as some of the less demonstrable water-borne virus-caused epidemics enumerated by Weibel, et al. (1964).

TABLE 1. ENTERIC VIRUSES AND ASSOCIATED DISEASES
(Grabow, 1968 and Andrewes, 1964)

Poliovirus	3	Paralytic poliomyelitis, aseptic meningitis
Coxsackie virus		
Group A	25	Herpangia, aseptic meningitis
Group B	6	Pleurodynia, aseptic meningitis, acute infantile myocarditis
ECHO-virus	32	Aseptic meningitis, rash and fever, diarrheal diseases, respiratory illness
Infectious hepatitis virus	?	Infectious hepatitis
Reovirus	3	Fever, respiratory infections, diarrhea
Adenovirus	30	Respiratory and eye infections

Conventional water and wastewater treatment processes do not remove all the soluble inorganic and organic matter, including viruses, from raw water sources or wastewater. Thus, advanced treatment processes ultimately must be included in the system to insure effective removals of these potentially harmful materials. One of the advanced treatment processes which shows considerable promise from a practical, economical standpoint is reverse osmosis.

The use of this process to desalt seawater and brackish water has been studied in both its basic and applied aspects for more than a decade. More recently, limited investigations have been directed toward the applicability of reverse osmosis to water renovation for reuse and the primary emphasis has been on the removal of chemical constituents.

The primary objective of this study was to determine the ability of asymmetrical cellulose acetate membranes commonly used in ultrafiltration and reverse osmosis operations, to reject viruses. The development of quantitative techniques for enumerating extremely low titers of virus was essential to meeting this objective. These techniques permit the use of virus titers which are normally

found in polluted natural waters, and eliminate the need for adding viruses to the samples at titers far in excess of that found under natural conditions.

Results of this study indicate that viruses can penetrate asymmetrical cellulose acetate membranes as employed under conditions common for reverse osmosis operations. Quantitative techniques employed for the detection of extremely low titers of virus are relatively accurate and dependable.

CHAPTER II

Evaluation of the Literature

Virus Levels in Natural Waters

Limited information relating to the quantitative enumeration of viruses in natural waters is available in the literature. Clark, et al., (1964) have calculated coliform bacteria-enteric virus ratios of about 92,000: 1 for sewage and about 50,000: 1 for polluted surface water. Gilcreas and Kelley (1955) found coliform indices of 95,000/ml, 75,000/ml, and 4,300/ml in a sewage treatment plant effluent, in a secondary sewage treatment plant outfall, and in a sample collected downstream from a secondary sewage treatment plant outfall. Conditions such as these could result in virus levels as high as 0.1 to 1 virus per milliliter in polluted waters. In fact, Grinstein, et al. (1970) recently demonstrated virus titers in the range of 2×10^{-2} to 2×10^{-1} pfu/ml in the Brays Bayou near Houston, Texas.

Detection of Viruses

One of the most difficult problems associated with virus and water supply has been the inability to quantitatively detect the presence of virus at low titers. This problem is even more

significant for surface waters which carry suspended particulate and colloidal materials.

The Committee on Environmental Quality Management of the Sanitary Engineering Division, American Society of Civil Engineers (1970) and the Committee on Viruses in Water of the American Water Works Association (1969) have indicated that considerable improvements in detection techniques must be achieved before reliable information regarding the presence or absence of viruses, in water supplies can be determined. Various methods of concentrating viruses including hydro-extraction (Shuval, et al., 1967), ultra-centrifugation (Anderson, et al., 1967), electrophoresis (Bier, et al., 1967), adsorption to precipitable inorganic salts (Wallis and Melnick, 1967), membrane chromatography (Cliver, 1968), soluble ultrafiltration (Gartner, 1967), two-phase polymer separation (Shuval, et al., 1967), fiber swab technique (Duff, 1970), and insoluble polyelectrolyte technique (Johnson, et al., 1967), have been proposed and evaluated. All of these methods result in manyfold virus concentration. However, most of these techniques are subject to interferences when large volumes of water are concentrated or when the water contains significant quantities of soluble organics or suspended solids. One of the most promising virus concentration methods

is the insoluble polyelectrolyte technique. Wallis, et al., (1969) have demonstrated the effectiveness of this technique in the presence of high concentrations of soluble organic materials and suspended solids. Employing poliovirus, Type 1, (LSC strain) they reported recoveries of up to 93 percent from virus seeded sewage over a pH range of five to six. Although no data were presented, these authors indicated that echovirus, Type 7, and coxsackie viruses A9 and B3 were tested and were found to "adsorb to the polyelectrolyte" under the same conditions as poliovirus. Wallis, et al., (1970) reported that for the same poliovirus used in the previous study, 94 - 99 percent of the seeded virus was recovered from tap water over a pH range of 5.0 to 8.4. Berg (1970), however, reported that this technique had not yet been efficient in the hands of others.

Virus Removal by Water and Wastewater Unit Processes

Grabow (1968) and Sproul (1970) have conducted extensive literature reviews on the effectiveness of virus removal by water and wastewater unit processes. More recently virus removal by activated carbon and by coagulation with alum and ferric chloride have been reported. A summary of these investigations is presented in Table 2. The data include the original citations, virus employed, processes used and general results obtained.

TABLE 2. SUMMARY OF RESEARCH IN VIRUS REMOVAL BY WATER AND WASTEWATER UNIT PROCESSES

Unit Process	Virus Investigated	Initial Titer (pfu/ml)	Virus Removal (%)	Reference
Primary Clarification	Polio 1 (Mahoney)	2×10^5	26 - 55	Clarke
	ECHO 1-14, Polio 1, 2, 3	(a)	(b)	Bloom
	Enterovirus (various strains)	(a)	(d)	Mack
	Coxsackie A13	2×10^8 (c)	(d)	Bush
	Coxsackie, Polio, ECHO (various strains)	(a)	(d)	Kelly
	Reovirus, Enterovirus	2×10^2 (e)	(b)	Malherbe
	Polio 1, 2, 3 (Sabin)	(f)	0 - 12	England
Trickling Filtration	Coxsackie A13	2×10^8 (c)	(d)	Bush
	Reovirus, Enterovirus	$<2 \times 10^2$ (e)	(b)	Malherbe
	Polio 1, 2, 3	(f)	(d)	England
Activated Sludge	Polio 1 (Mahoney)	7×10^4	79 - 94	Clarke
	Coxsackie A9	3×10^5	96 - 99.4	Clarke
	ECHO 1-14, Polio 1, 2, 3	(a)	(b)	Bloom
	Enterovirus (various strains)	(a)	(d)	Mack
	Coxsackie A13	2×10^8 (c)	(d)	Bush

Unit Process	Virus Investigated	Initial Titer (pfu/ml)	Virus Removal (%)	Reference
Activated Sludge (cont'd).	Coxsackie, Polio ECHO (various strains)	(a)	(d)	Kelly
	Reovirus, Enterovirus	$<2 \times 10^2$ (e)	(b)	Malherbe
	Polio 1, 2, 3 (Sabin)	(f)	76 - 90	England
	Polio 1, 2, 3 (Sabin)	(f)	(d)	Theios
	Polio 1 (MK 500)	$.02\text{-}2 \times 10^5$	64 - 78 (g)	Kelly
	Bacteriophage T2	$.3\text{-}5 \times 10^3$	0 - 98 (g)	Kelly
Oxidation Ponds	Reovirus, Enterovirus	$<2 \times 10^2$ (e)	(b)	Malherbe
	Polio 1 (attenuated)	5.6×10^5 (e)	92	Malherbe, Strickland Cholmley
	Polio 2 (attenuated)	7×10^7 (e)	(d)	Malherbe, Strickland Cholmley
Sand Filtration	Reovirus, Enterovirus	$<2 \times 10^2$ (e)	(b)	Malherbe
	Bacteriophage T4	1×10^4	36 - 99 (h)	Gilcreas, Kelly
	Coxsackie A5	(i)	10 - 99 (h)	Gilcreas, Kelly
	Polio 1 (attenuated) (Mahoney)	$1\text{-}6 \times 10^4$	1 - >99 (h)	Robeck
Coagulation/Flocculation	Coxsackie A5	(i)	40	Gilcreas, Kelly

Unit Process	Virus Investigated	Initial Titer (pfu/ml)	Virus Removal (%)	Reference
Coagulation/Flocculation (cont'd.)	Bacteriophage T4	1×10^4	83	Gilcreas, Kelly
	Bacteriophage F2	1×10^6	76 - 99.9(g)	York
	Coxsackie A2	1×10^3 (e)	≤99 (g)	Chang
	Bacteriophage	$2\text{-}6 \times 10^5$	≤99 (g)	Chang
	Polio 1 (Sabin)	$10^6 \times 10^7$	≤86 (j)	Thorup
	Bacteriophage T2	$3\text{-}7 \times 10^4$	≤94 (j)	Thorup
	Bacteriophage MS2	$3\text{-}5 \times 10^5$	≤99.8 (g)	Chaudhuri
	Bacteriophage MS2	$3\text{-}4 \times 10^5$	≤99.7 (g)	Manwaring
	Bacteriophage T3	1.3×10^9	(d)	Mack
Softening	Bacteriophage T2	1×10^6	60 - 99.9 (k)	Thayer
Carbon Adsorption	Polio	-------	(d)	Carlson
	Bacteriophage T4	1×10^8	≥99	Cookson
	Bacteriophage T2	$6\text{-}9 \times 10^5$	≥75	Sproul

NOTES:

a. Used naturally-occurring levels only
b. Techniques employed were not quantitative and provided virus isolation only
c. Approximately calculated from information in source
d. Techniques employed were not quantitative and only indicated absence of presence of virus although some removal was indicated
e. Values expressed in Tissue Culture Infectious Dose - 50/ml
f. Detected natural levels in sewage following immunization program
g. Removal efficiencies varied with test conditions
h. Removal efficiencies depended on flow rate, type of sand, and type of pretreatment. Maximum removal occurred at slow sand filtration flow rates.
i. Initial level of virus expressed as 8.0 x median effective dose required to produce limb paralysis in test mice
j. A coagulant aid was used also
k. Maximum removal occurred at a pH greater than 10.5

Inactivation of viruses by chlorination has long been studied. Experimental results of Weidenkoph (1958), Clarke and Kabler (1954), and Clarke, Stevenson and Kabler (1956) are compared with the inactivation of E. coli as determined by Butterfield, et al., (1943) in Figure 1. Significantly, hypochlorous acid is the active viricide during the chlorination process and its effectiveness is dependent upon the type of virus present. Recently, Morris (1971) has suggested that these results might not be extrapolated to naturally occurring levels of virus in water because of the protection afforded by even extremely low concentrations of small particulates found in finished waters.

Rejection of viruses by membrane processes has not been as extensively studied. Most of the work in this field has been limited to concentrating and purifying virus stocks (Wang, et al., 1969), and Chian and Selldorff, 1969). Because of the size of viruses and the generally accepted membrane transport theory, manufacturers of reverse osmosis equipment and membranes have long insisted that no viruses should appear in the product water. In a study of reclamation of wastewater by reverse osmosis which was primarily devoted to investigating chemical complex passage of membranes, Hindin, et al., (1968) innoculated the feedwater of a

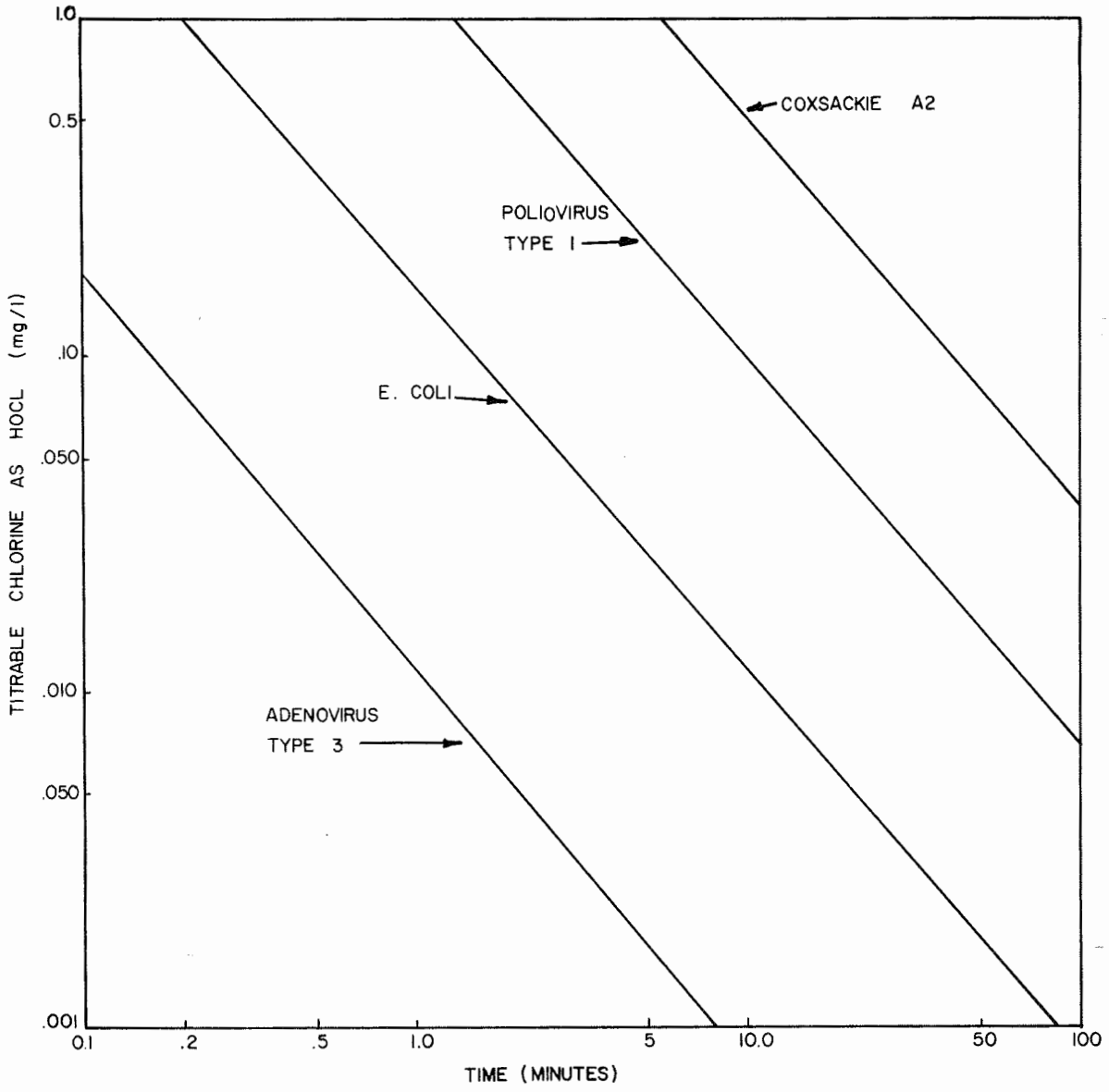

FIGURE 1. CONCENTRATION- TIME RELATIONSHIP FOR 99% DESTRUCTION OF E.COLI AND SEVERAL VIRUSES BY HOCL AT 0-6°C.

reverse osmosis unit in specified titers of the coliphages T7 and ΦX 175. Operating the unit with cellulose acetate membranes at a pressure of 100 atmospheres, a temperature of 25°C and a product flux of 15 to 20 gpd/ft^2, a few milliliters of product water was collected. These workers then attempted to isolate virus by directly plating part of the product water without concentrating the virus. No virus was found in the product water. In a related study, Ironside and Sourirajan (1967) indicated no E. coli penetration of laboratory-cast membranes. These membranes were annealed at 88°C and provided a product flux of about 30 gpd/ft^2 at an operating pressure of 68 atmospheres.

