A QUANTITATIVE STUDY OF THE ENTERIC VIRUSES IN SEWAGE

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James R. Frey

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THESIS

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W. M. Mack
Major professor

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ABSTRACT

A QUANTITATIVE STUDY OF THE ENTERIC VIRUSES IN SEWAGE

by James R. Frey

The purpose of this study was to determine step by step (quantitatively) the reduction of the enteric viruses in sewage during the process of sewage treatment.

A total of 616 pad, dip and sludge samples were collected from the East Lansing sewage treatment plant from June 6, 1959, through November 7, 1959. Fifty-four samples were positive for the presence of virus when the samples were tested in monkey kidney epithelial monolayers. The pad sampling method proved to be the most satisfactory for the recovery of virus from sewage followed by the sludge and dip samples. Plaque counts were made on each of the original positive samples to determine the number of virus particles per unit volume. A new, improved, semi-microplaque technique for counting enteric viruses was developed and used in counting the virus samples.

An increase in virus particles as expressed in plaque forming units per ml (pfu/ml) resulted from the treatment process of sewage through the disposal plant up to and including

the final settling tank. The final effluent was exposed to an average of 0.5 parts per million of free residual chlorine for 15 minutes. The virus concentration entering the sewage plant was reduced 94.7 per cent as a result of the treatment process.

The potential reservoir of virus disease is great in raw sewage, contaminated water supplies, inadequately treated sewage and activated sludge materials. Chlorine residual should be increased if destruction of viral pathogens is desired.

A QUANTITATIVE STUDY OF THE ENTERIC VIRUSES IN SEWAGE

By

James R. Frey

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INTRODUCTION

The discovery of antibiotics, correlated with the knowledge that many multiplying viruses produce degenerative changes in cultured cells, has given the virologist a new tool with which to study the viruses in greater detail. The use of tissue cell culture in clinical and epidemiological studies has increased over the last ten years. The rapid development of new methods for growing various primary tissue cell lines is further proof of the practicability of this method for use in studying viruses.

The purpose of this study was to determine step by step (quantitatively) the reduction of the enteric viruses in sewage during the process of sewage treatment. The enteric group of viruses at present consists of the polioviruses, Coxsackie viruses, the enteric cytopathogenic human orphan (ECHO) viruses and possibly other unknown viral agents. The presence of these viruses in sewage has been established. Studies have shown a marked seasonal incidence of the enteric viruses in sewage with the highest recoveries occurring during the summer months. Little was known concerning the quantitative nature of the enteric viruses in sewage and the number that survive modern day treatment procedures. The efficiency of the sewage treatment process in removing the enteric

viruses is of practical public health and epidemiological significance. The possible spread of disease by continuous discharge of treated sewage effluents into surface water is aesthetically disagreeable and important to every individual.

REVIEW OF LITERATURE

Today there is one factor on which virologists and epidemiologists agree-----the consistency with which poliomyelitis, as well as the other enteric virus diseases seem to avoid falling into patterns normal to other transmissible diseases. Clinical manifestations of these diseases vary from the usually unrecognized carrier to frank paralysis. One of the first possible answers to this problem was suggested by Sawyer (1915-1916) who demonstrated the active virus of poliomyelitis in rectal washings from a patient 14 days after the onset of paralysis. Prior to this report Kling et al. (1912) infected monkeys with colonic flushings from patients suffering from infantile paralysis.

Since the publication of these initial reports, numerous investigators have isolated many types of enteric viruses from fecal materials. Among these are: Trask et al. (1938), Howe and Bodian (1939), Kramer et al. (1939), Lepine (1939), Lepine et al. (1939), Vignac et al. (1939), Gard (1940), Kramer (1940), Piszczek et al. (1941), Silverman (1941), McClure and Langmuir (1942), Dalldorf and Sickles (1948), Melnick et al. (1949), Robbins et al. (1951), Riordan et al. (1952), Melnick and Agran (1952), Steigman et al. (1953), Ramos-Alvarez and Sabin (1954), Honig et al. (1956), Ramos-Alvarez and Sabin (1956) and Gelfand et al. (1957).

Trask et al.(1938) isolated poliomyelitis virus from 4 cases of the disease. Three isolations were made from the nasopharynx in 3 different cases and 3 more isolations were made from stool specimens in one case. The virus remained viable for 10 weeks in one of the stool samples that was stored in the refrigerator at 4 C. Such findings suggested that these hitherto unrecognized sources of poliomyelitis virus might have been responsible for a high degree of pollution of sewage with the virus. As a result investigators turned to the study of sewage as a possible reservoir and means of naturally transmitting the enteric virus diseases. This led to the first isolation of poliomyelitis virus from sewage by Paul et al. (1939). These results supported the hypothesis that improper disposal of feces from patients might have serious public health consequences. Trask (1939) stated, "Authorities are now ready to admit other avenues to the central nervous system besides the nose, since lesions are not found in the olfactory bulbs in fatal human cases, and the disease has been produced accidentally in man by the subcutaneous inoculation of vaccine, and monkeys (except the common rhesus) may be infected by placing virus in their food."

Following the initial isolation of poliomyelitis virus from sewage, Levaditi (1940) isolated virus from sewer

water in Paris. This was quickly followed by an anonymous report in the Journal of American Medical Association (1940) stating Kling had inoculated a number of monkeys with samples of the contents of Paris city waters. After repeated inoculations the animals developed paralysis thus confirming the previous reports concerning the presence of the poliomyelitis virus in sewage. In a report during the same year (Paul et al., 1940), poliomyelitis virus was recovered from urban sewage 6 times in 50 attempts. McDaniel (1940) recovered poliomyelitis virus from sewage in South Carolina. Paul et al. (1940) demonstrated that poliomyelitis virus survived in the sewage flow for several miles.

Trask and Paul (1942) established the fact that the poliomyelitis virus was not detectable in urban sewage during non-epidemic times. This conclusion was subsequently refuted by Mundel et al. (1947) who reported the recovery of poliomyelitis virus from sewage in a treatment plant in Johannesburg, South Africa. No case of poliomyelitis had been reported for the previous two months in the area prior to the initial virus recovery. This finding possibly indicated that continued infection of poliomyelitis was still occurring.

A method for concentrating and detecting poliomyelitis virus in sewage was devised by Melnick (1947). This method

consisted of ammonium sulfate precipitation followed by ultracentrifugation. Using this method virus was detected in sewage from Imhoff settling tanks, in raw sewage and in sewage leaving a poliomyelitis hospital. Virus was also recovered from the sewage of a residential area in which no persons with poliomyelitis were living and in which no poliomyelitis subsequently developed. Virus was recovered only in late summer and fall months.

Dalldorf and Sickles (1948) isolated an unidentifiable agent from the feces of a child with paralysis. Isolation of the virus was dependent upon the utilization of day old mice rather than the more expensive monkey. This agent was later found not to be poliomyelitis virus but a new virus which was called Coxsackie virus. Melnick et al. (1949) reported finding a filterable agent similar to that reported by Dalldorf. This agent was isolated from sewage and produced characteristic paralysis in day old mice.

Clark et al.(1951) studied the Coxsackie virus in urban sewage and reported that the virus was probably excreted by healthy carriers. Francis et al. (1953) detected poliomyelitis and Coxsackie viruses from privy contents in Texas, thus supporting the presence of these viruses which had previously been isolated from sewage.

Melnick et al. (1954a) studied two sampling methods

in collecting sewage specimens for virus isolations. Gauze pad and dip sampling methods were compared. The gauze pad technique yielded a significantly higher percentage of positive viruses than did the dip sampling method. These methods are described in detail under materials and methods.

The seasonal distribution of Coxsackie viruses in urban sewage was investigated by Melnick et al. (1954b) by testing a total of 1,926 samples which yielded 269 viruses. The viruses appeared mostly in samples collected in the summer and fall, but rarely during the winter and spring.

Kelly et al. (1955) demonstrated again the presence of Coxsackie virus in sewage. The gauze pad device was used as the method of sampling and these data supported anew, the concept that "acceptable" sewage treatment facilities did not always destroy pathogenic agents.

Mack et al. (1958) tested sewage for 24 consecutive months. Viruses were recovered 118 times from 1,403 tests resulting in 8 per cent positive recovery. The greatest per cent of recoveries was made during the months of July, August, September, and October. Tests on the non-chlorinated final effluent from the sewage plant showed that some of the viruses were not removed by the sewage treatment. Types I and III poliomyelitis, ECHO, Coxsackie and 17 unknown viruses were isolated during this investigation.

Giovanardi and Berganani (1959) found type I poliomyelitis virus to be the most prevalent of all as the cause of paralytic poliomyelitis in Italy. These investigators isolated this agent during inter-epidemic periods from sewage.

