Data presented in this report indicate that the activated sludge process effectively removes at least two enteric viruses from sewage, and probably removes more agents.

REMOVAL OF ENTERIC VIRUSES FROM SEWAGE BY ACTIVATED SLUDGE TREATMENT

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QUALITATIVE recovery from urban sewage of a variety of human enteric viruses has been reported by several investigators.[1-3] However, the effectiveness of sewage treatment processes on removal of these viruses has had little study, although the effectiveness of conventional water treatment processes in removing or destroying these agents has received some attention.[3]

Gilcreas and Kelly[4] reported that the level of Coxsackie A virus was reduced about 60 per cent in sewage treated by the trickling filter processes. In a subsequent publication, Kelly, et al.,[5] reported that sedimentation in an Imhoff tank having a two-hour retention time had no destructive action on virus and that secondary treatment on a trickling filter, followed by sedimentation and chlorination, was not always effective in destroying virus in sewage. Mack, et al.,[6] noted a higher per cent recovery of virus from settled activated sludge than from the liquid phase of sewage, indicating perhaps that activated sludge may be effective in removing virus from sewage.

Carlson, et al.,[7] have reported data on the efficacy of activated sludge in removing poliovirus from mixed activated sludge liquor. Their experiments were of the batch type; virus in the form of a suspension of infected mouse cords and brains was added to mixed activated sludge liquor in a ratio of one part virus to 300 parts of sludge and the mixtures aerated zero, six, and nine hours. Using sludge concentrations of 1,100, 2,200, and 3,300 ppm, their data indicate that activated sludge in amounts as low as 1,100 ppm, with six hours aeration, did remove or inactivate the virus to a point where infectivity for mice was greatly reduced.

The investigations of Carlson, et al., were carried out long before accurate quantitative methods for virus assay were developed, and although their data indicate that activated sludge does remove or inactivate poliovirus, the per cent removal, the effect of continually feeding large quantities of virus to sludge, and many other questions remain unanswered. Accordingly, a study was initiated to determine the quantitative aspects of the reaction between activated sludge and certain enteric viruses as carried out by both batch type and continuous feed experiments.

Methods

Viruses—Two enteric viruses were used in these studies, a strain of Coxsackie A9 virus and a strain of Type I poliovirus. The Coxsackie virus, designated CME 456, was isolated in a monkey kidney cell culture from a rectal

swab specimen obtained from a "normal" child. The virus was received in this laboratory as monkey kidney cell passage 1. A large quantity of monkey kidney passage 2 virus was prepared in cell cultures and was used in all experiments. The poliovirus was obtained from Dr. Albert Sabin. It was designated Type I poliovirus (Mahoney) monkey kidney cell passage 37. A large pool of this virus was prepared (monkey kidney passage 38) and used in all experiments.

Activated Sludge Unit—The bench model activated sludge unit has recently been described in detail elsewhere.[8] The unit was operated in most experiments with a six- to seven-hour detention time. Sewage was fed at an average rate of 650 ml per hour, and virus was fed at an average rate of 30 ml per hour. Figures 1 and 2 present details of the sludge unit, sewage feed, and virus feed. In initial experiments both sewage feed and virus were mixed in the same container in a ratio of 95 per cent sewage to 5 per cent virus suspension. However, it was observed that the simple mixing of sewage and virus resulted in a loss of plaque-forming units (PFU) as high as 75 per cent or more of the original virus within five minutes of its addition to the raw sewage. This large loss precluded the feeding of virus-seeded raw sewage to the activated sludge unit, because it was technically impossible to add sufficient virus to raw sewage to have a measurable quantity of virus in the activated sludge unit effluent. A virus feed unit was therefore constructed with a 1,000-ml separatory funnel which fed virus into a constant level feed device. The feed device was fitted on one end with a 25-gauge ⅜-in. hypodermic needle. This apparatus allowed virus to drip into the activated sludge unit at an average rate of 30 ml per hour. The separatory funnel was surrounded by cooling coils in order to maintain virus viability and prevent bacterial growth in the virus suspending medium, Hanks's balanced salt solution (BSS). Antibiotics were not added to the virus suspending medium because of the effect they would have on the activated sludge.

Sewage Feed—Settled urban sewage was used as the basic sewage feed in all experiments. The COD of this sewage was quite low, and the sewage was routinely "fortified" with gelatine and homogenized dog food to yield a sewage with an average COD of approximately 300 mg per liter in the continuous feed experiments.