As shown in Table 2, all the previous studies have been conducted at virus titers of 10^4 to 10^9 plaque forming units (pfu)/ml. These high levels have been required to permit quantification by counting techniques in spite of any loss of virus within the process. Unless an effective virus concentration technique is employed, statistically realistic counting levels require minimal titers of 10^2 or 10^3 pfu/ml. Some obvious problems encountered with high virus levels are the clumping of virus, the lack of random dispersion in the solute and protection by organic materials from the medium in which the virus is normally maintained.

Cellulose Acetate Membranes and Transport Theory

The basic structural units of cellulose-type membranes are polymers about 15,000 A° in length and 10 to 20 A° in width (Ott, et al., 1954). These polymers have a random orientation in the membrane and crystalline regions are established when the chains run parallel to each other. Conversely, the spaces or holes (amorphous regions) between polymers are greatly enlarged when the chains lie in a disordered manner. Cellulose acetate exhibts the ability to hold a large percentage of the polymer chains in highly ordered regions (Baker, et al., 1942).

Reid and Breton (1959) have proposed two different mechanisms of transport through cellulose acetate membranes. Ions and molecules that can combine with the membrane through hydrogen bonding are presumed to be transferred from one hydrogen bonding site to another. This phenomenon as expanded by Vincent, et al., (1965) is considered to be the major water diffusion mechanism through "tight" cellulose acetate membranes. On the other hand, ions and molecules that cannot enter into hydrogen bonding are transported across the membrane through holes. This transport mechanism depends upon the probability of hole formation in the membrane. If

the basic pore structure of the cellulose acetate is filled with tightly bound water, the probability of hole formation is greatly reduced. Application of heat and pressure to cellulose acetate membranes increases the amount of "primary" bound water (Vincent, et al., 1965), thereby increasing the "tightness" of the membrane. In practice, real membranes will have characteristics of both of these mechanisms (Menten, 1966).

Friedlander and Rickles (1966), after a review of work in this field, have pointed out that transport through a cellulose diacetate membrane may be correlated by an equation of the form:

$$J = K_1 (\Delta P - \Delta \pi) + K_2 \Delta P$$

where J is the flux, ΔP is the differential operating pressure, $\Delta \pi$ is the differential osmotic pressure, and the constants K_1 and K_2 represent characteristics of the membrane which depend upon diffusion coefficients, solute concentrations and effective membrane thickness. The pressure dependent solute flux is generally attributed to very fine pores in the membrane and the presence of larger pores or imperfections more widely distributed throughout the membrane (Goff and Gloyna, 1970).

CHAPTER III

Materials and Methods

Suspending Media

Phosphate buffered saline (PBS) at pH 7.2 or natural surface water were used as suspending media. Surface water was obtained from Town Lake, a shallow man-made impoundment of the Colorado River at Austin, Texas. Town Lake receives storm drainage, urban runoff and some rural runoff in addition to the releases of six other man-made lakes upstream on the Colorado River.

Viruses

The coliphage T2 was used in the first part of this study. This bacteriophage is one of the most structurally complex viruses and consists of a polyhedral-shaped head about 95 mμ long and 65 mμ wide and a tail complex about 25 mμ in diameter and 100 mμ long (Adams, 1959). The tail is composed of a rigid central core and an outer sheath to which is attached a hexagonal baseplate containing six spikes and six long slender tail fibers (Hayes, 1968). The tail fibers are associated with the recognition of specific receptor sites on the wall of the host cell and, upon attachment to the cell, the sheath retracts pushing the central core through the cell wall.

Coliphage deoxyribonucleic acid is injected into the host cell for replication and ultimate virus reproduction. Coliphage T2 was selected for the initial investigative phases of this study because the cultures can be handled and maintained easily and their plaques may be enumerated reliably.

Stock cultures were prepared by the plate method using Tryptose-Phosphate Broth* solidified by one percent agar. Plaque assays omitting bottom layer agar were used to titrate the virus (Rizi and Nova, 1963). Two-tenths ml of a 24-hour E. coli B culture in 7.5 ml of Tryptose-Phosphate Agar were inocculated with samples ranging from 0.1 ml to 0.5 ml. Plaques were counted after 15 hours incubation at 37°C.

Coliphage T2 labeled with ^{3}H-thymidine was prepared in M9 medium (Eisenstark, 1967). The lysate was clarified by centrifugation at 2500 times gravity (2500 x g) for 15 minutes at 4°C. The virus was concentrated from the supernatant by high speed centrifugation (21,000 x g) in the cold for 60 minutes. The pellet was allowed to resuspend overnight in 1/10 the original volume of phosphate buffered saline. After an additional low speed centrifugation, 2 ml of virus was layered on a discontinuous sucrose

*A product of DIFCO Laboratories, Detroit, Michigan

gradient (4 ml each of 37.5 percent, 33.8 percent, 30.0 percent, 26.3 percent, 22.5 percent, 18.8 percent, and 15 percent) and centrifuged in the cold at 21,000 x g for 45 minutes. The virus banded at the interface between the 18.8 percent and 22.5 percent sucrose layers. It was removed in 4 ml of sucrose with a Pasteur pipette. The plaque titer was 1.5×10^{10} pfu/ml and the radioactivity 2.8×10^{4} cpm/ml as determined by counting in Cabosil with a Nuclear-Chicago Scintillation counter, Model Mark I.

Poliovirus has cubic symmetry and is roughly spherical in shape with a diameter of about 25 mμ (Andrewes, 1964). Attachment to the host cell can be accomplished at any of a large number of attachment sites located on the exterior portion of the virus. The major chemical constituent of this virus is ribonucleic acid. The poliovirus virulent strain Mahoney Type I (PIM) was selected for use in this study because it can be a causative agent for paralytic poliomyelities, it is similar in size and shape to the smallest enteric viruses listed in Table 1 (page 2) (the hepatitis viruses being a possible exception), it is very stable over a pH range of four to ten, it is stable over the temperature ranges used in the study, and it is relatively easy to assay.

Stock suspensions of the virus were prepared in HeLa cells which were grown in suspension, centrifuged and washed twice with Hank's Balanced Saline solution (HBS). The cells were diluted with warm HBS minus serum so as to provide a concentration of approximately 5×10^5 cells/ml. Two milliliters of virus suspension were used to inocculate the HeLa cell suspension providing about 10 pfu/cell. The virus was allowed to adsorb for 30 minutes at 37°C with periodic shaking. These cultures were then incubated in suspension for 24 hours. The virus was harvested by employing three cycles of rapid freezing and thawing followed by low speed centrifugation for ten minutes to remove cell debris. The resulting stock virus suspension had a titer of 25×10^9 pfu/ml.

Virus assay was performed by inocculating a monolayer of HeLa cells with either 0.5 or 1.0 ml of virus suspension. Virus was allowed to adsorb for 30 minutes at 37°C with periodic shaking. The HeLa cell monolayer with adsorbed virus was then overlayed with eight to ten ml of Eagles-Minimum-Medium containing one percent Bacto-agar* and incubated for 48 hours at 37°C in a humidified atmosphere of five percent CO_2. Plaque development was observed after staining viable cells with nine ml of neutral red in HBS for two hours at 37°C.

*A product of DIFCO Laboratories, Detroit, Michigan

Virus Concentration Method

The insoluble polyelectrolyte technique for concentrating virus suspensions employed in these studies has been described previously by Wallis, Grinstein, Melnick and Fields (1969). The polyelectrolyte PE 60* was used in the water-washed form in all experiments. A constant concentration of 100 mg/l of the polyelectrolyte was used and all samples were stirred mechanically for one hour. The pH was adjusted with 1N HCl immediately after the addition of the polyelectrolyte. After stirring, the suspension was filtered through 0.8 micron filters when PBS was the suspending medium and fiberglass filters when lake water was the suspending medium. The polyelectrolyte was then lifted from the filter with a spatula.

The recovered polyelectrolyte was suspended in five ml borate buffer, pH nine to which ten percent calf serum was added for one liter samples. Eight ml borate buffer-calf serum solution was used for five liter samples. The polyelectrolyte-borate-serum suspension was shaken on a horizontal mechanical shaker for five minutes to affect maximum elution of the virus from the polyelectrolyte. The suspension was then centrifuged at 500 x g for five minutes. If

*A product of the Monsanto Company, St. Louis, Missouri

sterile artificial medium was used for initial virus suspension, the supernatant was transferred directly for plating. When natural waters were used for initial virus suspension the supernatant was filtered through 0.45 micron filters to remove bacteria which could contaminate plaque assay cells.

Experimental Control and Replication of Results

For each run a control or working stock virus suspension was prepared by diluting the concentrated virus stock to the desired working level. The virus titer of the control was determined by plating four to ten plates each with a volume of from 0.1 to 1.0 ml. The plating volume was selected so that 30 to 80 plaques would develop on each plate. These limits were chosen for three reasons:

a. To provide ease of counting

b. To minimize the opportunity for multiple infectivity which results at high plate counts.

c. To minimize excessive variation from the mean of a plating series resulting from low plate counts.

Experimental data which did not fall within the established limits was considered unacceptable and therefore not used for analysis. An illustration of a typical control plating series is shown in Table 3.

TABLE 3. RESULTS OF PLATING A CONTROL COLIPHAGE T2 SUSPENSION

Series and Volume Plated (ml)	A 0.5	B 0.1	C* 0.5
	145	39	10
Individual	159	37	17
Plate Counts	162	32	18
Plaques	191	37	22
	183	33	11
Sum (Σ)	840	178	78
Mean ($\bar{x}$)	168	35.6	15.6
Standard Deviation (σ)	16.6	2.6	4.5
Titer (pfu/ml)	336	356	312

*Series C is a 10:1 dilution of the working stock

After concentrating the virus from test samples, the borate-calf serum-virus suspension separated from the polyelectrolyte was either diluted for plating for high titers or, when only up to few hundred virus were expected in the eluate, the total separated fraction was plated. In the latter case, calculation of total virus concentrated was accomplished as follows:

$$\text{Total number of Virus Concentrated} = \text{Number of Plaques Counted} \times \frac{\text{Volume of Borate-Calf Serum Added}}{\text{Volume of Borate-Calf Serum Plated}}$$

Results of an experiment designed to demonstrate replication and dependability of the insoluble polyelectrolyte concentration technique are shown in Table 4. Replicate one-liter samples of PBS were dosed with 1.0 ml of a coliphage T2 working suspension having a titer of 126 ± 12 pfu/ml for 90 percent confidence limits. Total plaques concentrated from the samples were 28 and 29, resulting in a total calculated number of virus of 31 and 32, respectively.

Reverse Osmosis-Ultrafiltration Apparatus

Investigations of the rejection of virus by membranes were conducted on a bench-scale reverse osmosis-ultrafiltration unit*. The unit was composed of two high-pressure test cells, pressure

*A product of Universal Water Corporation, Del Mar, California

TABLE 4. CONCENTRATION OF COLIPHAGE T2 FROM REPLICATE 1-LITER SAMPLES OF PBS

Sample	A	B
Virus Added (pfu)	126 ± 12	126 ± 12
Virus Counted (pfu)	28	29
Borate-Calf Serum Added (ml)	5.0	5.0
Borate-Calf Serum Plated (ml)	4.5	4.5
Calculated Virus Recovered (pfu)	31	32
Percent Virus Recovered (%)	24.6 ± 2.3	25.4 ± 2.2

gauges, a back pressure regulator, accumulator, feed solution tank, pressurizing pump, and associated plumbing and valves. A schematic flow diagram is presented in Figure 2.

The high pressure test cells which are illustrated diagramatically in Figure 3 are constructed of stainless steel and are of the basic plate and frame design accommodating a single flat membrane with an effective surface area of 2.0 square inches.

Membranes

Membranes employed in this study were of the modified Loeb type cellulose acetate*. The nomenclature and pertinent characteristics of these membranes are summarized in Table 5. Membranes are manufactured to achieve a thin, dense surface layer less than one micron thick backed by a thicker porous layer approximately 99 microns thick. The dense layer is in contact with the feed solution and provides the primary barrier for solute passage while the porous layer permits the diffusion of water and solutes into the collecting channels.

The membrane most frequently employed in this study is not annealed when received from the manufacturer. Annealing at

*Products of Eastman Chemical Products, Inc., Kingsport, Tennessee.