Kelly and Sanderson (1959) studied 140 samples of raw and treated sewage collected in the summer and fall of 1957. Samples were obtained from the influent, effluent and activated sludge only. Viruses were isolated from these samples 166 times. The frequency of isolation and the concentration of infectious particles in the samples indicated that sewage treatment did not destroy the viruses. Chlorination for disinfection, often but not always, reduced the frequency of isolation and concentration of the infectious particles.

Prior to this report, Casey and Aymond (1940) correlated the distribution of poliomyelitis in Louisiana with case rates in communities with and without water supplies and sewage systems. Data indicated that communities without a water supply and sewage system had 39.7 cases per 100,000 inhabitants. Communities with a water supply and a sewage system had 26.6 cases per 100,000 population while communities with a water supply but no sewage system had a total of 83.6 cases per 100,000 population. Ellsworth (1940) also noted that certain bathing beaches in Massachusetts were affected by sewage pollution which may have resulted in a higher than

normal incidence of poliomyelitis. It was suggested that the virus might have been present in sewage polluted waters in sufficient concentrations to cause infection as a result of repeated contact of the water with the nasal passages.

Hargreaves (1950) discussed a severe outbreak of poliomyelitis in Cornwall, England, which occurred in 1949. There was an incidence of 0.3 per cent per 1,000 population, 20 cases occurring in 18 days. Investigation showed that the cases were evenly distributed over the city. One interesting aspect of this report was the fact that the city water supply had been augmented by tapping a stream heavily polluted with Escherichia coli. After increased chlorination of the stream supplement, only one additional case was noted. Little (1954) demonstrated that a possible reservoir of infection was present in a town upstream from which inadequately treated sewage had access to the water supply. The poliomyelitis virus was demonstrated in sewage at the time the disease was present in the town.

Along with the increased interest in the study of sewage as a source of enteric virus disease transmission the period of viral excretion by infected patients has been investigated. Trask et al. (1938) found that the poliomyelitis virus remained viable for 10 weeks in a stool sample that was stored in the refrigerator. Virus was also recovered

from the feces of a child with abortive poliomyelitis on the second, fourteenth and twenty-fifth day after the onset of the disease. In 1940 Kramer reported that the virus of poliomyelitis survived for 6 months in the feces of a contact which had been stored in the refrigerator at 3 to 4 C.

Piszczek et al. (1941) isolated poliomyelitis virus from the feces of 3 of 4 patients about one month after the onset of the disease and also isolated the virus from stools of 6 of 38 contacts. Feces from 14 normal residents in the community gave negative results. Horstmann et al. (1944) studied the persistence of virus excretion in the stools of poliomyelitis patients and concluded that the period of virus excretion extended into the seventh and eighth weeks. The virus was isolated from the feces of 61 patients. Again in 1946 Horstmann et al. studied the excretion of virus in the stools of patients following acute poliomyelitis. The virus was present in 70 per cent of the cases in the first two weeks, 50 per cent during the third and fourth weeks, 27 per cent at the end of the fifth and sixth weeks, and 13 per cent at the end of the seventh and eighth weeks. In a single instance virus was demonstrated at the twelfth week after the onset of the disease. Rhodes et al. (1950) demonstrated that poliomyelitis virus in a stool specimen, after addition of river water, was infective for monkeys for at least 188 days.

The stool sample was diluted in river water at a dilution of 1:200 and stored in the refrigerator at 4 C. These data again suggest the possibility that improperly treated sewage could play an important role in the transmission of enteric viral diseases.

Due to the ease with which the enteric viruses could be isolated from feces and sewage materials, several investigators undertook studies involving the so-called carrier state and viruses present in normal individuals. Melnick and Agren (1952) isolated both poliomyelitis and Coxsackie viruses from normal infants in Egypt. They obtained samples by rectal swabs and used monkey testicular tissue cultures for the isolations. They reported finding an unidentifiable cytopathic agent; however, it failed to produce disease in monkeys, mice and hamsters. The characteristics of this unidentifiable agent were similar to others previously reported in the literature. Prior to this work Robbins et al. (1951) isolated two unidentifiable agents from two patients with diseases diagnosed as non-paralytic poliomyelitis. Riordan et al. (1952) reported that three agents out of a total of 24 isolations from the feces of both paralytic and non-paralytic poliomyelitis patients could not be typed. Kibrick et al. (1953), Melnick et al. (1953), Steigman et al. (1953) and Horstmann (1955), all isolated unidentifiable agents in the

stools, blood or rectal swabs from healthy people or patients ill with poliomyelitis, non-paralytic poliomyelitis or aseptic meningitis. These viruses were tentatively called "orphan viruses" by Melnick et al. (1955).

The recovery of large numbers of new cytopathogenic viruses in different laboratories from the intestinal tract of man prompted the National Foundation for Infantile Paraylsis to establish the Enteric Cytopathogenic Human Orphan (ECHO) Virus Committee to classify these viruses (1955). In 1957 the Committee on ECHO viruses changed its name to the Committee on Enteric viruses. This name change was based on the fact that the poliomyelitis, Coxsackie and ECHO viruses may all inhabit the alimentary tract of man. Finally the enteric viruses included the 3 types of poliomyelitis viruses, the 19 Group A and 5 Group B Coxsackie viruses and the 19 ECHO viruses. Needless to say, the task of continued classification of new enteric viruses is continuing.

Ramos-Alvarez and Sabin (1954) recovered 31 cytopathogenic agents from 1,566 healthy American children who had no contact with clinically recognized cases of poliomyelitis. Rectal swabs were used as the sampling method. Poliomyelitis, Coxsackie and ECHO viruses were recovered from normal children with no apparent disease manifestations. In view of these findings Honig et al. (1956) investigated endemiologically

the enteric virus infections among 136 normal children in two socio-economic groups over a 29 month period. A repeatable seasonal incidence of enteric virus excretion was noted with over 90 per cent isolations occurring during the months of June through October. Again in 1956, Ramos-Alvarez and Sabin (1956) studied the viral flora of 3,336 healthy children in this country and in Mexico using rectal swabs as the sampling method. These data indicated that the highest recovery of the viruses occurred during the months of June, July and August. Gelfand et al. (1957) studied the enteric viral flora of normal children in southern Louisiana by testing extracts of fecal specimens. A marked seasonal association was evident with a peak in the summer and autumn months. Melnick et al. (1953) concluded that stool samples were superior to rectal swab samples for virus isolations. During this study viruses were isolated that were not identifiable as poliomyelitis virus or Coxsackie virus. These agents were found to belong to the ECHO group of viruses.

As a result of the ease with which enteric viruses could be recovered from stool samples and sewage, investigators searched for improved methods of isolation and concentration of these viruses. Kling et al. (1939) devised a method for concentrating poliomyelitis virus from fecal material by means of ether extraction. The treated stool

sample materials could be kept in the refrigerator in ether up to 53 days before the virus was destroyed. Virus was recovered by use of this method from patients with the abortive form of poliomyelitis. Howe and Bodian (1939) investigated the efficiency of the intranasal inoculation in rhesus monkeys as a means of recovering poliomyelitis virus from stools. By using this route of inoculation, virus was demonstrated in 63 per cent of 174 stool specimens. By testing each specimen with two or more monkeys instead of one, the virus recovery could be increased to 90 per cent. Gard (1940) designed a method for detecting poliomyelitis virus in sewage and from stool samples. The method consisted of making aqueous suspensions of human stools or sewage and precipitating the suspensions with 50 per cent saturation of ammonium sulfate. The precipitate was dialyzed against water to remove the ammonium sulfate without loss of viral activity. Melnick (1947) in studying the various techniques used for isolating enteric viruses from sewage found the most sensitive method to be a combination of ammonium sulfate precipitation followed by concentration in the ultracentrifuge. Kelly (1953) reported the recovery of virus from sewage employing a simple, rapid and comparatively safe method using absorption on, and elution from, ion exchange resins. Stevenson et al. (1956) developed a method for concentrating dilute virus

suspensions byalum flocculation. The method proved to be more sensitive than the ion exchange method described previously and possessed the additional advantage of being economical and simple.