Virus Feed—Stock virus suspensions were diluted in Hanks's or Earle's BSS so that the feed averaged about 8 x 10^6 PFU per ml for the Coxsackie A9 virus and about 2 x 10^6 PFU per ml for the poliovirus strain. Virus feed titers remained relatively stable for the 48-hour periods during which the experiments were conducted, providing the feeds were held at approximately 5° C.

Virus Titrations—All virus titrations were conducted by using the plaque technic. African green monkey (Cercopithecus aethiops sabaeus) or rhesus (Macacus rhesus) kidney cell cultures, grown in 6-oz bottles, were prepared by essentially the technic described by Hsiung and Melnick.[9] Fourfold dilutions of the suspension being titrated were prepared in BSS containing penicillin, dihydrostreptomycin, nystatin, and tetracycline, and each of four plaque-bottles inoculated with 0.5 ml of each dilution. Plaque counts were made on the first day plaques became clearly visible, and for one or two days thereafter to insure time for the appearance of slow-developing plaques.

Coliform and Fecal Streptococci Counts—Coliform counts were determined by the membrane filter technics as described in the tenth edition of Standard Methods, except that m-Endo broth MF* was used in medium preparation. Fecal streptococci counts were

* Difco Laboratories, Inc., Detroit, Mich.

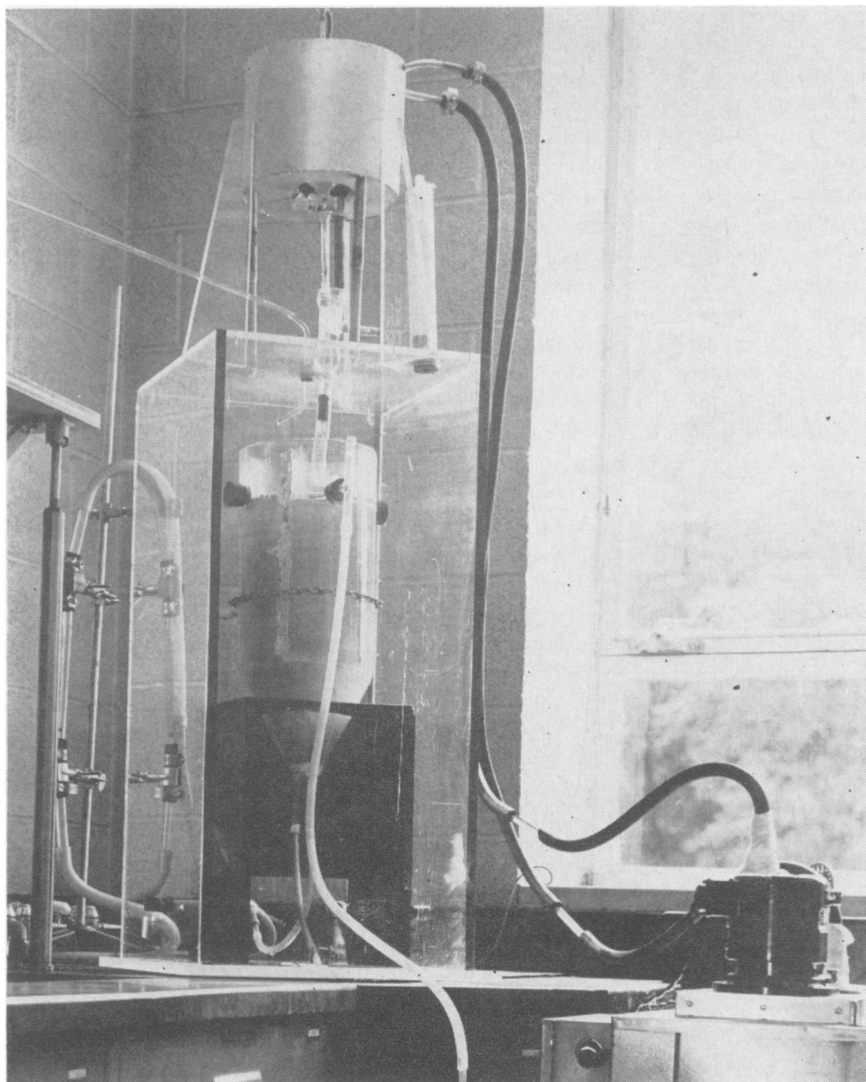

Figure 1—Bench Model Activated Sludge Unit

made using the membrane filter procedure described by Kenner, et al.[10]

Activated Sludge—The sludge used in this study was obtained from a stock unit maintained by F. J. Ludzack of this center. For the purpose of these experiments, three parameters were measured on the sludge obtained for a particular experiment, the sludge density index, which ranged from 1.07 to 3.44, with a mean value of 2.06; the per cent volatile solids, which ranged from 67 to 88, with a mean value of 78; and the per cent nitrogen in the volatile solids, which ranged from 5.3 to 11.9, with a mean value of 9.2. The amount of sludge used to seed the sludge unit was varied according to the particular information desired, and is noted in the experimental details.