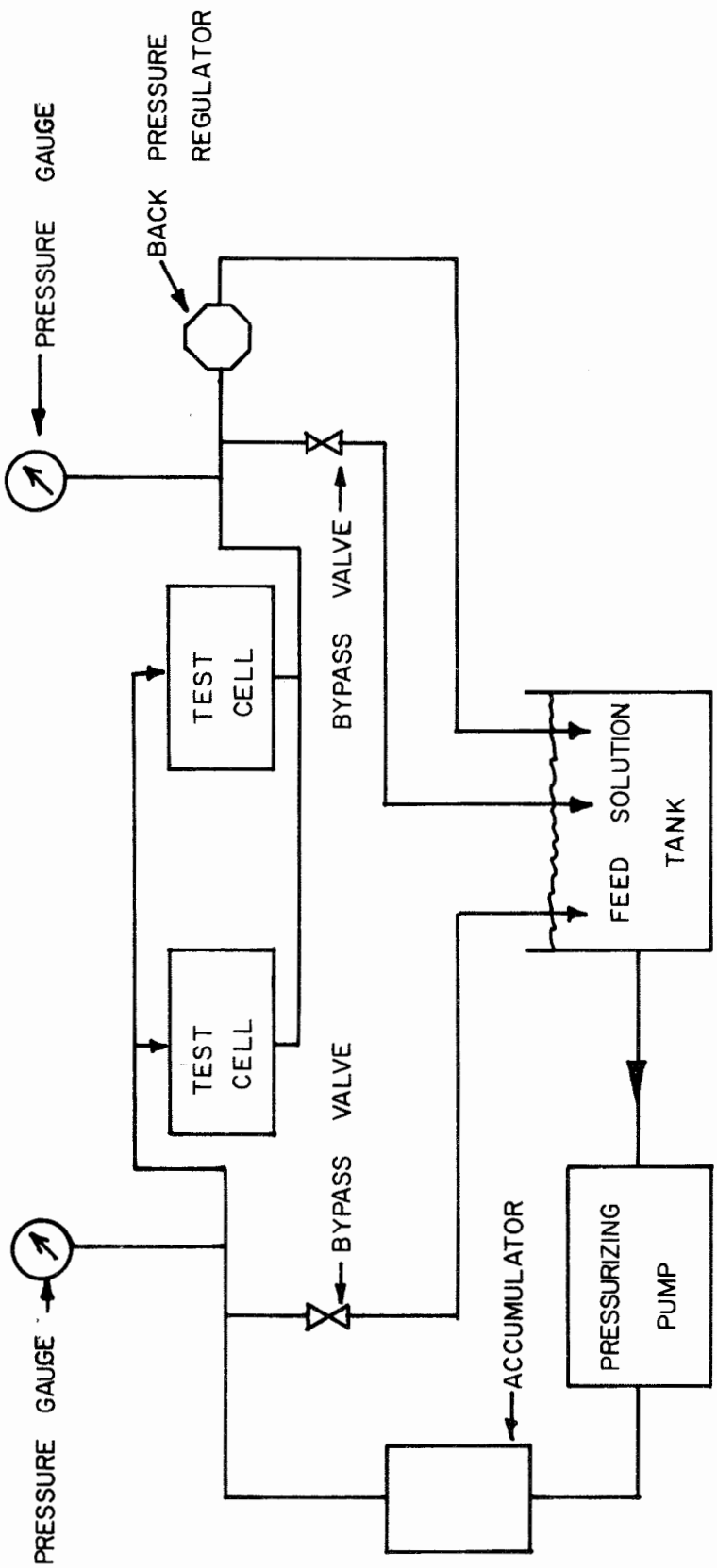

FIGURE 2. FLOW SCHEMATIC OF REVERSE OSMOSIS – ULTRAFILTRATION UNIT

TABLE 5. MEMBRANE DESIGNATIONS AND CHARACTERISTICS AS DETERMINED BY THE MANUFACTURER

Membrane Designation	Food Solution	Concentration Feed Solution (mg/l)	Percent Rejection %*	Temperature of Food (°F)	Approximate Flux (gpd/ft^2)
RO-97	NaCl	5250	96-97	84	18-19
RO-89	NaCl	5250	90-92	84	28-30
HT-00	----	----	-----	77	150-200
UF-10	Sucrose	10	35-45	80	58-62

*Flux and rejection measured during second 30 minutes of test at feed flow of 800 ac/minute and operating pressure of 600 psi.

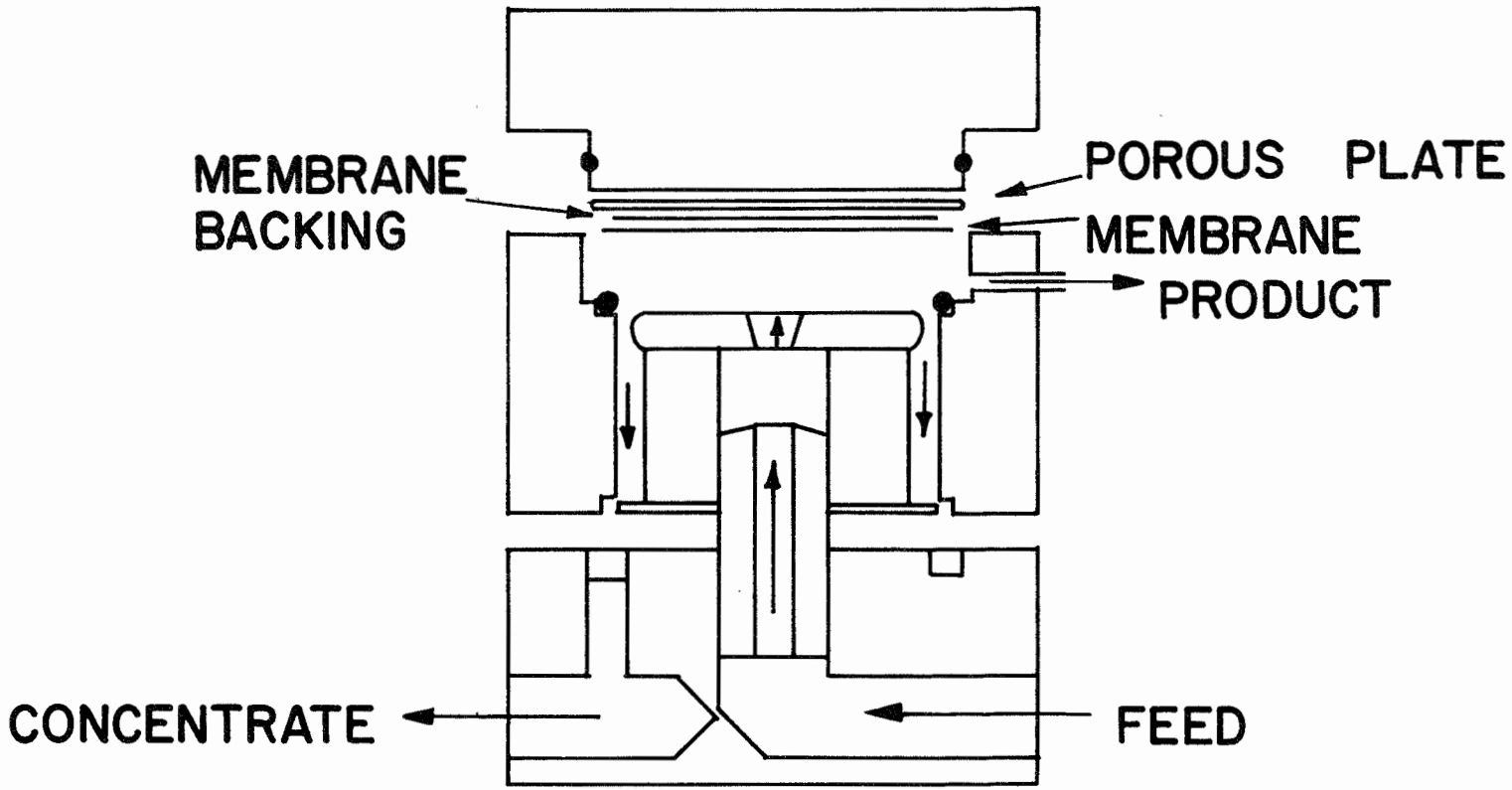

FIGURE 3. HIGH PRESSURE TEST CELL

at various temperatures permitted the selection of any desired solvent flux and degree of solute penetration of the membrane. All membranes were annealed in a constant temperature bath of distilled and deionized water at the selected temperature for four minutes, cooled in distilled water at room temperature for two minutes, and installed in the reverse osmosis unit which was pressurized to operating pressure in less than three minutes. Typical operating flux and rejection of conductivity in tap water over a wide range of annealing temperatures are presented in Figure 4.

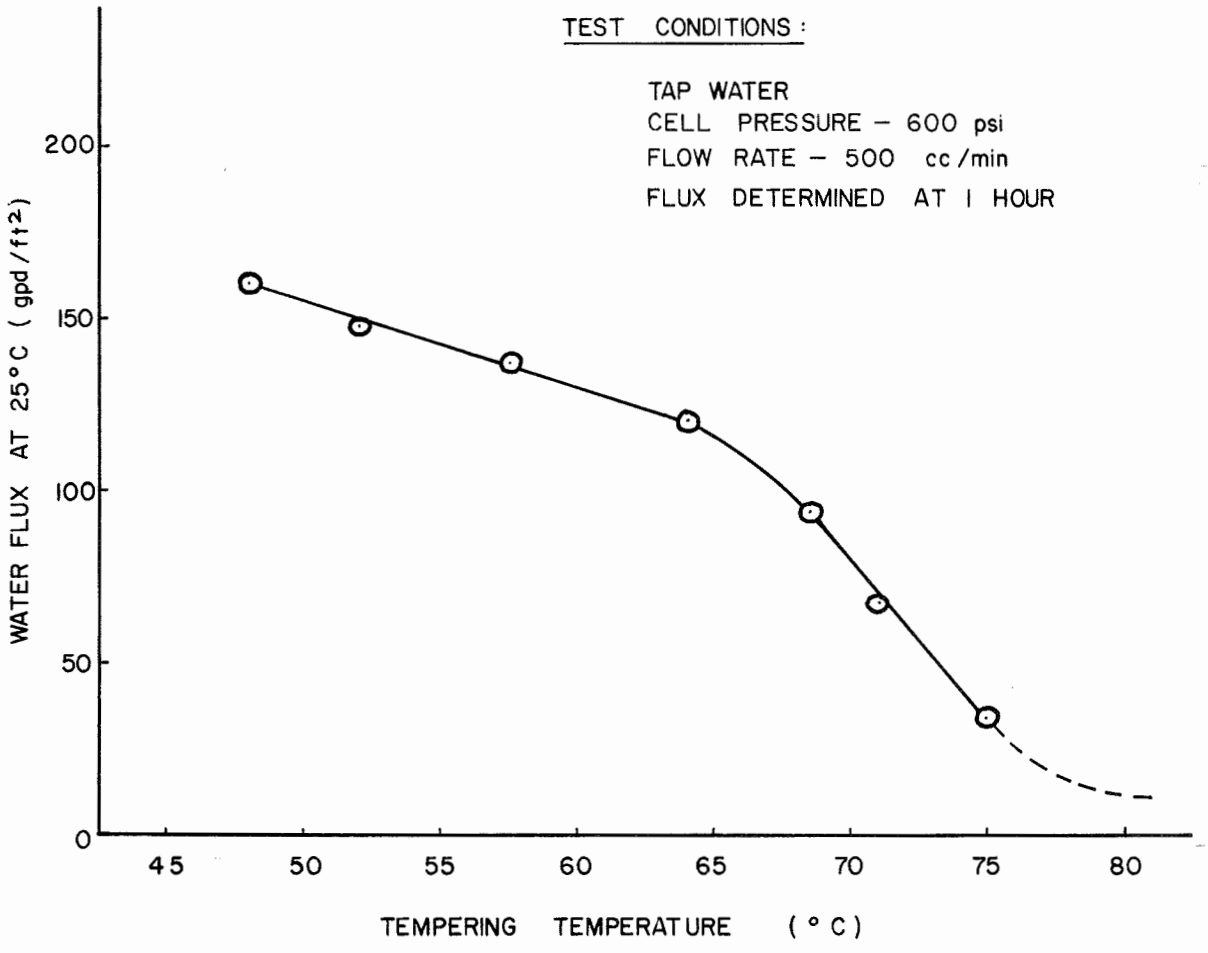

FIGURE 4. RELATIONSHIP BETWEEN WATER FLUX AT 25° C AND ANNEALING TEMPERATURE FOR HT00 MEMBRANE.

CHAPTER IV

Detection of Viruses at Low Levels

Coliphage T2 Recovery from PBS

The efficiency of virus recovery from PBS was evaluated in terms of the number of viruses recovered as compared to the number of plaques forming units (pfu) of T2 added to the PBS. Initial concentrations of T2 added to one liter volumes of PBS ranged from 17 to 6.5×10^6 pfu/ml. The results are tabulated in Table 6 and are presented in Figure 5 and demonstrate a relatively constant 25 percent recovery rate. Significant variability from calculated virus dosages were encountered at extreme dilutions as indicated by the data in Table 7 and as shown in Figure 6. At two dilutions of consequence virus recovery was not achieved. In both these cases the total calculated virus count expected in a five-liter quantity of PBS was 3.3 pfu. However, the adjusted pH of the PBS was 5.75 or 0.5 units above optimum for recovery. The effectiveness of this technique for recovery of serially diluted virus suspensions at a constant pH of 5.25 can be seen in Figure 7.