Tissue culture methods have greatly facilitated the isolation and identification of the enteric viruses. Enders et al. (1949) first utilized tissue culture methods by cultivating the 3 strains of poliomyelitis virus in human embryonic tissues. As a result of these techniques the viruses could be studied on a larger scale with less expense than was formerly possible. Milzer et al. (1950) succeeded in confirming the propagation of poliomyelitis virus by cultivating the Lansing strain in various human embryonic tissue cultures. Following this report, Robbins et al. (1951), using tissue culture methods, isolated poliomyelitis virus directly from the patient's feces. In 1952 Robbins et al. cultivated poliomyelitis virus in roller-tube cultures of embryonic skin, muscle and fascia, mature human uterus, kidney and testes, and kidney and testicular tissues from rhesus monkeys. Riordan et al. (1952) reported isolating and serologically typing strains of poliomyelitis virus from human stools and spinal cords in roller-tube cultures of monkey testes. Scherer et al. (1953) demonstrated propagation of all 3 types of poliomyelitis viruses in human uterine

carcinoma cell cultures (HeLa) established by Gey *et al.* (1952). The virus multiplied and caused degeneration and destruction of the cancer cells within 12 to 96 hours after inoculation. These experimental results demonstrated the usefulness of the HeLa cells for quantitating the poliomyelitis virus, measuring poliomyelitis antibodies and general laboratory production of the virus. Syverton and Scherer (1954) found that monkey kidney epithelial cells were more susceptible to infection by poliomyelitis virus than was the HeLa cell line. Weller (1953) stated that conditions observed *in vitro* were different from those obtained *in vivo* and that human tissue culture systems promised an alternative, in certain cases, to the study of the infective entities of man that possess a high degree of host specificity.

Melnick *et al.* (1954) compared standard suspensions of monkey kidney epithelial monolayers and fibroblast monolayer cultures of monkey testes with suspended fragments of kidney epithelial tissue for sensitivity in primary isolation of poliomyelitis virus. Results indicated that the monkey kidney epithelial monolayer tissue culture was more sensitive than the other methods and tissues used in this study. With the development of a procedure by Dulbecco and Vogt (1954) for the preparation of cell suspensions of monkey kidney by trypsinization, an important step in tissue culture methods

was achieved. Younger (1954) improved this original technique thus enabling investigators to prepare standard suspensions of monkey kidney cells which could be used to prepare large numbers of replicate cultures for use in isolation and identification of the enteric viruses.

In 1954 Dulbecco and Vogt (1954) devised a plaque method for counting the enteric viruses. Monkey kidney and testicular monolayer cells were used in this study with pure lines of the 3 types of poliomyelitis viruses. The authors concluded that each plaque originated from a single virus particle, defined as the virus unit that was inseparable by dilution and that the number of plaques was equivalent to the virus concentration.

Wenner and Miller (1954) compared monkey testicular cell cultures, HeLa cell cultures and monkey kidney epithelial cell cultures for recovering poliomyelitis viruses from human sources. Under the conditions of the study, monkey epithelial cell cultures were superior to HeLa or monkey testicular cultures in the primary isolation of poliomyelitis virus from oropharyngeal specimens.

Kibrick et al. (1953) compared the sensitivity of various human cell cultures with monkey kidney epithelial cell cultures in the detection of poliomyelitis virus from human sources. They found the monkey epithelial cell cultures

to be more sensitive than the other cell cultures.

Hsuing and Melnick (1955) simplified Dulbecco's method of plaque formation by growing the monkey kidney epithelial cells in ordinary prescription bottles with rubber stoppers. These same authors (1957a) were able to group provisionally 70 isolates of the ECHO viruses by plaque morphology. Serological procedures were then used to type the various groups of viruses. In the hands of the authors this plaque procedure eliminated the time consuming serological grouping that would have been necessary before individual antigenic types could be determined. In the same year Hsuing and Melnick (1957b) compared the susceptibility of kidney cells from different species of monkeys to the enteric viruses. They found that the Macaca cynamalogus kidney cells were highly susceptible to infection by the enteric virus group as were the rhesus monkey cells. As a result of these findings the monkey kidney epithelial cell monolayer is the cell line of choice for use in enteric virus investigations.

The present program of isolating viruses from sewage is best summarized by Kabler (1959) who stated, "Enteric viruses and pathogenic fungi have also been recovered from sewage, but it is recognized that numerical results are only useful approximations because of the lack of sensitivity and wide variability in the procedures along with the unpredictable

effects of interferring organisms and other organic components." In this work to be presented quantitative recoveries of virus have been obtained.

MATERIALS AND METHODS

Collection of samples:

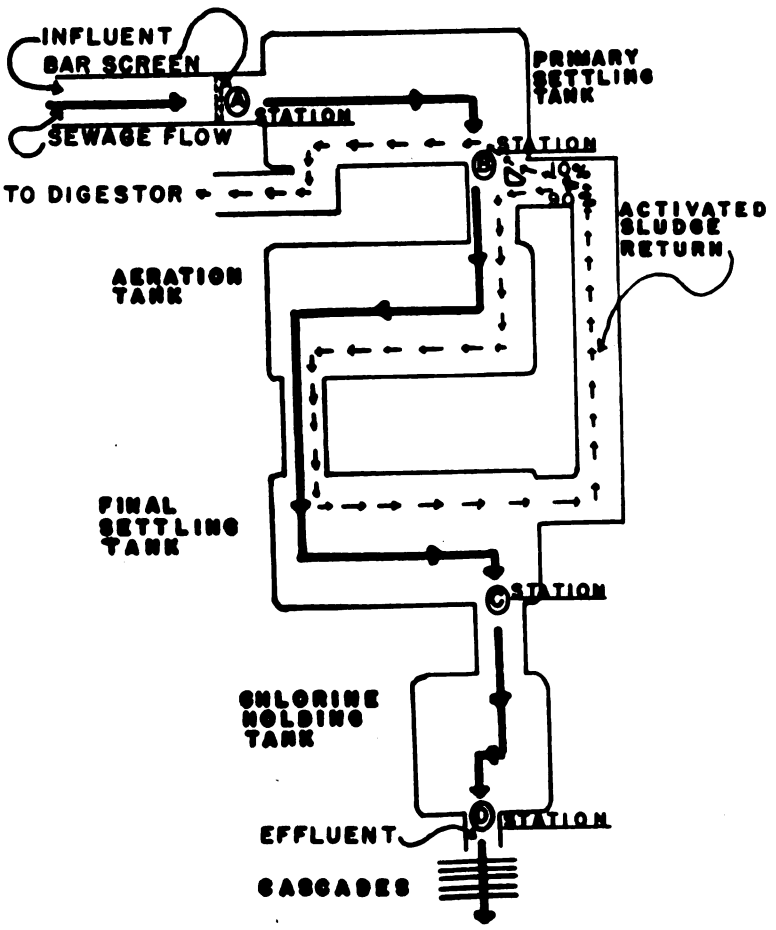
East Lansing's treatment plant is of the activated sludge type and is unique in the fact that there is little or no industrial waste material processed by the plant. Previous studies by Mack et al. (1958) have established the presence of viral agents in the sewage of this treatment plant. The next course of study was to determine the stepwise virus concentration in sewage during the process of sewage treatment. Based on the above criteria this plant is ideally suited for the exacting methods that are required in the quantitative recovery of viruses from sewage.

The designed flow of this particular sewage treatment plant is 3.7 million gallons daily (mgd). Maximum daily flow through the plant occurs between 9:00 a.m. and 12:00 noon. The plant normally serves a residential area of 30,148 people when the university is in session. During the summer months the number of persons decreases by approximately 20,000 people. The final effluent was exposed to an average of 0.5 parts per million (ppm) of chlorine residual for 15 minutes. The concentration varied from 0.2 ppm at night to 4.0 ppm during the heavy flow period. The average hydrogen ion concentration (pH) of the final effluent was between 7.2 and 7.5.

Sewage samples were collected three times a week during the period of June 6, 1959, through November 7, 1959. A total of 685 samples were collected during this period. Pad, dip and raw sludge samples were collected at the same period. The pads consisted of 4 1/2 x 4 1/2 inch absorbent cotton placed between two layers of cheese cloth and sewed together around the periphery. The pads were placed in the flowing sewage and upon extraction yielded approximately 150 ml of liquid. Dip samples were collected by permitting 200 ml of sewage to flow into a wide mouth bottle. The raw sludge was accumulated in a holding tank and pumped to the digestor daily. Raw sludge samples represented a 24 hour collection of 200 ml of sludge. All samples were brought to the laboratory immediately after collection and stored at -20 C.

In order to determine the amount of enteric viruses in sewage during the process of sewage treatment, samples were collected (figure 1) stepwise throughout the processing procedure. Samples taken from the influent channel directly behind the bar screen were labeled station A. Samples taken from the weir of the primary settling tank were labeled station B. Samples taken from the spillway of the final settling tank were designated as station C and samples obtained from the chlorinated effluent, at the

FIGURE I. A DIAGRAMATIC STEPWISE COLLECTION BY STATIONS OF SEWAGE SAMPLES THROUGHOUT THE PROCESSING PLANT.



outfall to the river, or cascades were labeled station D.