A. Preliminary Experiments

Pilot experiments were conducted to determine (I) the effect of primary sewage treatment (settling) on the virus, (II) the effect of aeration on the virus, and (III) the effect of activated sludge (batch type process) on the virus.

I. Primary Treatment—Twenty gallons of raw sewage (not fortified) were placed in a 30-gallon polyethylene drum and seeded with Type 1 poliovirus. The sewage-virus mixture was stirred for about 15 minutes and aliquots were then poured into four-liter serum bottles and allowed to settle for the duration of the study. Samples were carefully removed from the top quarter of the liquid at specified time intervals and checked for virus titer, coliform count, five-day BOD, and suspended solids. The results of

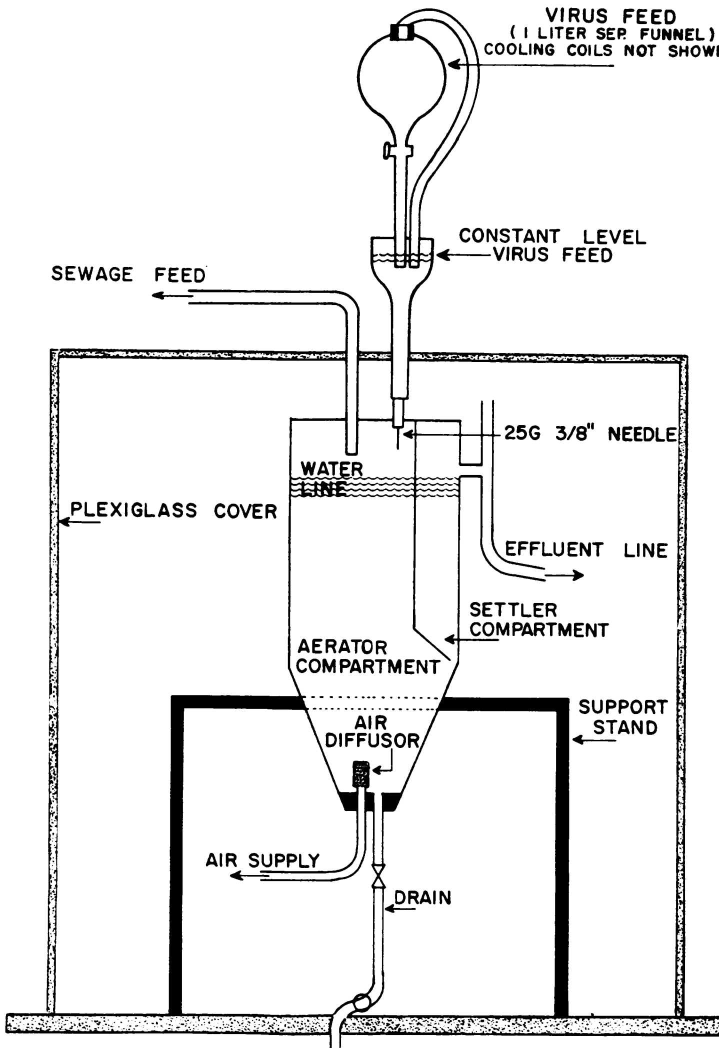

Figure 2—Bench Model of Activated Sludge Unit and Virus Feed System (Schematic Diagram)

Table 1—Effect of Primary Sewage Treatment (Settling) on Poliovirus Titers, Coliform Counts, BOD, and Suspended Solid Determinations

Settling Time*	Average Plaque Counts/0.5 ml Exp. 1	Average Plaque Counts/0.5 ml Exp. 2	Coliform Counts/ml Exp. 1	Coliform Counts/ml Exp. 2	Five-Day BOD (mg/l) Exp. 1	Five-Day BOD (mg/l) Exp. 2	Suspended Solids (ppm) Exp. 1	Suspended Solids (ppm) Exp. 2
0	155	185	1.6×10^5	3.7×10^4	64	22	72	19
45 min	150	190	6.3×10^4	3.3×10^4	56	13	49	12
1.5 hr	150	206	6.1×10^4	2.7×10^4	46	9	44	7
3 hr	165	180	1.1×10^5	3.4×10^4	42	10	40	10
6 hr	115	90	1.6×10^5	7.6×10^4	37	8	28	9
12 hr	110	100	2.6×10^5	6.7×10^4	28	9	20	8
24 hr	110	70	1.3×10^5	4.5×10^4	27	6	18	5