Minor differences in pH significantly affected the efficiency of T2 recovery. A series of experiments were conducted

TABLE 6. RECOVERY OF COLIPHAGE T2 FROM 1-LITER SAMPLES OF PBS BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE

Run Number	Total Virus Added (pfu)	Total Virus Recovered (pfu)
3 - R - T2	210	52 *
	21	6.3*
	21	7.6*
4 - R - T2	7500	1400
	1600	320
	170	34 *
	17	3.6*
5 - R - T2	6.5×10^6	8.6×10^5
	6.5×10^5	1.0×10^5
	6.5×10^4	1.2×10^4
	6500	890
	650	97 *
	65	10 *

*Total available volume eluate plated and value calculated on the basis of:

$$\text{Total Virus Recovered} = \frac{\text{initial volume borate}}{\text{volume borate plated}} \times \text{total plaques counted}$$

All other values calculated from dilution series

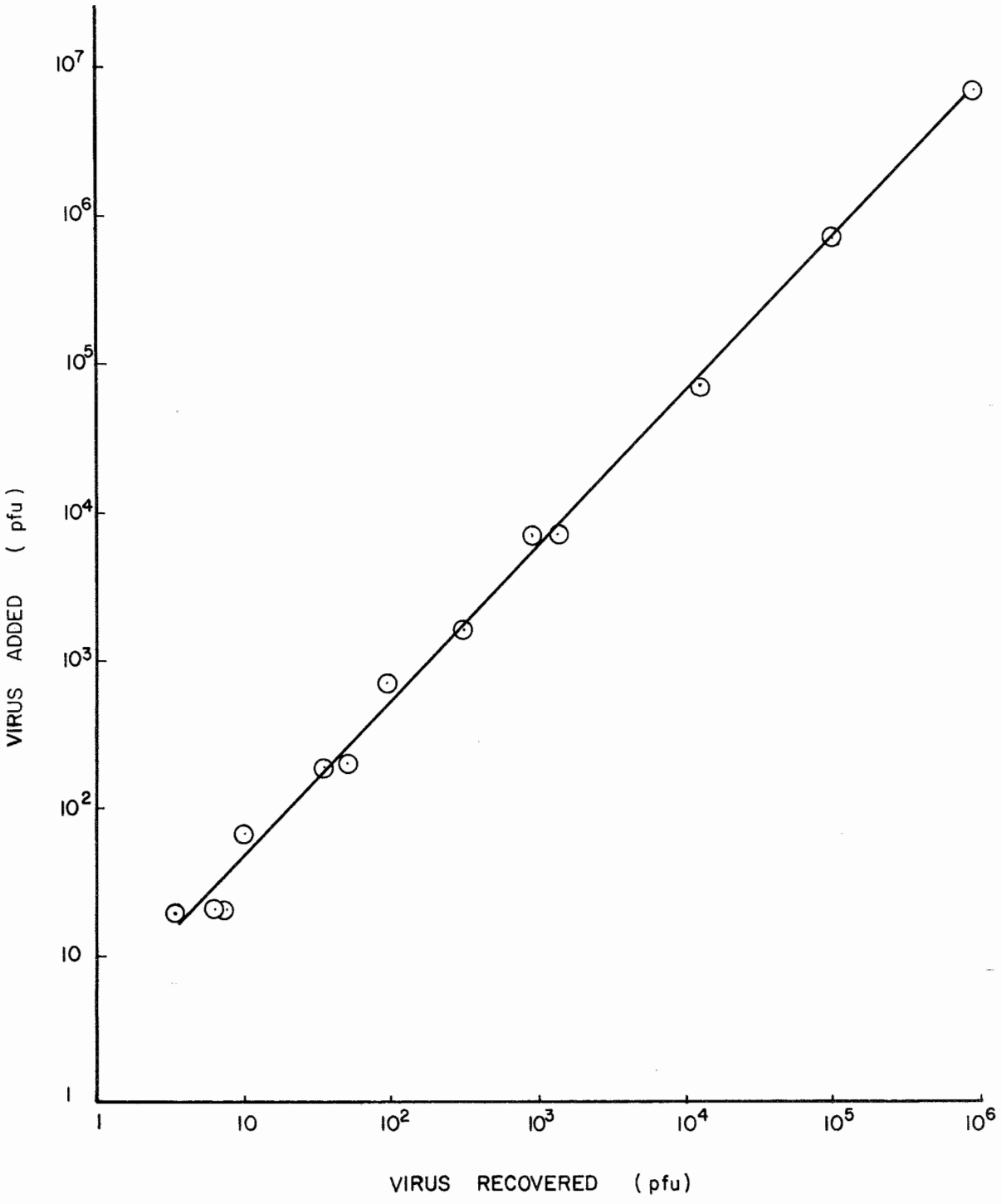

FIGURE 5. RECOVERY OF COLIPHAGE T2 FROM 1-LITER SAMPLES OF PBS BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE.

TABLE 7. RECOVERY OF COLIPHAGE T2 FROM 5-LITER SAMPLES OF PBS BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE

	Control Data			
Run Number	Total Count (Plaques)	Titer at 90% CI (pfu/0.5 ml)	Calculated Virus Added (pfu)	Observed Virus (pfu)
2 - LL - T2	310	31 ± 2.9	31	10.3
			19	4.5
			12	2.3
			6.2	2.3
			3.1	1.1
			1.6	0
3 - LL - T2	126	32 ± 4.7	32	8.0
			25	5.3
			12	9.1
			6.3	4.7
			3.2	0
			1.6	1.2
			0.8	0
4 - LL - T2	128	32 ± 4.7	32	9.1
			16	3.9
			13	5.7
			3.2	0
			1.6	1.3

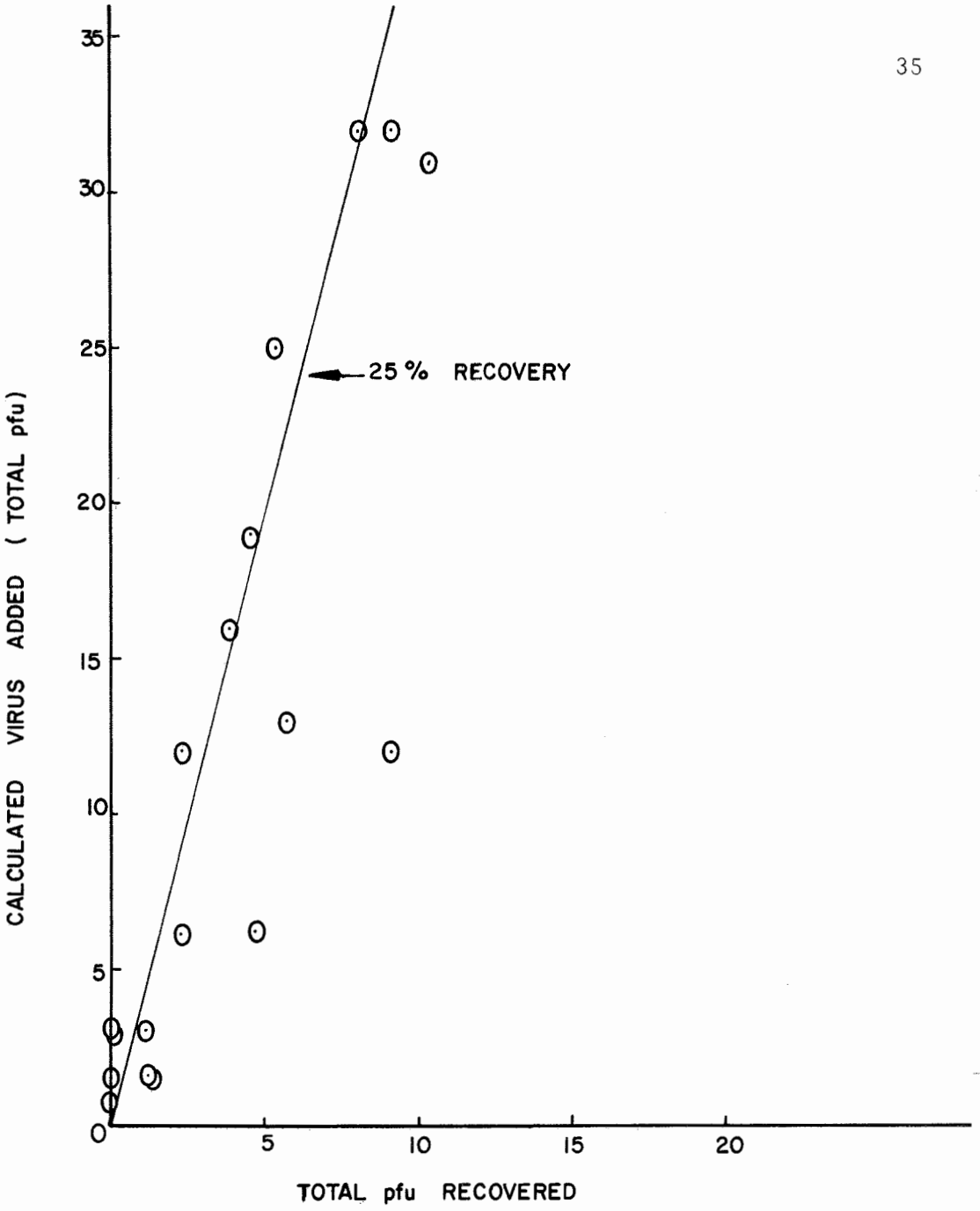

FIGURE 6. RECOVERY OF COLIPHAGE T2 FROM 5- LITER SAMPLES OF PBS BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE

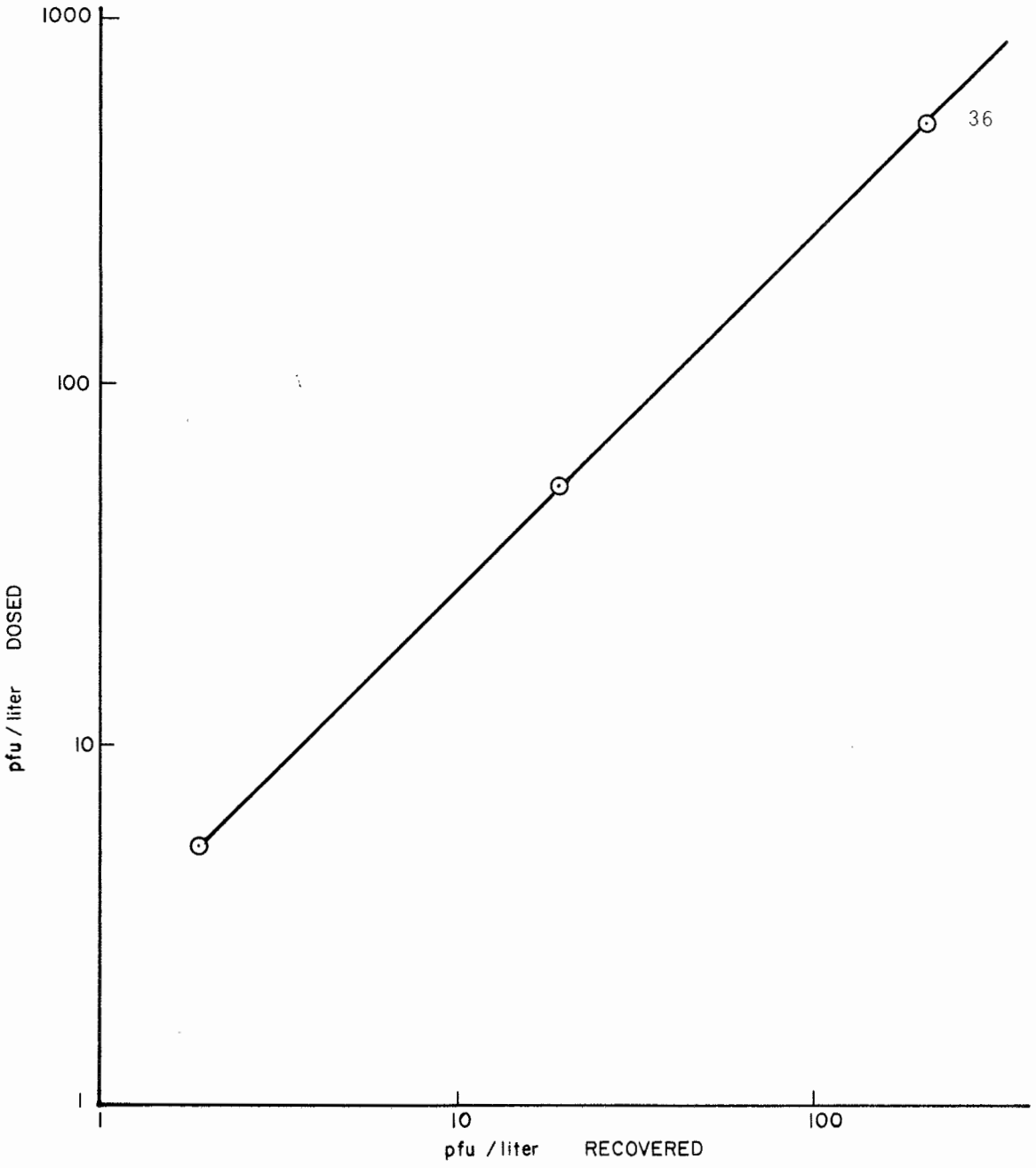

FIGURE 7. RECOVERY OF SERIALLY DILUTED COLIPHAGE T2 FROM 1-LITER SAMPLES BY THE POLYELECTROLYTE TECHNIQUE

to evaluate this variable. A known amount of virus was added to several one-liter containers, 100 mg polyelectrolyte added, and while mixing, the pH was adjusted to various levels by 0.25 pH increments. Typical data are presented in Table 8 and illustrated in Figure 8 and they indicate that the optimal pH for T2 recovery occurs in a very narrow range. Shifting $\pm$ 0.3pH units from the optimum can result in a significant reduction in recovery efficiency. These data are in conflict with the results reported by Wallis, et al. (1969 and 1970) who indicated a much broader range of pH for optimal recovery of poliovirus by this method.