Laboratory processing of samples:

The collecting bottles containing the frozen pad samples were placed in cool tap water to hasten thawing. After thawing, the pad was removed from the bottle and placed in a large funnel. The liquid content of the pad (approximately 50 ml) was removed into a 25 x 150 mm test tube. To remove the remaining fluid from each pad, the pad was cut in half with a sterile instrument and the pieces of pad were placed into the barrel of a 100 cc syringe. Pressure was applied to the plunger of the syringe removing approximately 50 ml of additional fluid. To prevent accidental loss of a single sample in processing, each sample after mixing, was divided into two equal parts in separate tubes. The duplicate samples were centrifuged at 3,000 rpm (1,800 x g) for 15 minutes in a horizontal centrifuge to remove gross particles. Ten milliliters of the supernatant fluids were removed from each of the tubes and placed in two 16 x 125 mm screw-capped test tubes and labeled. One-hundred and twenty ug of streptomycin and 30,000 units of penicillin were added to each duplicate sample to eliminate viable bacteria. The samples remained at room temperature for 4 hours before they were tested for sterility. Three tubes each containing 10 ml of brain heart infusion broth in 16 x 125 mm screw-capped tubes were

inoculated with 0.1 ml of each sewage sample. The specimen was considered free of viable bacteria if the brain heart infusion broth indicated no bacterial activity at the end of a 72 hour incubation period at 37 C. If the specimen contained viable bacteria it was centrifuged at 9000 rpm (6000 x g) for 15 minutes in an International refrigerated multi-speed centrifuge, model PR-1. The supernatant fluid was removed and an additional 20,000 units of penicillin and 100 ug of streptomycin were added per sample. The samples were again allowed to remain at room temperature for 4 hours and the sterility tests repeated. A sample was discarded after three such cycles if it continued to show the presence of viable bacteria in sterility tests.

The dip and sludge samples were treated in a similar manner.

Tissue culture methods:

Macaca cynamalogus kidney epithelial monolayer tube cultures were obtained from Microbiological Associates, Bethesda, Maryland. These tubes were used to isolate and confirm the presence of virus in the processed sewage specimens. Monkeys of the same species weighing approximately 1 to 2 kilograms were obtained from Trefflick's Animal and Bird Company, New York City. Kidneys from these animals were

processed in the laboratory and served as a source of cells for preparing monolayers for plaque determinations.

Lactalbumin hydrolysate medium (Melnick, 1952) was used to grow and maintain the kidney cells. The medium was prepared in the laboratory and consisted of Hank's basal salt solution, lactalbumin hydrolysate, sodium bicarbonate, antibiotics and inactivated calf serum. A ten times concentrated stock solution of Hank's basal salt solution was prepared and consisted of the following components:

Hank's basal salt solution 10X

$CaCl_2$	1.4 g
Glucose	10.0 g
NaCl	80.0 g
KCL	4.0 g
$MgSO_4 \cdot 7HOH$	2.0 g
KH_2PO_4	0.6 g
$Na_2HPO_4 \cdot 2HOH$	0.6 g
Phenol red	0.2 g

These components were dissolved in 1100 ml of triple distilled water, dispensed in screw capped bottles and autoclaved at 10 lbs. pressure (115 C) for 10 minutes. This was one of the components used in preparing the lactalbumin hydrolysate medium.

The formulation of the lactalbumin hydrolysate medium is as follows:

Lactalbumin hydrolysate medium

	per cent
Hank's BSS 10X	10.0
Lactalbumin hydrolysate	0.5
$NaHCO_3$	0.875
Penicillin	100 units
Streptomycin	100 ug
Inactivated calf serum	1.0 (maintenance)
	8.0 (growth)

Blood was obtained aseptically from two-month-old healthy calves. The blood was allowed to clot and was then stored at 3-4 C overnight. The serum was removed and placed in 16 x 125 mm screw-capped tubes and inactivated at 56 C for 30 minutes. Serum sterility tests were made in brain heart infusion broth in triplicate and the sterile serum was stored at -20 C.

Virus isolation and confirmation:

The standard tissue culture tubes of monkey kidney cell sheets from Microbiological Associates were used in isolating and confirming the presence of viruses in the processed sewage specimens. Upon arrival at the laboratory the kidney cells were observed and if growth was not confluent, the medium was changed and the tubes were held at 37 C until the cell sheets were fully developed. The cultures were inoculated, in triplicate, with 0.5 ml of the processed sewage and observed daily under the microscope for an average of 14 days for signs of cytopathic effect (CPE).

Inocula exceeding this amount proved to be, in many instances, toxic for the supporting cell sheets. The first visible sign of viral activity was the degeneration of the epithelial cells shown by small clusters of cells becoming rounded and transparent. In this early stage, a partial release of the cells from the glass surface of the tubes could be observed. Some of the cells became stellate-like and as viral multiplication continued a characteristic rounding of the cell population developed. Finally the cells began to detach from the glass surface which interrupted the continuity of the monolayer. The culture fluid was harvested when the majority of the cells was released from the surface.

Cytopathic effect was usually observed from the second to the ninth day after inoculation with the sewage specimens. All three tubes of a series did not always show CPE. The nutrient fluids from tubes demonstrating CPE were harvested. Confirmation of virus isolation consisted of inoculating a fresh monolayer of cells with 0.5 ml of undiluted harvested nutrient fulid. These cells were observed for CPE. A sewage specimen was considered confirmed and ready for plaque count determinations if CPE occurred in this culture within 14 days. In most cases CPE could be observed within 4 days.

The nutrient fluids from all positive cultures were stored at -20 C until plaque counts were made.

Preparation of cell cultures for plaque count determinations:

Kidneys were aseptically removed from exsanguinated M. cynamalogus monkeys and placed in a sterile Petri dish. The kidneys were decapsulated and placed in maintenance medium. The cortices of the kidneys were removed and the remaining portions discarded. The tissue was cut into pieces about 1 cm^2, transferred to a trypsinization flask and covered with 0.25 per cent trypsin solution prewarmed to 37 C. The trypsin flask was incubated for 1 hour at 37 C. After incubation the trypsin was decanted and fresh, prewarmed trypsin was added to the flask. The tissue fragments and trypsin were stirred with a magnetic stirrer for 8 minutes at 37 C. The speed of the stirring was sufficient to allow rapid mixing without foaming. After 8 minutes of stirring the flask was removed from the magnetic stirrer and the tissue fragments were allowed to settle. The trypsin solution, containing the free cells and tissue debris, was decanted into a sterile 50 ml conical centrifuge tube. Fresh prewarmed trypsin was added to the trypsinizing flask and the stirring procedure repeated. Similar cycles were continued until the tissue was exhausted.

The centrifuge tube containing the trypsin, free cells and cellular debris was centrifuged in a horizontal centrifuge at 600 rpm (75 x g) for 4 minutes. The trypsin

solution was removed and the packed cellular material was washed twice with maintenance medium. All the cells obtained from the trypsinizing procedure were accumulated in a sterile 50 ml centrifuge tube. The suspended cells were filtered through two layers of sterile cheese cloth to remove connective tissue and cellular debris. Following filtration the cells were centrifuged and resuspended in 15 ml of lactalbumin hydrolysate growth medium.

A Spencer, bright-line hemacytometer was used to determine the number of kidney cells obtained as a result of trypsinization. One-tenth milliliter of the cell suspension was added to 0.9 ml of a crystal violet staining solution. The staining solution consisted of 2.1 per cent citric acid and 0.1 per cent crystal violet dissolved in double distilled water. The 1:10 dilution of the kidney cell suspension was thoroughly mixed and a portion of this suspension was used to fill the counting chamber of the hemacytometer. Only those cells showing both nuclei and attached cytoplasm were counted. The cells appearing in the four large corner squares, normally used when counting white blood cells, were counted and an arithmetic average was obtained. This figure was multiplied by the dilution factor and the number of monkey kidney cells per ml of the original suspension was determined. The cell suspension was then diluted in lactalbumin hydrolysate nutrient

growth medium to give 300,000 cells per ml. Three ml of this suspension were seeded into each French square culture bottle and after 5 days incubation in a stationary, inclined position at 37 C a confluent sheet of growth developed. After the fifth day of incubation the growth medium was replaced and the cell sheets were incubated an additional 2 days to allow for maximum cell sheet development. The cell sheets were then considered ready for use.