The data indicated that about 25 percent of the added T2 was recovered from the polyelectrolyte. It was possible that all of the virus were not adsorbed on the polyelectrolyte during the mixing phase of the concentrating technique. However, when the filtrate was successively reconcentrated under ideal pH conditions, 22 percent of the coliphage was recovered on the first polyelectrolyte addition and no additional virus was detected on subsequent concentration attempts. Under less favorable conditions, where pH was 4.75 as compared to an optimum of 5.25, only 15 percent of the initial phage titer was recovered on the initial concentration

TABLE 8. RECOVERY OF COLIPHAGE T2
FROM 1-LITER SAMPLES IN DIFFERENT ENVIRONMENTS
AS A FUNCTION OF pH

Run Number Environment and Number of Virus Added (90% Confidence Limits)	pH	Total Virus Recovered (Plaques)	Confidence Limits (+ or -)	Percent Maximal Reocvery (%)
6 - pH - T2 Unfiltered Lake Water 1790 ± 208 pfu/l	4.00	158	21	37
	4.50	241	26	56
	4.75	275	27	64
	5.00	311	29	73
	5.25	430	34	100
	5.50	278	28	65
	5.75	265	27	62
	6.00	256	26	60
	6.50	216	24	50
6 - pH - T2 Filtered Lake Water 1790 ± 208 pfu/l	4.00	162	21	37
	4.50	290	28	67
	4.75	285	28	64
	5.00	271	27	61
	5.25	321	30	74
	5.50	433	34	100
	5.75	341	31	79
	6.00	317	30	73
	6.50	226	25	52
4 - pH - T2 PBS 1820 ± 202 pfu/l	4.25	228	25	53
	4.50	250	25	58
	4.75	298	29	69
	5.00	336	31	78
	5.25	431	34	100
	5.50	203	24	47
	6.00	194	23	45
	6.50	190	23	44

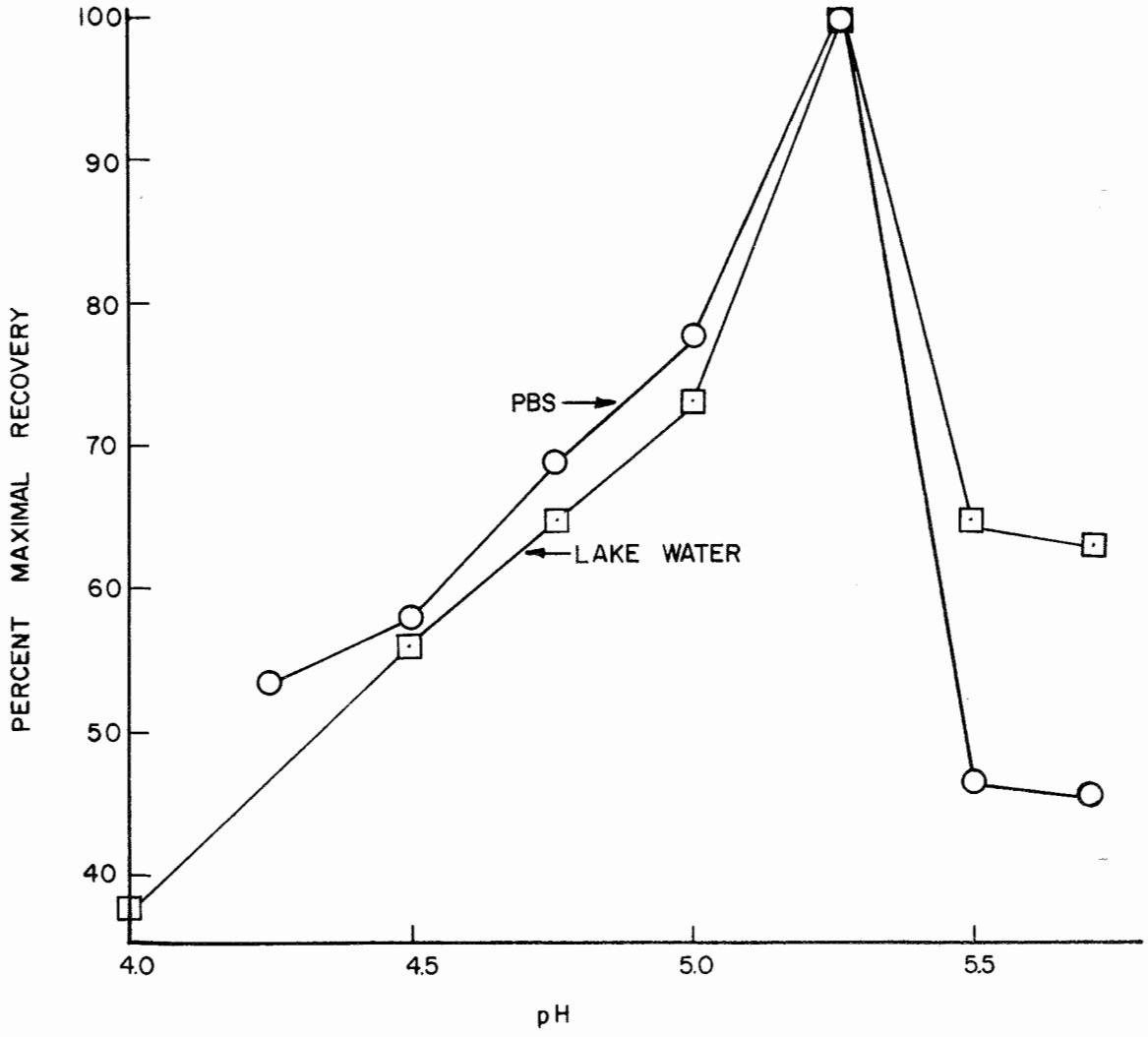

FIGURE 8. THE EFFECT OF pH ON THE RECOVERY OF COLIPHAGE T2 BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE

attempt while 1.2 percent was recovered during the second concentration. After the initial separation of the borate buffer from the polyelectrolyte, the polyelectrolyte was resuspended in another five ml of borate buffer and plated directly. Less than two percent of the added phage were found. This recovery was expected since 0.5 ml of the initial five ml borate remained with the polyelectrolyte.

An experiment was conducted to determine the relationship between shaking time of the virus-polyelectrolyte-borate buffer suspension and recovery efficiency. Three one-liter samples of PBS were dosed with equal amounts of virus and concentrated at pH = 5.3. The recovered polyelectrolyte from the samples was shaken for three, five, or ten minutes. The data are presented in Table 9 and indicate essentially no variation in virus recovery as a function of shaking time. Each time the suspension was shaken the polyelectrolyte was separated from the borate, resuspended in fresh borate and reshaken for the original time period. Again, no variations from expected results were observed.

Coliphage isotopically labeled with ^{3}H thymidine were used in a further attempt to determine the fate of unrecovered virus. This stock was concentrated from PBS with the polyelectrolyte

TABLE 9. EFFECT OF DIFFERENT SHAKING PERIODS FOR ELUTION OF COLIPHAGE FROM THE POLYELECTROLYTE

Sample	Shaking Time (minutes)	Total Virus Counted (pfu)
1	3	175
Resuspension of Sample 1	3	18
2	5	186
Resuspension of Sample 2	5	20
3	10	180
Resuspension of Sample 3	10	17

as in other experiments and the results appear in Table 10. Essentially all of the phage DNA-associated radioactivity was attached to the polyelectrolyte. Twenty-eight percent of the ^{3}H was recovered in the eluate while 56 percent remained attached to the polyelectrolyte after borate elution. Infectivity plating indicated that 15 percent of the initial titer of viable phage was recovered in the eluate and only 1.5 percent of the viable virus remained associated with the polyelectrolyte after elution.

Coliphage Recovery from Natural Water

Numerous attempts to recover indigenous coliphage from water samples collected from Town Lake were made. Recoveries from volumes of five and 18 liters indicated coliphage titers varying from 1.5×10^{-3} to 8.5×10^{-3} pfu/ml. These data along with some of the chemical, physical, and biological characteristics of Town Lake water are summarized in Table 11. It is interesting to note that while the coliform levels decreased drastically between April 19 and April 27, the coliphage titer remained essentially constant. These conditions most likely reflect the fact that the coliphage are propagated in the presence of large numbers of coliform at least to the extent of counteracting their natural inactivation in the environment. Quantites of lake water were filtered through a glass fiber filter* to

*Reave Angel #934 AH

TABLE 10. RESULTS OF TRACER STUDIES TO DETERMINE THE FATE OF UNRECOVERED VIRUS

State of Polyelectrolyte Virus Complex	Radioactivity		Total Viable Virus	
	Net Counts (cpm)	% of Total	pfu	% of Total
Polyelectrolyte + Coliphage	16,000	--	1.5×10^{10}*	100
Eluate (with coliphage)	4,400	28	2.3×10^{9}	15.3
Polyelectrolyte after elution of coliphage	8,900	56	2.2×10^{8}	1.5

*Amount of coliphage added

TABLE 11. CHEMICAL, PHYSICAL AND BIOLOGICAL CHARACTERISTICS OF TOWN LAKE WATER

Characteristic* (date)	Sample #1 (3/3/71)	Sample #4** (4/19/71)	Sample #5 (4/22/71)	Sample #6 (4/27/71)
Volume Sampled (liters)	5.0	18.0	6.0	18.0
pH (units)	8.3	8.2	8.2	8.2
Specific Conductance (μmhos/cm)	------	570	------	560
Alkalinity (mg/l as $CaCO_3$)	------	160	------	160
Chemical Oxygen Demand (mg/l)	12	10	------	-------
Filterable Solids (mg/l)	8	10	------	------
Standard Plate Count at 35°C (organisms/ml)	5,000	13,000	------	------
Coliform (Organisms/100 ml)	600	3,100	1,540	740
Coliphage (pfu/ml)	1.5×10^{-3}	8.0×10^{-3}	8.5×10^{-3}	7.0×10^{-3}
Coliform: Coliphage (ratio)	4,000:1	3,900:1	2,000:1	1,100:1

* All analyses except those outlined in the Methods section were performed according to Standard Methods for the Examination of Water and Wastewater, American Public Health Association, 12th Edition, New York, 1965.

**Sample #1 was collected from the lake after several months of essentially no precipitation in the watershed while sample #4 was collected three days following a one-inch rainfall, the first since sample #1 was collected. Sample #6 was collected eight days later.

remove suspended solids and ten coliphage/ml were added. In parallel, containers of unfiltered lake water were dosed with identical amounts of coliphage. The samples were concentrated at pH values ranging from 5.0 to 7.0. Maximal recovery was about 20 percent of the initial titer in both samples, however, the filtered water had an optimal recovery in the filtered water occurred at pH = 5.5 and that of the unfiltered water at pH = 5.25.

Poliovirus Recovery from PBS

Poliovirus recovery from PBS was relatively inefficient at the concentrations studied, namely 4×10^{-3} pfu/ml to five pfu/ml. Maximum recovery efficiency was found to be 13.5 percent at pH = 4.0.

The pH markedly affected the efficiency of recovery and these data are presented in Table 12 and shown in Figure 9. The low pH required when using PBS and the relatively inefficient recoveries resulted in abandonment of this medium in favor of natural waters.

Poliovirus Recovery from Natural Water

The efficiency of recovery of poliovirus from inocculated

TABLE 12. RECOVERY OF POLIOVIRUS FROM 1-LITER SAMPLES IN DIFFERENT ENVIRONMENTS AT VARYING pH

Run Number Environment and Number of Virus Added (90% Confidence Limits)	pH	Total Virus Recovered (Plaques)	Confidence Limits (+ or -)	Percent Maximal Recovery (%)
	3.50	175	22	26
	3.75	516	38	72
3-pH-Polio	4.00	672	43	100
PBS	4.50	248	26	37
	4.75	134	19	20
5080 ± 592 pfu/l	5.00	118	18	15
	5.50	68	14	10
	6.00	54	12	8
	6.50	55	12	8
2-pH-Polio	3.5	38	10	23
	4.0	167	21	100
PBS	4.5	62	13	37
	5.0	28	9	17
	5.5	2	>2	1
1220 ± 164 pfu/l	6.0	8	>5	5
	6.5	8	>5	5
	7.0	10	>6	6
	5.50	528	38	71
	5.75	545	39	73
	6.00	683	43	91
6-pH-Polio	6.25	749	45	100
	6.50	705	44	94
Unfiltered Lake	6.75	616	41	82
Water	7.00	396	33	53
1670 ± 193 pfu/l				
	5.50	203	23	73
	5.75	206	24	74
7-pH-Polio	6.00	222	25	80
	6.25	278	28	100
Filtered Lake	6.50	223	25	84
Water	6.75	220	25	79
	7.00	178	22	64
612 ± 53 pfu/l				

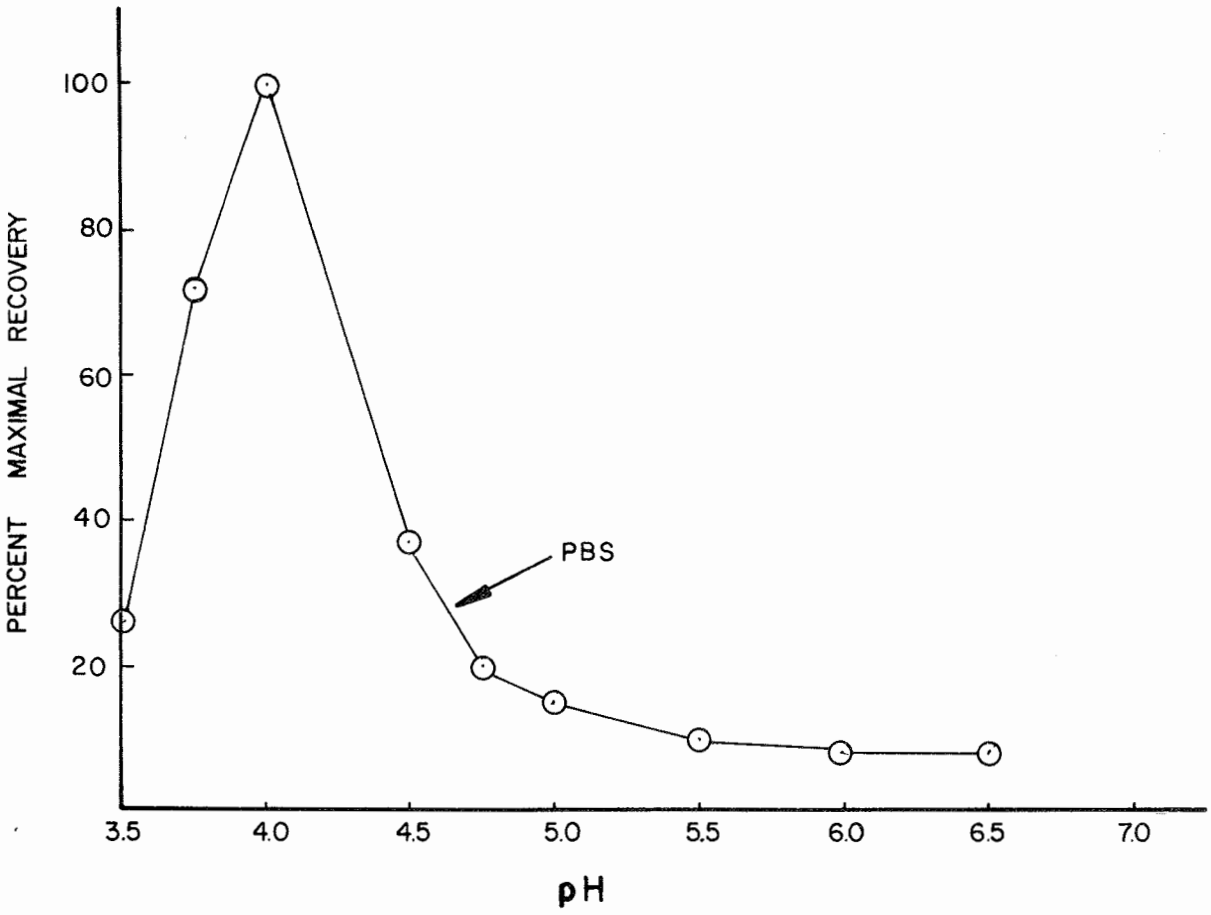

FIGURE 9. THE EFFECT OF pH ON THE RECOVERY OF POLIOVIRUS FROM PBS BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE.

lake water samples was as high as 45 percent. The optimal recovery occurred at pH = 6.25 and minor variations in pH resulted in significant reductions in recovery efficiency. These results are illustrated in Figure 10.

Efforts were made to determine if all of the virus were adsorbed on the polyelectrolyte. At optimal pH no virus was detected in successive concentrations of the filtrate. However, at pH = 6.0 (0.25 units from the optimum), 0.6 percent of the initial titer was reconcentrated from the filtrate of the first concentration attempt. Upon resuspending the polyelectrolyte with fresh borate buffer and calf serum, 6.2 percent of the amount of virus first recovered was detected. This concentration was slightly less than the expected value since 0.5 ml borate and calf serum remained with the polyelectrolyte.

The relationship between optimal pH for poliovirus recovery and presence of suspended material in lake water was investigated in much the same manner as for coliphage. The data presented in Figure 10 illustrate that the filterable solids exerted less influence on poliovirus recovery than it did for coliphage recovery. This is most probably due to the higher optimal recovery pH

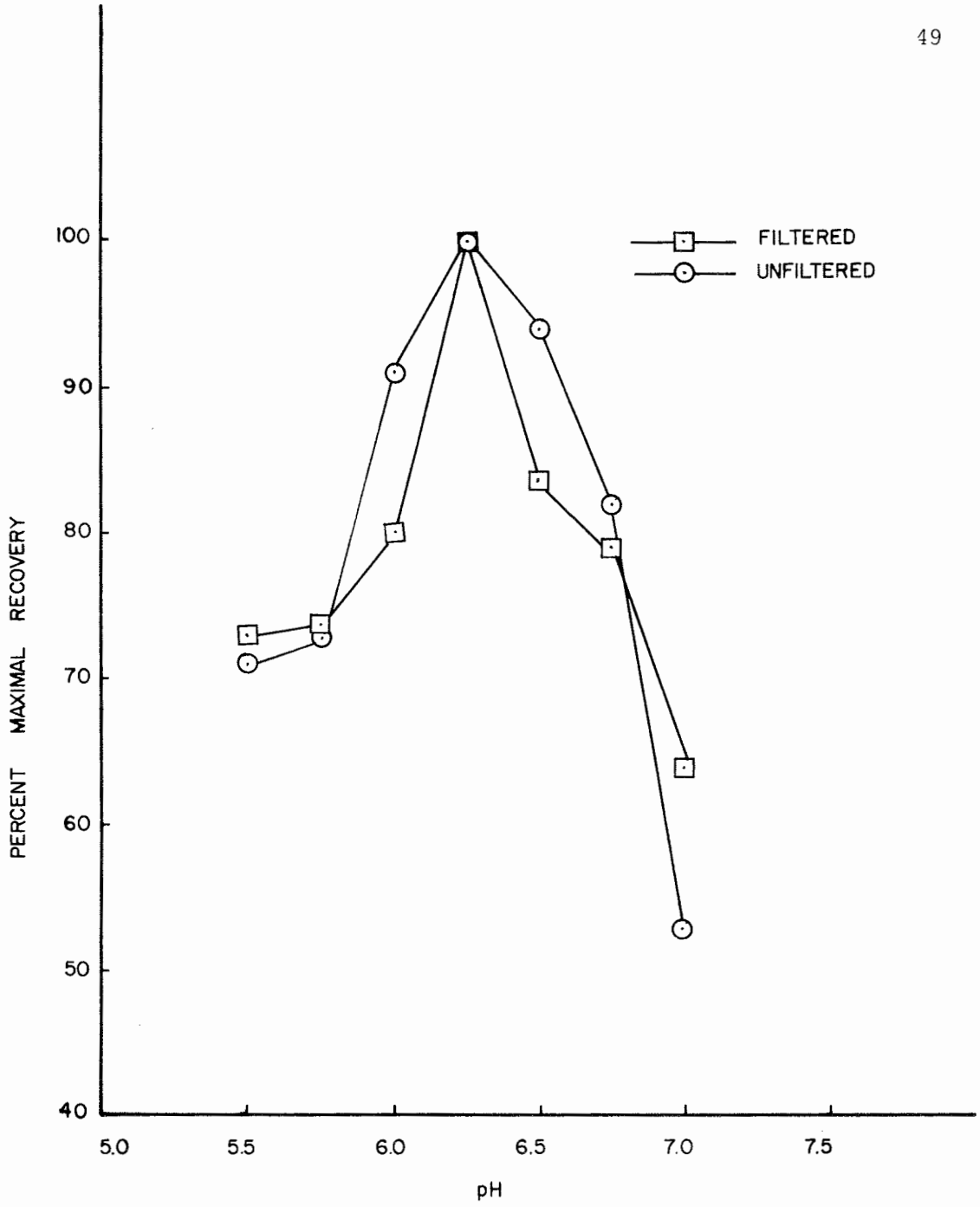

FIGURE 10. THE EFFECT OF pH ON THE RECOVERY OF POLIOVIRUS FROM LAKE WATER BY THE INSOLUBLE POLYELECTROLYTE TECHNIQUE

for the poliovirus thereby minimizing the buffer capacity of the filterable material in the lake water.

The effect of removing suspended material from raw lake water before concentration was investigated since it is awkward to handle large quantities of lake water during the filtration steps of the concentration technique. Four replicate one-liter samples were dosed with virus and stirred with a magnetic mixer for ten minutes to effect dispersion of the virus. The suspended material was removed from two of the samples by filtration through glass fiber filters*. Recovery of virus from the unfiltered samples averaged 275 plaques while an average of 191 plaques were recovered from the filtered samples. These data indicated that significant numbers of virus (31 percent) were attached to the suspended solids or lost in the glass fiber filter. Therefore, pretreatment to remove suspended material markedly reduces virus recovery efficiency.

Several attempts to recovery indigenous poliovirus or other enteric viruses from 18-liter volumes of lake water were made. The results of two of these runs and the total coliform density at the time of sampling are presented in Table 13. It is interesting

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*Reeve Angel #934 AH

TABLE 13. RECOVERY OF INDIGENOUS ENTERIC VIRUSES FROM TOWN LAKE

Date	6/10/71	7/28/71*
Volume Samples (liters)	18	18
Coliform (organisms/100 ml)	800	9500
Virus Recovered (pfu/ml)	6.9×10^{-4}	9.2×10^{-4}
Coliform: Virus (ratio)	11,000:1	100,000:1

*This samples was taken a few days after the shoreline was dragged and cleaned

to note the coliform to virus ratios determined in light of the 50,000:1 value estimated by Clarke, et al. (1964) for all enteric viruses. As was observed with indigenous coliphage, the enteric virus titer was relatively constant while there was considerable variation in coliform levels.

CHAPTER V

Rejection of Viruses by Cellulose Acetate Membranes

Membrane Characteristics

As previously stated, the transport of water through a cellulose acetate membrane most probably occurs by a combination of molecular diffusion and passage through pores or imperfections widely distributed throughout the membrane. The operation of these transport mechanisms may be illustrated best by Figure 4 (page 30) where unannealed cellulose acetate membranes were annealed at various temperatures and operated under constant conditions with tap water. The linear portion of the curve from 47°C to 67°C represent the area in which pore flow is the predominant transport mechanism while the abrupt shift in slope at about 67°C indicates that diffusion predominates. At this temperature, approximately the glass transition temperature for cellulose acetate (Mark and Tabolsky, 1950), crystalline regions are extensive and the amount of "primary" bound water held in the membrane is increased. Figure 11 indicates a similar but more subtle shift for conductivity rejection from tap water over the same annealing range.

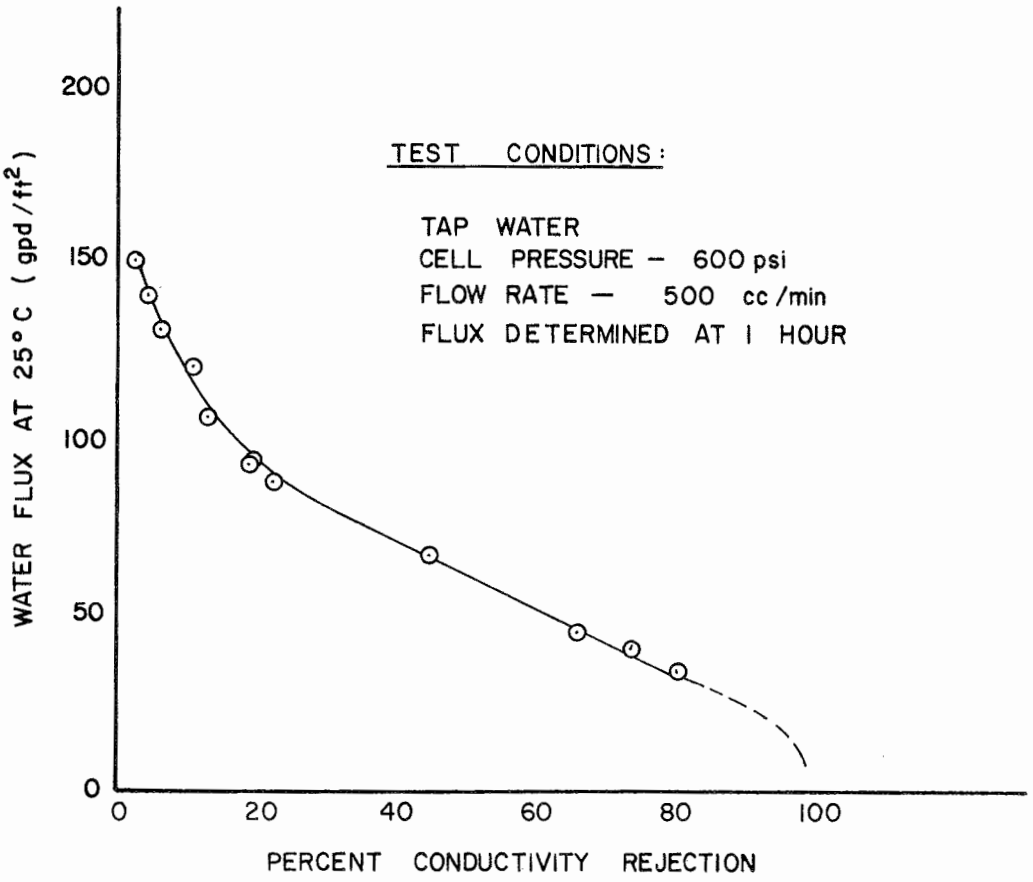

FIGURE 11. RELATIONSHIP BETWEEN WATER FLUX AT 25°C AND CONDUCTIVITY REJECTION FOR HTOO MEMBRANE ANNEALED AT VARING TEMPERATURES

Effects of the Reverse Osmosis-Ultrafiltration Unit on Viruses

The effects on viruses of the mechanical properties of the reverse osmosis-ultrafiltration unit were determined prior to quantitative evaluation of the passage of viruses through the membranes. Quantities of PBS were inocculated with virus, the passage of product water from the unit was blocked by cellophane, and the unit was operated for extended periods with recirculation of the feed. Virus titer determinations were conducted at various intervals by drawing small quantities of PBS-virus mixture from the feed solution tank.

A summary of the data obtained for the coliphage T2 and poliovirus are tabulated in Table 14 and shown in Figure 12. Comparative information reflecting minimal virus inactivation in PBS at room temperature can be found in Table 15. A rapid unpredictable inactivation of the coliphage T2 occurred while poliovirus infectivity was not affected. The most likely explanation for this difference is the sensitivity of the T2 tail structure to mechanical damage. On the other hand, poliovirus is spherical with multiple receptor sites thereby minimizing the potential for random damage. Consequently, maintaining a relatively constant titer of coliphage required frequent addition of fresh virus to the feed solution.

TABLE 14. RESULTS OF UNIT DIE-OFF STUDIES

Run Number	Time into Run (hr)	Total Count (plaques)	Virus Concentration (pfu/ml)	90 percent Confidence Limit (+ or -)	Initial Titer (pfu/ml)
U - 1 - T2	0	742	1480*	90	8.9×10^0
	1.0	420	840*	67	
	3.0	471	186*	14	
	4.0	285	114*	11	
U - 2 - T2	0	423	850	68	8.5×10^3
	0.5	644	640	42	
	1.0	556	560	39	
	2.0	1218	2430*	110	
	4.0	531	1060*	76	
U - 3 - T2	0	477	950	72	9.5×10^5
	0.5	315	790	42	
	1.0	322	640	59	
	2.0	421	420	34	
	4.0	80	80	14	
U - 1 - Polio	0	246	82	8.6	8.2×10^4
	0.5	289	96	9.3	
	1.0	233	78	8.4	
	2.0	224	76	8.3	
	3.0	233	78	8.4	
	4.0	209	70	7.9	

*No dilutions required. All other values required dilution before plating.