Plaque studies:

Preliminary studies were performed in order to determine which plaque method would prove to be most satisfactory for use in this study. The procedure of Dulbecco and Vogt (1954) was first applied using 1 ounce French square bottles in place of unsealed Petri dishes. Dilute samples of the three known groups of enteric viruses were placed on kidney monolayer cells. The cultures were incubated at 37 C for 1 hour to allow the virus particles to attach to the cells. An overlay medium consisting of 1.5 per cent Noble agar in Earle's salt solution with 0.0017 per cent neutral red was added to each bottle to cover the cell sheet. A commercially prepared stock solution, five times concentrated of Earle's salt solution was used and consisted of the following components:

Earle's salt solution 5X

	g/l
NaCl	34.0
KCl	2.0
$CaCl_2$	1.0
$MgSO_4 \cdot 7HOH$	1.0
$NaH_2PO_4 \cdot HPH$	0.625
Glucose	5.0

After the agar solidified, the bottles were inverted and incubated at 37 C for plaque development. The agar confined the virus infection to the immediate area of the cells that were exposed to each infective particle introduced in the inoculum. The neutral red was employed to outline the area of infected cells. With this method the cell sheet degenerated within 48 hours. Decreasing agar concentration, varying the amount of neutral red in the overlay and staining the developing plaques with neutral red after 72 hours incubation improved results, but some of the plaques of the various species of virus used had not developed by this time. The neutral red dye precipitated in some instances resulting in the fomration of crystals on the cell sheets. Trypan blue was substituted for neutral red under the same conditions but gave equally poor results.

Klein and Goodgal (1959) found that monkey kidney monolayer cell cultures underwent degeneration within 24 hours after exposure to white light in the presence of neutral red. Prior to this work Darnell et al. (1958) studied the effect

of neutral red on plaque formation. They used monolayers of HeLa cells in Petri dishes incubated with type I poliomyelitis virus for 75 minutes at 37 C in 5 per cent carbon dioxide to allow the virus particles to attach to the cell sheet. Half the plates were then overlaid with nutrient agar containing neutral red and the other half with the same medium lacking neutral red. The plates were incubated in carbon dioxide atmosphere for 72 hours. At this time the plates, without dye, were stained with Earle's saline solution containing neutral red. Although the plaque size was unaffected, the number of plaques was reduced 50 per cent in the plates containing neutral red. These studies suggested that the use of a plaque technique which did not incorporate neutral red in the procedure would be more advantageous.

Attempts were made to follow Holland and McLaren's (1959) plaque technique. Known samples of the three groups of enteric viruses were inoculated into monkey kidney monolayers grown in 1 ounce French square bottles. A low agar concentration (0.6%) without neutral red was employed in the overlay medium. When the French squares were inverted so that the cell monolayer could be observed microscopically, the agar overlay slipped off the cell sheet surface. The procedure was originally designed for use with viruses whose period of plaque development was known. After plaques had attained

optimal size the agar overlay could be removed and the firmly adherent non-cytopathically affected cells of the monolayer could be fixed and stained with crystal violet. Originally this procedure did not necessitate daily observation of the cell sheets for determining the presence of developing plaque areas. A second disappointing feature of this plaque method was that the cell sheets started to degenerate on the third day after the addition of the agar overlay medium. This was probably due to the lack of available nutrient materials. It was necessary in this present work to keep the cell monolayers viable for a minimum of 7 to 8 days and preferably for 14 days so that the slow plaque forming viruses such as the ECHO viruses and some of the Coxsackie viruses could develop and produce plaques.

In 1959 Sommerville developed a microplaque method for counting enteric virus particles. His procedure was based on the small isolated foci of rounded degenerating cells preceding the appearance of a fully developed plaque. No dyes were used and the method did not depend upon an agar overlay. In the hands of the author the formation of these microplaques gave an accurate and reproducible method for counting enteric viruses. The cell sheets were washed three times with maintenance medium to remove unattached virus particles prior to incubation and subsequent plaque development.

The monolayers were observed with the high powered lens of the light microscope. The microplaques developed rapidly and in some instances secondary microplaques developed as a result of prolonged incubation. This method was promising, but the rapid development of secondary microplaques combined with the requirement of frequent microscopic examination of the cell sheets rendered the method unsuitable for use in this study.

For the purpose of this work a plaque technique was needed in which the cell sheet would remain viable for a minimum of 14 days and not be subject to the disadvantages of the previously mentioned methods. Preliminary studies resulted in an improved semi-microplaque technique for determining the virus concentrations in the sewage samples. This technique was found to be reproducible and is described as follows:

The maintenance medium was removed from the French square bottles containing confluent sheets of epithelial cells. The cells were inoculated with varying volumes of the sewage samples containing the unknown viruses to insure the formation of plaques that were separate and discrete. Immediately following inoculation the bottles were placed in the incubator at 37 C for 1 hour to allow the viruses to attach to the cells. The bottles were gently rotated every 15 minutes to assure

even distribution of the inoculum over the entire surface of the monolayer. The excess inoculum was removed from the bottles and the cell sheets were covered with 2.5 ml of 1.5 per cent Noble agar containing lactalbumin hydrolysate medium with 2 per cent inactivated calf serum. After the agar overlay solidified, 3 ml of fluid maintenance medium was added to each culture bottle to nourish the cells. The maintenance medium was changed, depending on the condition of the cell monolayers and variations observed in the indicator system in the medium. The inoculated cultures were inverted for incubation at 37 C and observed daily for 14 days for plaque development. Using this method the cell sheets remained viable. A Bausch and Lomb series B, stereomicroscope with a variable power pod of 7X to 30X total magnification was used to observe the cell sheets for the presence of developing plaques.

The first visible signs of plaque formation occurred between the first and the ninth day after inoculation. Small areas of degeneration of the cells were the first signs of plaque development. This was followed by small individual clustering of cells throughout the cell sheets as observed in figure 2. The number of developing plaques was dependent upon the size of the inoculum and the concentration of virus in the specimen. The developed plaques (see figure 4) could

Figure 2. Individual clustering of cells throughout the cell monolayer prior to full plaque development, fixed and stained with crystal violet (11X).

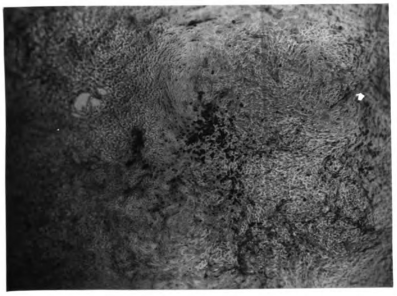


Figure 3. Normal control cell monolayer fixed and stained with crystal violet (11X).

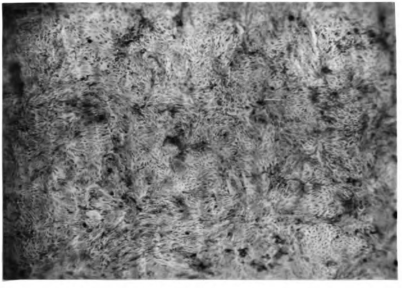


Figure 4. Full plaque development 48 to 72 hours after the appearance of small areas of degeneration, fixed and stained with crystal violet (11X).

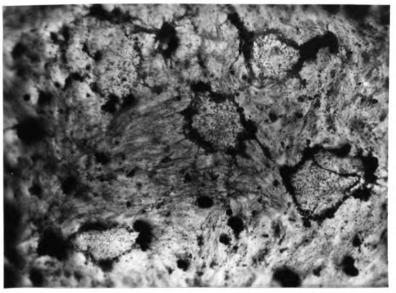
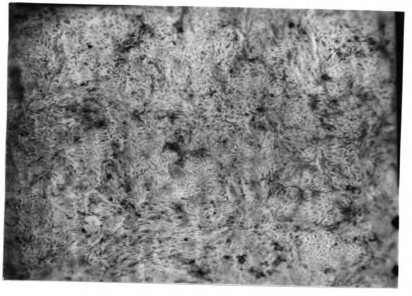


Figure 5. Normal control cell monolayer fixed and stained with crystal violet (11X).



be observed within 48 to 72 hours after the appearance of the small areas of degeneration. These areas of degeneration and plaque development were compared with normal control cell monolayers (figure 3 and 5).

To prevent confluent lysis of the cell sheet, 96 hours after the first visible appearance of a plaque, the cultures were fixed with 70 per cent ethanol for five minutes. After the alcohol was removed the cells were stained with 0.1 per cent crystal violet for five minutes and washed three times with tap water. By permitting the fixed and stained cultures to remain at room temperature overnight the stain penetrated the agar and stained the cell sheets. This staining procedure greatly facilitated plaque counting and preserved the plaques until counts could be made. A typical French square overlaid with agar and stained with crystal violet is presented in figure 6.

Plaque counts:

Four culture bottles each were inoculated with the undiluted sewage samples containing virus. After development, the plaque areas in each culture were counted and an arithmetic mean of the counts was determined for each sample. The volume of the inoculum varied in samples because of the amount of virus present in each sample. The final counts were computed to represent plaque forming units per milliliter of sample.

Figure 6. A typical French square bottle overlaid with agar and stained with crystal violet.

Ultracentrifuge concentration of samples:

Viruses were not found in samples from the chlorinated effluent when tested by the methods above. Because of the treatment and the presence of chlorine this was to be expected. To determine whether the effluent samples were completely free of viable viruses, all pad and dip samples collected in July were pooled into two separate groups. The total volume of the pad pool was 175 ml and the dip pool contained 125 ml. Both pools were concentrated in the ultracentrifuge at 42,040 rpm (114,610 x g) for 1 hour in the Spinco model E ultracentrifuge. The supernatant fluids were removed and the sediment from each sample was resuspended in 5 ml of sterile distilled water. Reasons for using this volume of diluent were to permit sufficient volume for virus isolation followed by repeated plaque counts should a virus be isolated. Virus was not recovered from these samples. Additional pools were made of pad and dip specimens collected in August which were concentrated in the ultracentrifuge. Since virus was not recovered in any of the concentrated pooled samples, further pools were made of the concentrates. All effluent samples for the months of July and August that had been concentrated in the ultracentrifuge were combined in a single sample of approximately 40 ml. This pool was ultracentrifuged at 110,660 x g for 1 hour and reconstituted in 5 ml of sterile

distilled water. In addition to the effluent samples collected during July and August two additional pools consisting of 355 ml each were made from effluent samples collected on the same day that virus was isolated from other stations in the plant. These two pools consisted of both dip and pad samples and were also concentrated in the ultracentrifuge. As a result of this treatment all the samples collected from the chlorinated effluent for the months of July and August and on those days when virus was isolated from other stations in the plant were contained in 3 concentrated samples. The 3 pooled ultracentrifuged samples were tested for the presence of virus in the conventional manner.

To have a valid basis for determining the amount of concentration obtained by ultracentrifugation, a known suspension of poliomyelitis virus type II diluted in sterilized sewage was studied. The virus suspension was concentrated in the ultracentrifuge as described previously. Plaque counts of the known suspension before and after ultracentrifugation were used to determine the degree of concentration.

RESULTS

During 5 consecutive months, June 6, 1959, through November 7, 1959, a total of 616 sewage specimens were tested in monkey kidney tissue culture. Virus was isolated a total of 54 times resulting in 8.8 per cent recoveries. The total per cent virus recoveries per month is illustrated in Table I. As can be seen, the largest per cent of virus isolations occurred in July when 13.1 per cent of the samples contained virus. This was followed by 9.5 per cent for August, 9.5 per cent for September, 6.5 per cent for October and 3.4 per cent for June. Only 24 samples were tested during November and viruses were not encountered.

A comparison is made in Table II of the per cent of virus recovered by the three sampling methods. Although the largest number of specimens tested were dip samples (293) only once was virus recovered from this type of sample. When the 268 pad samples were tested, virus was recovered 44 times. In 55 sludge samples, virus was recovered 6 times. The per cent virus recovery from the dip, pad and sludge samples was 0.3, 16.4 and 10.8 per cent respectively. The results show that the pad sampling was superior to dip sampling under the experimental conditions. The dip sampling method detected only the virus present at the time of collection. The pads on the other hand, were exposed to the sewage flow for 48 to

Table I. Total monthly per cent recovery of virus from sewage.

Month of collection 1959	No. positive/No. tested	Per cent virus isolations
June	4/117	3.4
July	18/137	13.1
August	10/105	9.5
September	12/123	9.5
October	7/107	6.5
November	0/24	0.0
Total	*51/616	8.8

*This total does not include the three positive pooled and concentrated dip and pad effluent samples.

Table II. Per cent virus recovery from dip, pad and sludge samples.

Sampling method	Total samples tested	Total positive samples	Per cent positive
Dip	293	1	0.3
Pad	268	44	16.4
Sludge	55	6	10.8
Total	616	*51	8.8

*This total does not include the three positive pooled and concentrated dip and pad effluent samples.

72 hours. The dip sampling method proved disappointing. Originally it was planned to compare the dip and pad sampling methods.

After virus was isolated from any sewage sample the original sample was set aside until all samples had been tested for the presence of virus. The original samples that were previously shown to contain virus were then tested to determine the number of virus particles per ml. Varied volumes from 0.3 to 0.75 ml were used to determine the number of plaque forming units (pfu) contained in each sewage sample. The volume of inoculum depended on the concentration of virus contained in each original sewage sample. Four culture bottles each were inoculated with a given volume of the undiluted sewage sample containing virus. The plaque areas in each culture were counted and an arithmetic mean of the counts was determined for each sample. The final counts were computed to represent plaque forming units per milliliter (pfu/ml) of the original sample. Each plaque represented at least one infectious virus particle. Duplicate plaque counts were determined whenever possible to verify results. The average pfu/ml for each sample for each collecting station each month is presented in Table III. Average plaque counts on the four viruses isolated in June were 22.7 in the influent, 28.7 in the primary settling and 28.7 in the final settling tank.

Table III. Total monthly average in pfu/ml at each collecting station.

Month collected	Number of samples	STATIONS A influent	B primary sett.	C final sett.	sludge	D effluent
June	4	22.7	28.7	28.7	0.0	0.0
July	18	17.2	51.4	43.3	28.4	0.0
August	10	28.6	40.6	195.0	76.0	0.0
September	12	62.8	48.5	36.0	26.5	0.0
October	7	48.5	96.6	0.0	34.2	0.0

There was a slight increase in virus particles per ml progressively through the disposal system. No virus was recovered from the sludge samples collected in June. In July there was a greater number of virus particles present in the primary settling samples (51.4) as compared to the influent samples (17.2). The virus particle concentration decreased slightly in the final settling samples (43.3) and the average sludge samples for July contained 28.4 pfu/ml. In August the trend seen in the previous two months continued and 28.6, 40.6 and 195.0 pfu/ml were found in the influent, primary and final settling stations respectively. A total of 76.0 pfu/ml was obtained from the sludge samples for the month of August.

During September and October the virus particles per ml were 62.8 and 48.5 pfu/ml in the respective influent samples and in September the virus particles per ml decreased as treatment was continued. There was 48.5 and 36.0 pfu/ml in the primary and final settling samples. In October there were large numbers of virus particles per ml (96.6) in the primary settling, but none was found in the final settling. The number of pfu/ml in the sludge for these months decreased respectively to 26.5 and 34.2 pfu/ml.

In general the data in Table III do not correlate with the crude percentages as given in Table I. This is

understandable as two different methods of analysis were used. The trend presented in Table III illustrates that as the sewage was processed there was a release of individual virus particles, and the sampling of the influent did not necessarily represent the total virus particles present in the other locations or sampling stations.

Since no virus was recovered from the effluent when the samples were tested for the presence of virus in the conventional manner, the pad and dip samples collected from the effluent for the month of July were combined into four separate pools containing 70, 105, 105 and 120 ml respectively, totaling 400 ml. The samples were concentrated by ultracentrifugation at 114,610 x g for 1 hour. All but 5 ml of the supernatant fluid was removed from each sample and 5 ml of water was added to each concentrated pool. The water was added to the concentrated sewage to give a volume with which to make repeated plaque counts. When these samples were tested in tissue culture no viruses were isolated. The August pad and dip samples were made into four pools containing 95, 70, 100 and 60 ml respectively (total 325 ml). After concentration, each pool was tested for virus in tissue culture. Again no virus was recovered. The concentration was 5 volumes for the July pools and 4 for the August pools. Since virus was not encountered by the above concentrations,

the 5 ml remaining in each of the eight pools for July and August were combined, giving 40 ml. The 40 ml of this pool was combined with 690 ml of the original supernatant fluid and ultracentrifuged at 114,610 x g for 1 hour. The supernatant fluid was removed permitting 5 ml to remain on the pellet. An additional 5 ml of water was used to wash the walls of the tubes, giving 10 ml total volume. This concentrated pool, when tested in tissue culture, was positive for the presence of virus. The total concentration, by volume, was 19.7 times for the four original July samples and 16.1 times for the August samples. The combined concentration was 18.3 times for the pool of the two month samples.

When virus was recovered from the effluent samples by pooling and concentration, additional pools were made and concentrated. Tests on the unconcentrated samples in the plant were not found to contain viruses each day and the effluent would probably not contain large amounts of virus on those days. Pad and dip samples from the effluent were pooled only on those days when viruses were already known to be present in the plant. Twelve pad and 12 dip effluent samples collected on June 6 through September 6 (excluding July and August) were combined in a 355 ml pool. A second pool of 17 pad and 16 dip effluent samples of September 12 through October 24 contained 355 ml. The pooled samples

were ultracentrifuged at 114,610 x g and all but 11 ml of the supernatant fluid was removed from the sediment. Three ml of water was then added to give a sufficient volume of material with which to fill the ultracentrifuge tubes for further concentration. The two 14 ml pooled samples were ultracentrifuged again at 110,660 x g for 1 hour. The supernatant fluid was removed permitting 1 ml to remain on the pellet. An additional 9 ml of water was used to wash the tube walls giving a total volume of 10 ml. These concentrated pooled samples were positive for the presence of virus when tested in tissue culture. The total concentration for each pool was 35.7 times by volume.