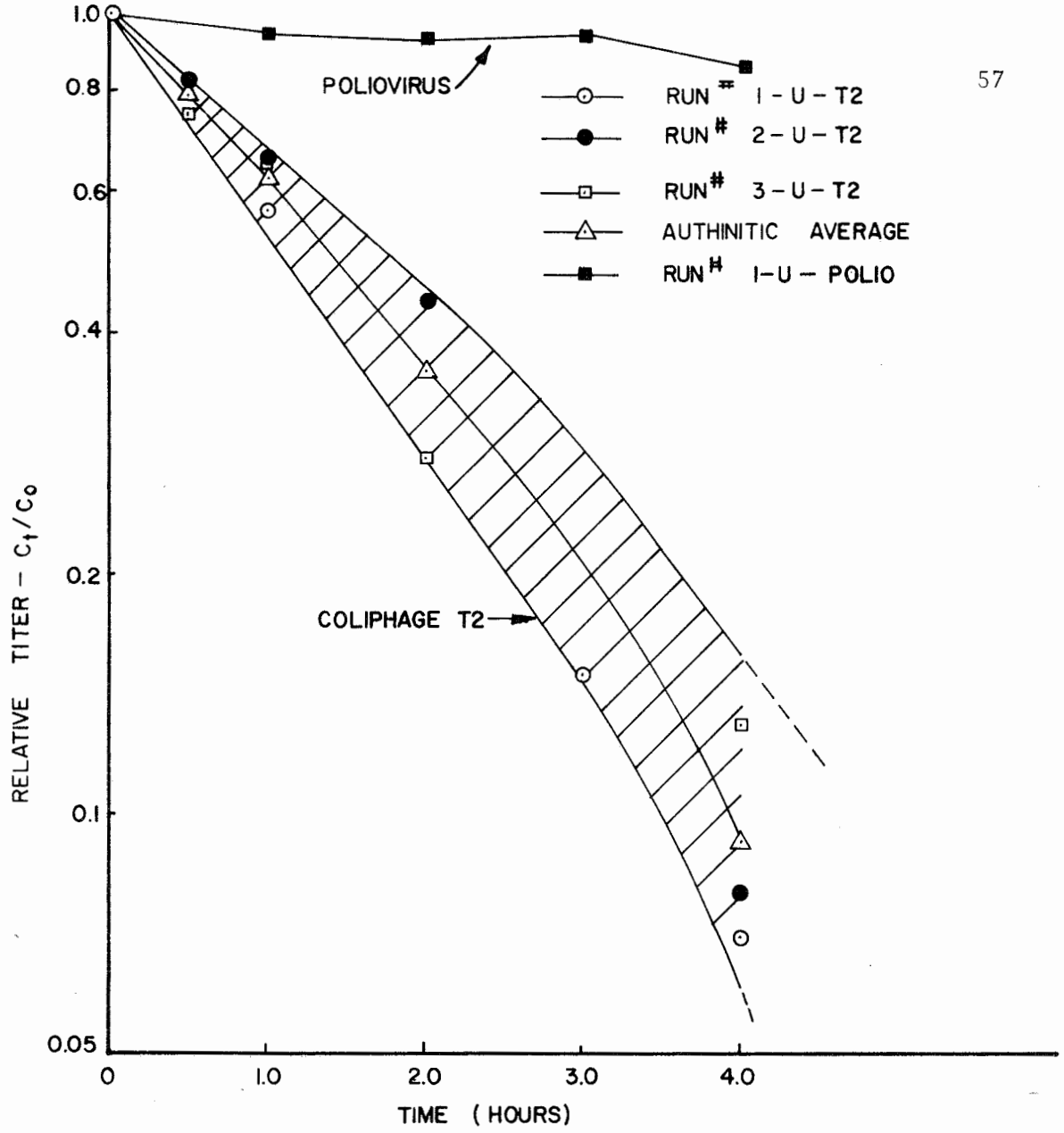

FIGURE 12. EFFECT OF REVERSE OSMOSIS UNIT ON COLIPHAGE T2 AN POLIOVIRUS SUSPENDED IN PBS AND RECIRCULATED THROUGH THE UNIT AT 600 psi AND 500 cc/min

TABLE 15. COLIPHAGE T2 INACTIVATION IN DIFFERENT ENVIRONMENTS AT 20-25°C

Run Number and Environment	Time (hr)	Total Count (plaques)	Virus Concentration (pfu/ml)	90 percent Confidence Limit (+ or -)
1-D-T2 PBS	0	216	1080	121
	0.7	210	1050	120
	3.3	201	1010	118
	5.0	184	920	112
2-D-T2 Borate Buffer @ pH = 9 with 10% calf serum	0	421	840	68
	1.0	415	830	67
	2.0	410	820	67
	3.0	412	820	67
	4.0	398	800	66
3-D-T2 Lake Water (Row)	0	158	530	69
	0.1	174	580	73
	1.0	177	590	73
	2.0	228	730	83
	4.3	244	810	86

Membrane Penetration by Viruses

Initial studies of virus penetration of the membranes were conducted with coliphage T2 at varying and relatively constant feed virus titers. The data presented in Table 16 summarize the conditions of the membrane, virus titers, fluxes, and results for all of these studies. Actual virus penetrations may be up to four times the number detected due to the efficiency of the virus concentration technique. Virus penetration does not appear to be dependent on concentration. In addition, the influence of "tightness" of the membrane as represented by flux does not appear to control virus penetration. Only in one run (#3-T2-PBS) did significant numbers of coliphage T2 get past the barrier. It would appear that either a leak around test cell seals or an extremely small hole was the cause of this penetration.

The ability of poliovirus to penetrate the membranes can also be found in Table 16. Initially, membrane runs were conducted in PBS and the three negative results most likely indicate the inefficiency of the virus concentration technique at an extremely low virus titer in PBS. As a result these tests were discontinued. The one major penetration resulted in PBS but with the UF 10 membrane.

TABLE 16. RESULTS OF VIRUS PENETRATION DETERMINATIONS FOR CELLULOSE ACETATE MEMBRANES

Run Number and Suspending Medium	Membrane Identification	Operating Flux Data (gpd/ft^2 @ 25°C) Range	Average	Duration of Run (hours)	Product Water Collected (liter)	Feed Water Virus Concentration (pfu/l) Initial (c)	Final (c)	Total No. of Virus Detected in Product Water (c)
1-T2 (PBS)	UF 10	315-210	256	2.0	2.00	1.6×10^4	2.7×10^3	1
3-T2 (PBS)	UF 10 (a)	211-120	163	3.0	1.93	3.6×10^9	4.1×10^8	150
4-T2 (PBS)	UF 10 (a)	186- 98	127	4.0	2.08	5.8×10^8	8.0×10^7	4
5-T2 (PBS)	HT 70 (a,b)	47- 40	43	4.0	0.69	2.4×10^8	7.8×10^7	1
6-T2 (PBS)	UF 10	296-144	218	2.5	2.19	8.6×10^8	5.0×10^8	3
7-T2 (PBS)	UF 10	287- 94	164	3.5	2.50	3.0×10^8	3.0×10^8	2
1-Polio (PBS)	UF 10 (a)	197-117	149	4.0	2.61	TNTC	TNTC	0
2-Polio (PBS)	UF 10 (a)	202-145	166	4.0	2.91	8.0×10^7	7.6×10^7	0
3-Polio (PBS)	UF 10 (a)	180- 50	81	24.0	8.60	8.8×10^7	1.8×10^7	1370
4-Polio (PBS)	HT 70 (a,b)	89- 44	59	24.0	6.14	1.2×10^8	1.7×10^7	0
5-Polio (Lake)	HT 75 (b)	52- 16	25	24.0	2.59	1.5×10^8	2.0×10^7	1
6-Polio (Lake)	HT 65 (b)	115- 28	49	24.0	5.09	9.0×10^1	1.7×10^2	3
7-Polio (Lake)	RO 89	48- 27	34	24.0	3.70	4.8×10^1	3.8×10^1	3
8-Polio (Lake)	RO 97	30- 25	27	24.0	2.86	5.1×10^1	4.0×10^1	0
9-Polio (Lake)	RO 89	51- 39	45	22.0	3.51	7.9×10^1	1.7×10^2	2
10-Polio (Lake)	RO 97	33- 28	31	24.0	3.32	9.2×10^1	3.2×10^1	1
11-Polio (Lake)	RO 97	34- 27	31	24.0	3.30	1.0×10^2	6.9×10^1	0
12-Polio (Lake)	RO 97	32- 26	28	24.0	3.13	3.1×10^2	1.8×10^2	1

NOTES:

a. Membrane was compressed one hour before virus was added to feed water
b. Number following HT refers to temperature at which HTOO membrane was annealed
c. Values not corrected for recovery efficiency

This penetration most likely represents the inability of the UF 10 membrane to withstand excessive pressures for extended periods and the ultimate development of minute holes or fissures. In this run, as with the others, product volumes were collected and concentrated independently throughout the run. Interestingly, virus penetration was relatively consistent over the five periods selected.

The performance of the membranes during the periods of virus penetration was monitored by observing their ability to reject dissolved solids as indicated by conductivity measurements. The data collected for some of these runs are presented in Table 17. The UF 10 membranes rejected only a small amount of dissolved solids represented by small changes in conductivity, however, this rejection was not considered significant because of the accuracy of the analytical technique used. The data in Table 17 indicate that the membranes were performing satisfactorily regarding rejection of inorganic salts.

TABLE 17. CONDUCTIVITY REJECTION OF MEMBRANES

Run Number	Membrane	Specific Conductance of Product (μmhos/cm)	Average Conductivity Rejection (%)
6-Polio-Raw Water	HT 65	450	30
7-Polio-Raw Water	RO 89	135	82
8-Polio-Raw Water	RO 97	53	93
9-Polio-Raw Water	RO 89	164	82
10-Polio-Raw Water	RO 97	76	92
11-Polio-Raw Water	RO 97	60	93
12-Polio-Raw Water	RO 97	60	93

CHAPTER VI

Discussion of Results

Detection of Viruses

The insoluble polyelectrolyte technique has been refined to provide an excellent means of quantitatively concentrating relatively low levels of viruses. However, the procedure is pH sensitive and consistant recoveries of a specific virus is possible only over a narrow pH range. The optimum pH range for different viruses must be determined and this limitation must be recognized. Therefore, quantitative determinations of specific viruses in natural waters can be made at levels as low as 0.1 pfu/liter (1×10^{-4} pfu/ml).

The correlation of virus recovery from low level concentrations (e.g. as low as 0.22 pfu/l) by the procedure used in this study was linear with a constant fractional recovery characteristic for each of the viruses studied.

Initially, Wallis, et al. (1969) reported that this procedure was effective in recovering over 90 percent of poliovirus, Type 1 (LSC strain), from polluted waters. In a subsequent study, Wallis,

et al., (1970) reported that 94 - 99 percent of the same virus was recovered from tap water over a range of pH = 5.0 to pH = 8.4.

The results of the study reported herein indicate that maximal efficiencies for the concentration and recovery of coliphage T2 from phosphate buffer solution (PBS) and lake water was 25 percent. Maximal recoveries of poliovirus, Type 1 (Mahoney strain) was 13.5 percent for PBS and 45 percent for lake water.

The recovery was dependent on the pH of the suspending medium during the concentration phase. Optimal recovery of coliphage T2 was achieved at pH = 5.25 for both the PBS and lake water. Varying the pH from the optimal by $\pm$ 0.25 pH units resulted in a 30 to 40 percent reduction in the total number of virus recovered. Maximal recovery of poliovirus in PBS occurred at pH = 4.0 and in lake water at pH = 6.25. The recovery of poliovirus from lake water was less sensitive to pH variations than was the coliphage, and a reduction in the total number of virus recovered of 18 to 27 percent resulted at a variation of $\pm$ 0.5 pH units from the optimum. The discrepancies between these results and those reported by Wallis, et al. (1969, 1970) may be attributable to the relative ease or difficulty by which different viruses can be eluted

from the polyelectrolyte. In addition, the data reported in this study indicate that the characteristics of the suspending medium significantly affects the recovery efficiency.

To further explore these differences, attempts were made to determine the fate of the coliphage upon attachment to the polyelectrolyte by employing ^{3}H labelled coliphage DNA. Results of these studies indicate that 56 percent of the DNA remained attached to the polyelectrolyte after elution of viable virus. These data indicate the possible discharge of DNA during attachment of the virus to the polyelectrolyte, since subsequent attempts to elute more virus were unsuccessful.

The fact that virus recoveries were not 100 percent indicate that additional understanding of virus attachment-detachment mechanisms is required. There is some evidence that the attachment phenomenon is charge associated. Attachment was near 100 percent under all conditions studied and may well be linked to the changing of electrophoretic mobility from a negative value toward neutrality. Limited information concerning the specific electrophoretic mobility of coliphage T2 is available in the literature. Puttman (1950) reported mobilities for the bacteriphage T6 of -7.3×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH = 8.6 and -3.6×10^{-5} cm^2 $volt^{-1}$ sec^{-1} at pH = 5.1. Assuming similarities between T2 and T6, a pattern

develops. This theory does not explain the inability to completely elute T2 from the polyelectrolyte at higher hydrogen ion concentrations. Maximum elution of viable coliphage T2 was only 25 percent. Evaluation of these factors was outside the scope of this research and additional investigations are required.

Sufficient information is presented to justify confidence in the linearity of the insoluble polyelectrolyte method for recovery of virus at a controlled pH, the polyelectrolyte concentration dosed, and the mixing conditions described. This confidence is valid in spite of the scatter of the data shown in Figure 6. The experimentation was conducted at extremely high dilutions of virus. A stock titer of virus containing about 60 pfu/ml was used in most cases. Dilutions were made by extracting an aliquot (0.035 to 0.5 ml) from the stock solution and mixing in a five-liter volume of PBS. The number of viruses added to the five-liter volumes was calculated as an average value (i.e., a 0.1 ml aliquot provided six pfu from a stock solution of 60 pfu/ml). At aliquots from 0.3 to 0.5 ml (or about 20-30 pfu) the linearity of virus recovery was maintained. However, for smaller aliquots scatter increased as illustrated in Figure 6. This condition was caused by the existing inability to accurately predict the number of particles in these smaller aliquots.

The data presented in Table 18 indicate the relatively low probabilities of extracting the number of particles nearest the calculated average as well as the probability of extracting zero and one or more particles for one low-level run. At the highest dilution the probability of extracting one or two particles ($p = 0.334$ and $p = 0.251$, respectively) approaches the probability of extracting zero particles ($p = 0.223$). The information in Table 18 provides a possible explanation for the scatter in Figure 6 at the lower concentrations. Calculated values for other runs are similar. The probability of extracting an aliquot with one or more particles is relatively high at even the highest dilution, thereby reflecting the fact that at least one virus was recovered from all but two dilutions of consequence.

The results of this study indicate that the basic requirements for dependable virus quantification using the insoluble polyelectrolyte technique, necessarily include:

a. Standardization of the polyelectrolyte for the specific virus and suspending medium employed.

b. Concentration of sufficient numbers of virus to justify statistical confidence in removal percentages.

The lower limit of virus detection is restricted only by the volume of media which can be handled conveniently for concentration. This study

TABLE 18. POISSON APPROXIMATION OF THE BINOMIAL PROBABILITY FUNCTIONS FOR LOW-LEVEL RUN #2-LL-T2

Aliquot Extracted from Stock Solution	Calculated Average Number of Particles in Aliquot (control titer = 31 ± 2.9/0.5 ml @ 90% confidence limits)	Probability of Aliquot Samples Containing			
		Zero Particles	One or more Particles	Number of Particles Nearest the Average Number	Probability
0.2	12	0.000	1.000	12	0.114
0.1	6.2	0.003	0.997	6	0.161
0.05	3.1	0.045	0.955	3	0.224
0.025	1.5	0.223	0.777	2	0.251
				1	0.334

demonstrated the feasibility of the detection of virus levels as low 0.1 pfu/liter from 20 liter volumes of natural water.

Penetration of Cellulose Acetate Membranes by Viruses

The results of these investigations indicate penetration of viruses through all membranes which otherwise exhibit excellent rejection properties. In other studies, Hindin, et al. (1968) were unable to isolate virus by direct plating of the product water from membranes subjected to coliphages T7 and X175. However, the results of the study reported herein indicate that virus concentrations of the product water ranged from zero to 7.6 pfu/liter for all of the membranes studied, exclusive of the two runs with high virus concentrations in the product water. Virus concentrations of the product water have been corrected for recovery efficiencies of 25 percent for coliphage T2 and 45 percent for poliovirus. The two high product water virus concentrations are attributable to mechanical deficiencies of the membrane or to feed water leakage around the cell seals. The normally low concentrations of virus in the product water make it almost impossible to detect viruses by direct plating of a few milliliters of sample, as was attempted by Hindin et al., (1968). This statement is supportable for these reasons:

a. Repeated runs with similar virus feed water concentrations and the same membranes did not result in high

product water virus levels. The data presented in Table 16 bear out this point.

b. After each run the reverse osmosis-ultrafiltration unit was operated for thirty minutes with a chlorine solution at a concentration of at least 200 mg/l to destroy the remaining virus and to prevent contamination from one run to another. The success of this procedure periodically was verified by the inability to isolate virus from feed water and product water which had not been spiked with virus.

c. Leakage of even a few tenths of a milliliter of feed water into the product water would result in the recovery of several hundred virus from the product water for all runs with feed water virus concentrations of more than 10^3 pfu/ml. Except in the two instances cited, only a relatively low concentration of viruses was detected in the product water.

The mechanism of virus penetration of asymmetrical cellulose membranes is not obvious. Insignificant penetrations of submicroscopic particulate matter such as viruses most likely would occur by either of the two mechanisms:

a. Random passage through a mechanical defect in the membrane (i.e. a very small hole) which is a defect in the membrane manufacturing process, or

b. Passage through large pores resulting from random imperfections in the crosslinkage of the cellulose acetate.

The theory of solute penetration of membranes leaves little room for penetration of 250 A^{o} diameter particles through the dense, active surface of the cellulose acetate membrane. Electron photomicrographs of similar membranes have demonstrated no pore structure in the dense layer down to 100 A^{o} (Merten 1966). Pore size in the porous sublayer was estimated to be about 400 mμ . Therefore, virus penetration should be impossible unless small fractures or fissures exist in the dense layer.

From a more theoretical viewpoint, however, Subcasky et al., (1969) indicated that in the case of cellulose acetate, desirable casting of membranes results in structures composed of individual solvated rods rather than supermolecular aggregates of rods. In actual practice, a mixture of structures exist capturing capillary water in the structure in areas of irregular crosslinkage of the cellulose acetate. The capillary

water widens the equivalent pore size resulting in decreased rejection over random areas of the membrane surface. Thus, it can be theorized that under high pressure, individual particles can be pushed through random areas of imperfect crosslinkage of cellulose acetate in the dense layer of the membrane. Observations during this research regarding frequency of virus penetration and the lack of penetration dependency on concentration of virus in the feed solution tend to support the latter mechanism. Consequently, results of this study should be extrapolable to any known virus particles with physical dimensions of the same order of magnitude as poliovirus or coliphage T2.

Rejection of Viruses by the Reverse Osmosis-Ultrafiltration Process

Average virus concentrations of the feed water ranged from 9.6×10^{1} to 6.8×10^{8} pfu/liter. No significant variations in the numbers of virus in the product water were observed. Viruses in the product water were consistently zero to 16 pfu/run resulting in a concentration range of zero to 7.6 pfu/liter for all of the membranes studied. However, the results of two runs were excluded since feed water leakage into the product water was evident. The data presented in Table 19 indicate that membranes with the higher flux rates consistently produced a product water with virus concentrations of 2.0 to 7.6 pfu/liter and the product water from the RO 97 membrane, which had a lower flux rate had

TABLE 19. AVERAGE PRODUCT WATER FLUXES, FEED WATER VIRUS CONCENTRATIONS PRODUCT WATER VIRUS CONCENTRATIONS, AND VIRUS REMOVAL EFFICIENCIES FOR VARIOUS MEMBRANES

Membrane	Average Flux (gpd/ft^2)	Average Total Number of Virus in Feedwater (a)	Average Virus Concentration of Feed Water (pfu/l) (a)	Number of Virus Detected in Product Water (pfu)	Virus Concentration of Product Water (pfu/l) (a)	Virus Removal Efficiency (%)
UF 10	256	2.6×10^5	3.7×10^4	1	2.0	> 99.999
UF 10	127	2.3×10^9	3.3×10^8	4	7.6	> 99.999
UF 10	218	4.9×10^9	6.8×10^8	3	5.4	> 99.999
UF 10	164	2.2×10^9	3.0×10^8	2	3.2	> 99.999
UF 10	166	6.3×10^8	7.8×10^7	0	0-2.4 (b)	> 99.999
HT 65	49	3.2×10^3	2.9×10^2	3	1.2	99.77
HT 70	59	8.0×10^8	7.0×10^7	0	0-1.1 (b)	> 99.999
HT 70	43	9.1×10^8	1.6×10^8	1	5.6	> 99.999
HT 75	25	6.5×10^8	8.5×10^7	1	0.85	> 99.999
RO 89	45	2.2×10^3	2.8×10^2	2	1.3	99.81
RO 89	34	8.2×10^2	9.6×10^1	3	1.8	99.20
RO 97	31	1.1×10^3	1.4×10^2	1	0.67	99.81
RO 97	31	1.5×10^3	1.9×10^2	0	0-0.61 (b)	100
RO 97	27	8.0×10^2	1.0×10^2	0	0-0.70 (b)	100
RO 97	28	4.4×10^3	5.5×10^2	1	0.71	99.50

NOTES:

a. Values corrected for recovery efficiency of 25 percent for coliphage T2 and 45 percent for poliovirus.

b. Range indicates possible maximum virus concentration due to virus concentration technique efficiency for the specific virus and medium employed, although no virus were actually detected in the product water.

virus concentrations of zero to 0.71 pfu/liter. The average virus concentration in the product water of four runs of the UF 10 membrane was 4.6 pfu/liter at flux rates of 127 to 256 gpd/ft^2. The RO 97 membrane yielded an average product water virus concentration of 0.35 pfu/liter for product water flux rates of 27 to 31 gpd/ft^2.

Figure 13 illustrates the relationship between the total number of virus in the feed water and the percentage rejection of virus by all of the membranes studied. Virus removal efficiencies of the different membranes varied with the virus concentration of the feed water, since the number of virus detected in the product water remained relatively constant at zero to 16 pfu for all membranes.

Calculated virus removal efficiencies are tabulated in Table 19. These data are calculated on the basis of the total average number of virus in the feed water compared to the actual number of virus detected in the product water (corrected for virus recovery efficiency). The average concentration of virus in the feed water varied from 9.6×10^1 to 6.8×10^8 pfu/liter; therefore, small changes in removal efficiencies could not be calculated. Virus removal efficiencies were essentially 100 percent at high feed water virus concentrations and as low as 99.2 percent at an average feed water virus concentration of 96 pfu/liter.

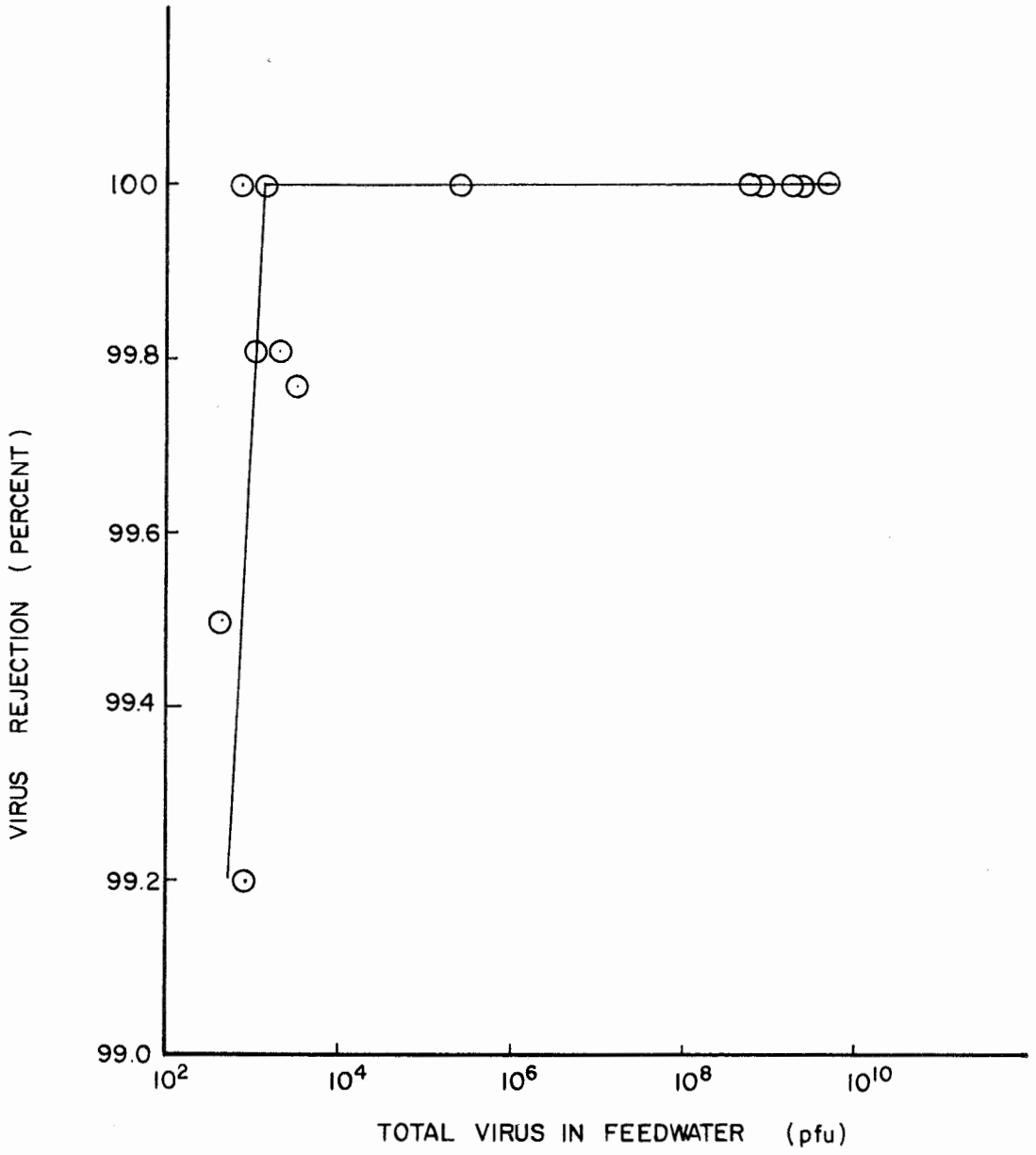

FIGURE 13. VIRUS REJECTION BY THE REVERSE OSMOSIS AND ULTRAFILTRATION UNIT PROCESSES

The results of these studies clearly demonstrate the need to evaluate the virus removal characteristics of water and wastewater unit processes at virus concentrations typical of those common to the natural environment. An evaluation of reverse osmosis membrane performance at the high virus concentrations traditionally used in research of water and wastewater processes would result in virus rejections of essentially 100 percent. This phenomenon is particularly significant because of the emphasis placed on "percent removal" frequently used to rate various unit processes.

The observed data indicate that at feed water average virus concentrations from 9.6×10^1 to 6.8×10^8 pfu/liter, the virus concentration in the product water was relatively constant, namely zero to 7.6 pfu/liter. Table 19 includes the virus concentrations of product water and the average virus concentration of the feed water. These data clearly indicate that only minor changes in product water virus concentrations (zero to 7.6 pfu/liter) accompanied very wide changes in feed water virus concentrations (9.6×10^1 to 6.8×10^8 pfu/liter). For the four data points where no virus was recovered from the product water, the range represents the maximum virus concentration which could be expected in the product water as a function of the recovery efficiency for the specific virus and

suspending medium employed. These data lend further support to the theory of random virus penetration of cellulose acetate membranes, especially at feed water virus concentrations below 10^8 pfu/liter. Specifically, at naturally occurring virus levels, viruses in the feed water to a reverse osmosis or ultrafiltration process employing asymmetrical cellulose acetate membranes can randomly penetrate imperfections in the membranes.

From a practical standpoint, cellulose acetate membranes such as those used in this study cannot be expected to provide a virus-free product water. On the other hand, since virus penetration is a random function, penetration of the high flux membranes (>100 gpd/ft^2) was almost the same as that of the tightest membranes (27-31 gpd/ft^2). Disinfection of the product water from any of these reverse osmosis and ultrafiltration membranes is essential to the production of virus-free water for drinking supplies. It should be noted however, that product disinfection would be more efficient after reverse osmosis or ultrafiltration since considerable quantities of particulate and dissolved materials exerting a disinfectant demand would have been eliminated from the product water of these unit processes.

CONCLUSIONS

The conclusions of this study can be summarized as follows:

1. The insoluble polyelectrolyte technique is effective for concentrating the quantitatively detecting low levels of virus in Phosphate Buffered Saline (PBS) and raw lake water. A maximal recovery from lake water of 25 percent of the coliphage T2 and 45 percent of the poliovirus was achieved. This procedure provided an effective method for evaluating the performance of the reverse osmosis - ultrafiltration process in the removal of poliovirus and coliphage T2.

2. The effectiveness of the insoluble polyelectrolyte concentration technique was sensitive to and directly dependent upon pH for the specific viruses and suspending media used in this study. Variations of $\pm$ 0.3 pH units from the optimum pH resulted in a 30 to 40 percent reduction in the numbers of coliphage recovered from lake water. Variations of $\pm$ 0.5 pH units resulted in an 18 to 27 percent reduction in the number of poliovirus recovered from lake water.

3. The cellulose acetate membranes used in this study rejected from 99.2 percent at over 99.999 percent of the coliphage T2 and poliovirus present in the feed solution. Penetration by viruses of asymmetrical cellulose acetate membranes was demonstrated to be a random phenomenon over a broad range of feed water virus concentrations.

4. The number of viruses penetrating the cellulose acetate membranes used in this study was zero to 16 virus (zero to seven pfu/L) and was relatively constant for all runs, although the average feed water virus concentration ranged from 5.5×10^{1} pfu/liter to 6.8×10^{8} pfu/liter. Exceptions were two runs where membranes exhibited characteristics of mechanical failure.

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