In order to determine the virus particle concentration resulting from ultracentrifugation of the pooled effluent samples a known suspension of poliomyelitis virus type II was diluted in sterilized sewage and concentrated in the ultracentrifuge. Two ml of a mouse brain suspension containing poliomyelitis virus was added to 98 ml of sterilized processed sewage. The suspension was thoroughly mixed and divided into two portions of 66 and 34 ml respectively. The smaller portion was used to determine the number of virus particles per ml in the original unconcentrated suspension. The 66 ml portion was concentrated in the ultracentrifuge in the same manner as those pooled effluent samples of June 6-30

and September 12 through October 24. The results of this experiment are presented in Table IV. The average pfu/ml in the 10^{-1} dilution of the unconcentrated original suspension was 6.7 as compared to 141.7 in the concentrated suspension. The virus was concentrated 21.1 times for each plaque forming unit contained in 1 ml of the unconcentrated original suspension as a result of ultracentrifugation. This represents an increase of 1.3 logarithmic units.

Small volumes of inocula (0.3 to 0.5 ml) of the concentrated effluent fluids were used in determining pfu/ml as compared to the larger volumes (0.75 ml) used in testing the other unconcentrated samples collected from other stations throughout the plant. The cell monolayers did not tolerate the toxic substances concentrated along with the viruses during ultracentrifugation when larger volumes of the concentrated effluent samples were used for inoculation. In order to determine the actual number of pfu/ml in the original effluent samples, the number of pfu/ml obtained from each of the 3 pooled and concentrated samples was divided by the number of times the viruses in the known suspension were concentrated (21.1) following ultracentrifugation. The total pfu/ml following concentration for the pooled samples for July and August was 50.6, 37.0 for June 6-30 and September 1-5 and 36.0 for September 12 through October 24. By dividing

Table IV. Concentration in pfu/ml by ultracentrifugation of known poliomyelitis virus in sterilized sewage.

Dilution	Original sample		Sample after ultracentrifugation	
	*Ave. count	pfu/ml	*Ave. count	pfu/ml
Original	30.0	50.0	**TNC	**TNC
10^{-1}	4.0	6.7	85.0	141.7
10^{-2}	1.3	2.2	10.1	16.8
10^{-3}	1.0	1.7	2.5	4.2

*Average of four individual plaque counts.
**Too numerous to count.

these counts by the concentration factor (21.1) the actual number of pfu/ml in the original unconcentrated effluent samples was 2.4, 1.7 and 1.7 pfu/ml for July and August, June 6-30 and September 1-5, and September 12 through October 24 respectively, or a total average of 1.9 pfu/ml. Based on the corrected average pfu/ml recovered from these samples less than 1 virus particle per ml was contained in the largest volume of inoculum used in determining pfu/ml of the effluent prior to concentration. This would probably account for the failure to recover virus from the chlorinated effluent samples prior to ultracentrifugation and the subsequent recovery of virus following such treatment. The completed sewage treatment reduced, but did not eliminate all of the virus particles present in the sewage undergoing treatment.

In order to show further the effect of sewage treatment on the removal of virus particles contained in the sewage undergoing treatment, the total average number of virus pfu/ml recovered from the sewage for the entire sample collecting period is presented in Table V. The influent contained 34.9 and the primary settling samples contained 52.1 pfu/ml. From the final settling tank a greater number of virus particles (75.7) was obtained as compared to the activated sludge (36.7). This was followed by a drop in the chlorinated effluent to a corrected 1.9 pfu/ml. The general trend of an

Table V. Total average of pfu/ml of viruses in sewage during processing.

Station	Total number of positive pad samples	Total number of pfu	pfu/ml
A-influent	15	524.4	34.9
B-primary settling	25	1303.0	52.1
C-final settling	4	303.0	75.7
Sludge	6	220.0	36.7
*D-effluent	3	5.7	1.9

*Represents pooled dip and pad samples from the effluent after concentration.

increase in virus particles as the sewage underwent treatment up to and including the final settling tank (Table V) correlated well with the similar increase in virus particles seen in Table III.

Based on the data tabulated in Table III (total monthly average in pfu/ml at each collecting station) and Table V (total average in pfu/ml of viruses in sewage during processing) it appears that there was a release of virus particles as the sewage material underwent treatment. The number was greatly reduced as the sewage passed through the chlorine holding tank.

In an attempt to determine if this trend of virus particle increase up to and including the final settling tank was affected by the day to day variations in virus concentrations entering the plant, the following comparisons were made. There was no single instance where virus was recovered from all collecting stations on the same day; however, virus was recovered 18 times from samples collected on 9 different days from two or more stations on the same day. The total monthly average in pfu/ml recovered from two or more samples collected on the same day is presented in Table VI. There was an increase in pfu/ml in each instance when the number of virus particles recovered from samples collected from the influent were compared with those recovered from

Table VI. Total monthly average pfu/ml of virus from samples collected on the same date from different stations.

Month	Number of samples	STATIONS A influent	B primary sett.	C final sett.	D effluent
July	10	17.6	57.2	43.3	
August	2	0.0	10.0	195.0	
					*1.9
September	4	64.2	91.7	36.0	
October	2	20.9	124.2	0.0	

*Represents pooled dip and pad samples from effluent after concentration.

the primary settling tank. When the number of virus particles recovered from the influent samples was compared with the number recovered from the final settling tank there was an increase in pfu/ml in the latter for the months of July and August, but a decrease for the months of September and October. In general the number of pfu/ml recovered from both the primary and final settling tanks was greater than the number recovered from the influent. By computing the total average number of virus pfu/ml recovered from each station for the entire collecting period for those days in which two or more viruses were isolated from different stations on the same day (Table VII), the virus particle increase was even more apparent. Total averages of 27.2, 65.0 and 91.4 pfu/ml were recovered from the influent, primary settling tank and final settling tank respectively. Based on the corrected average, a decrease to 1.9 pfu/ml was obtained from samples collected from the chlorinated effluent. These data indicated that the virus particles were released and increased in number as a result of sewage treatment up to and including the final settling tank. Following chlorination this number decreased to a minimum.

In order to study further the effects of the treatment on the viruses as they passed through the disposal system the per cent of viruses recovered from the pad samples

Table VII. Total average number of viruses in pfu/ml compiled from data where viruses were isolated from two or more stations on the same day.

Station	Total number positive samples	Total pfu	Average pfu/ml
A-influent	8	217.5	27.2
B-primary settling	7	454.7	65.0
C-final settling	3	274.3	91.4
*D-effluent	3	5.7	1.9

*Represents pooled dip and pad samples from the effluent after concentration.

at each collecting station throughout the plant, including the pooled dip and pad samples collected from the chlorinated effluent, were compiled and presented in Table VIII. As can be seen the per cent virus recovery based on the pad sampling method from the influent, primary settling tank, final settling tank and chlorinated effluent was 25.0, 24.3, 8.3 and 2.6 per cent respectively. It was apparent that the per cent recovery of virus decreased stepwise through the plant as the sewage underwent treatment while the virus concentration expressed in pfu/ml increased as indicated in Tables III, V, VI and VII.

By comparing the total average virus pfu/ml entering the disposal plant with the total number leaving the plant in the chlorinated effluent the total per cent reduction of virus particles resulting from the sewage treatment process could be determined. A corrected total average of 1.9 pfu/ml of virus was obtained from the original chlorinated effluent samples before concentration as compared to 36.0 pfu/ml obtained from the influent samples (Table III). Based on these comparative figures the virus concentration was reduced 94.7, or approximately 95 per cent, as a result of the treatment process. Approximately 5 per cent of the virus particles entering the plant survived the treatment process.

Table VIII. The per cent of viruses isolated by the pad sampling method from each station throughout the sewage treatment plant.

Station	Number positive / Number tested	Per cent positive
A-influent	15/60	25.0
B-primary settling	25/103	24.3
C-final settling	4/48	8.3
D-effluent	*3/117	2.6

*Represents pooled dip and pad samples from the effluent after concentration.

DISCUSSION

This was a quantitative study of viruses contained in samples collected from each step in the sewage processing plant. Sewage samples represent the fecal material from a population and this justifies a comparison of the results presented here with the results obtained by collecting stool samples directly from the population. In Table IX a comparison is made of the results of Gelfand et al. (1957), who isolated the enteric viruses directly from the population, to the work of Mack et al. (1958) who isolated the same viruses from East Lansing sewage. Further comparison is made to the results found in the present study. All data are presented in the number of specimens tested and the per cent viruses recovered. The results presented in the comparison (Table IX) were obtained by isolating the viruses in monkey kidney cell cultures. The work of Mack et al. (1958) was the result of ultracentrifuge concentration of all samples prior to testing for virus. This explains the lower recoveries of virus obtained in the present study. Because large volumes of inocula were required in a quantitative study, concentration of the samples would not have permitted a sufficient volume of sample to make numerous plaque counts on each sample.

Table IX. Virus isolations from sewage specimens as compared to isolations obtained from stool samples.

Month of specimen collection	Gelfand et al., 1957		Mack et al., 1958		MSU 1959	
	No. tested	Per cent positive	No. tested	Per cent positive	No. tested	Per cent positive
June	312	16.7	66	9.1	117	3.4
July	312	27.2	82	14.6	137	13.1
August	306	22.8	100	29.0	105	9.5
September	306	22.6	209	19.6	126	9.5
October	319	20.1	144	22.9	107	6.5
November	311	9.9	75	13.3	24	0.0
Total	3667	13.4	1018	14.9	616	8.8

The marked seasonal incidence of the enteric viruses in feces and sewage has been repeatedly shown by Melnick 1947, Melnick et al., 1954a, Honig et al., 1956, Gelfand et al., 1957 and Mack et al., 1958. The months of June to November were chosen for the present study because of the higher incidence of viruses present in sewage during this period.

The present study (Table II) showed a high recovery of viruses from the pad samples (16.4%) and compares favorably with the work of Mack et al. (1958), where concentration methods were used. The dip samples, however, produced a low recovery of viruses. This again emphasizes the fact that the pad sampling method produced favorable results without additional procedures.

The plaque count results obtained were surprising. It was thought that as the sewage was processed through the disposal system, less virus would be recovered at each step in the processing. This was not the case. The total average number of virus particles recovered from the sewage for the entire collecting period showed an increase in the number of virus particles as the sewage underwent treatment. The increase (Table V) in virus particles per ml continued from the influent (34.9 pfu/ml) through the final settling tank where 76.7 pfu/ml were recovered. The results clearly

indicate that there were more virus particles in the plant than was indicated by the recovery of virus at the influent. The data used in compiling Table V included all plaque counts from all samples containing viruses. To attempt to clarify the results obtained in Table V the data from which the table was made were arranged differently. The data of Table VII were compiled in pfu/ml of viruses contained in samples where viruses were isolated from two or more stations on the same day. When the results given in the two tables (Table V and Table VII) were compared, the trend was found to be the same. More virus particles were again found circulating within the plant than could be accounted for by the pfu/ml present in the influent. An explanation of the results obtained could be made by the activated sludge treatment in the plant. The procedures involved in the treatment process consisted of three basic steps: primary settling, aeration and final settling. Approximately 60 per cent of the suspended solid materials were removed from the sewage in the primary settling tank. The sewage was then treated by the aeration process to convert the organic materials by zoogloeal action into microbial flora. The activated sludge employed in the aeration process was composed of a gelatinous mass containing a stabilized flora of bacteria and protozoa. The sewage undergoing treatment was combined with the activated sludge and subjected to

mechanical aeration. Aeration was accomplished by agitating the combined activated sludge and sewage materials for 5 1/2 hours by means of mechanically driven propellers located near the bottom of the aeration tank. Following agitation the zoogloeal masses settled to the bottom of the aeration tank. The activated sludge was continually recirculated. Ninety per cent of the activated sludge was combined with the effluent of the primary settling tank and circulated back into the aeration tank. The remaining 10 per cent was returned to the primary settling tank and pumped to the digestor along with the settled solids of the primary settling tank.

The mechanical and biological action resulting from the aeration process would disperse the organic materials releasing individual virus particles. The released virus particles would recirculate resulting in larger numbers of viruses recovered from the final settling tank (Table VII).

The gauze pads were suspended in the sewage flow for 48 to 72 hours. Virus particles recovered in tissue cell cultures represented particles expressed from the pad samples and probably represented an accumulation. The pad did not represent a filter for the entire volume of flow in any station but was a sample. The almost complete lack of virus in the dip samples gave evidence that the pad accumulated viruses, although for how long and under what

physical and chemical conditions viruses survive in pads is not known. One reason that the influent did not show greater isolations of viruses may be that the virus particles were contained in organic material of a size that did not permit passage through the collecting pad. Thus, after the organic materials were acted upon in the various processes of the plant, the pad acted as a filter and accumulated the virus particles. There was a relationship between the number of virus particles recovered from the pads at different stations throughout the plant. A comparison of the total average number of viruses recovered from the influent with the corrected total average recovered from the effluent would give the total per cent reduction of viruses resulting from the treatment process. Based on this crude comparison the virus concentration entering the plant was reduced approximately 95 per cent as a result of the treatment process. This meant that approximately 5 per cent of the virus concentration survived the treatment process and was discharged in the effluent. Therefore, chlorination reduced but did not eliminate the viable virus particles in the discharged effluent.

Investigations have been carried out in the past to determine what effect, if any, conventional chlorination procedures have on the virus of poliomyelitis. It is recognized that the efficiency of chlorination is dependent on the

interrelation of co-existing conditions. Some of the factors that play prominent roles are the amount of organic material and amount of ammonia present, temperature and pH of the system, chlorine dosage and type of test used to determine residuals.

Weidenkopf (1958) studied the inactivation of type I poliomyelitis virus with chlorine. Highly purified virus suspensions containing a minimum amount of organic material were used to hold chlorine losses to insignificant levels. The plaque method of assay was used to determine the per cent survival. Results indicated that the rate of inactivation of poliomyelitis virus was independent of the virus concentration. These experiments were carried out in phosphate buffered saline solutions. The presence of great quantities of organic matter in sewage plant effluents would render the establishment of free residual chlorine difficult.

Kelly and Sanderson (1959) reported that effluents from secondary treatment plants that had been chlorinated for disinfection contained enteric viruses about a third of the time. Poliomyelitis virus type I, Coxsackie and ECHO viruses were all isolated from both the influent and effluent. Residual chlorine values reported for these samples were near the recommended value of 0.5 ppm. The chlorination practiced, however, had some destructive action on viruses, as plaque

counts of chlorinated effluents were lower than those obtained from the influent samples. Raw sewage which had been chlorinated to reduce nuisance in plants, involving concentrations of 0.1 and 0.3 ppm residual chlorine, contained viruses as often as non-chlorinated raw sewage.

The effluent was chlorinated throughout the course of this study. The average final effluent exposure in the chlorine contact chamber was 15 minutes. Attempts were made to maintain an average chlorine residual of 0.5 ppm; however, concentrations varied from 0.2 ppm to 4.0 ppm. As a result of this chlorination procedure the virus concentration of the sewage was reduced approximately 95 per cent. These results lend additional support to the results obtained by these previous workers.

The potential reservoir of virus disease is great in raw sewage, contaminated water supplies, inadequately treated sewage and activated sludge materials. Chlorine residuals should be increased if destruction of viral pathogens is desired. A total average of 31.9 pfu/ml recovered from the sludge samples (Table V and Table VII) suggest that wet sludge and settled solids are unsuitable for use in fertilizing vegetables, lawns and shrubbery. Proper chlorine dosages, retention times, and residual levels must be devised to fit the specific need. Continuous re-evaluation of the

standards for disinfection of sewage may be necessary. Many kinds of viruses have been isolated from sewage and each strain has its individual characteristics of resistance, virulence and stability. An adequate standard for disinfection should take into account the expected range of variation of these properties.

SUMMARY

1. A total of 616 sewage samples were tested for the presence of enteric viruses in monkey kidney tissue culture.

2. Gauze pad and raw sludge samples provided the best source of material for isolation of enteric viruses.

3. Fifty-four samples were shown to contain virus. Plaque counts were made on each of the original positive samples to determine the number of virus particles per unit volume.

4. A definite increase in virus particles expressed in plaque forming units per ml (pfu/ml) occurred in the treatment process of sewage through the plant up to and including the final settling tank.

5. The virus concentration in sewage entering the treatment plant was reduced 94.7 per cent as a result of the treatment and exposure of the final effluent for an average of 15 minutes to 0.5 parts per million of residual free chlorine at a pH of 7.2 to 7.5. This reduction occurred under actual field conditions.

6. A new, improved, semi-micro plaque technique for counting enteric viruses was developed and used in counting the viruses contained in each original positive sample.

7. Sewage samples provide a source of material from which the viral flora of a community may be determined.

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