* Samples stored at room temperature.

these experiments are summarized in Table 1, which presents the mean values obtained from duplicate samples in each of two separate experiments. From this table it appears that three-hour settling has little if any effect on the virus, although it does slowly reduce the BOD and suspended solids. The coliform results show an initial drop in count, followed by a rise due to bacterial multiplication and finally another reduction in count. The reason for the loss of virus between the three- and six-hour samples is not clear. The loss in experiment 1 was about 26 per cent, in experiment 2 about 55 per cent. It seems unlikely that the loss was due to settling of the virus, since the reduction was not gradual and consistent but occurred rather suddenly.

II. Aeration of Virus Suspensions—In order to determine if aeration as used in the bench model treatment unit would inactivate virus, the following experiment was conducted. Stock Type 1 poliovirus was added to phosphate buffered water (pH 7.2, 0.02 molarity) contained in a five-liter glass bottle. A sintered glass gas dispersion tube of medium porosity was introduced into the bottle, and air was bubbled through the water-virus suspension. Samples were removed periodically and checked for virus titer. Concomitantly, virus was added to buffered water but not aerated to determine the effects of simple storage on virus survival. The results of these tests indicated that aeration and storage for the length of time of these experiments (six hours) did not reduce virus titer significantly.

III. Removal of Virus by the Activated Sludge Process—Pilot experiments were designed to measure the per cent reduction in virus titer when virus was exposed to activated sludge and to determine the mechanisms involved in this reduction.

a. Effect of Aeration Time—Coxsackie A9 virus was added to a bottle containing three liters of water and one liter of activated sludge mixed liquor. The virus-sludge mixture was aerated at room temperature and samples removed periodically were allowed to settle 30 minutes and the supernatant titered for virus. The results of this investigation, presented in Table 2, indicate that about 99 per cent reduction of the added virus occurred in 45 minutes. As aeration time was increased, virus reduction also increased to 99.99 per cent in six hours. A control experiment, in which virus and water were aerated, showed practically no loss of virus in the six-hour aeration period.

These data indicated that the activated sludge process was apparently highly efficient in removing Coxsackie virus from water and additional studies were therefore conducted in an attempt to determine the mechanism involved in this removal. Two different experiments were designed to investigate this mechanism, in one the amount of virus was varied and the sludge concentration held constant; in the other the virus concentration was held constant, while sludge concentrations were varied.

b. Establishment of the Mode of Action of Activated Sludge on Virus—If the removal of virus in the activated sludge process is an adsorption phenomena, the removal pattern should conform to the Freundlich adsorption isotherm: $a/m = uC^n$ where a is the amount of adsorbate adsorbed per unit of adsorbent, m; C is the amount of adsorbate remaining; and u and n are constants. Applying this equation to the sludge-virus system, a becomes plaque-forming units of virus per inoculum (0.5 ml), m the amount of activated sludge as suspended solids in ppm, and C the PFU of virus per 0.5 ml remaining in the supernate after settling. The values of u and n would depend upon the nature of the sludge, virus type, and other variables in the system.

A series of experiments were conducted in which ten-fold serial dilutions of virus were added to two sets of bottles, one containing 2,320 ppm of activated sludge while the other contained 4,960 ppm of activated sludge. All bottles were held at 4° C to minimize microbiological activity were not aerated, but held for 45 minutes. At the end of this time, solids were settled by centrifugation at 1,000 rpm for ten minutes and the supernates titered for virus. The results of these experiments are presented in Figure 3 which shows the ratio of PFU of virus removed to the ppm of suspended solids (a/m) plotted against the PFU of virus remaining in the supernate. The curve for the 2,320 ppm of suspended solids was fitted by the least square method, the other curve was fitted by eye because of lack of sufficient points. The linear log-log relationship between (a/m) and C for both sludge concentrations clearly demonstrates the conformity of these data to an absorption isotherm and allows computation of the values for u and n which are 0.00091 and 0.996 for the 2,320 ppm and 0.00075 and 0.996 for the 4,960 ppm of suspended solids.

The values for n in both cases are close to unity, indicating that the per cent of virus adsorbed was constant over all initial virus levels tested, while the very small values of u, and the slight difference between them at the two different sludge concentrations indicates that the sludge floc at 4° C was a relatively poor absorbent and that a significant increase in virus removal could be obtained only by a very large increase in sludge concentration. To confirm this, a/c ratios were examined for homogeneity for each sludge concentration by a "between dilutions vs within dilutions" analysis of variance and were found to be constant within the limits of sampling and counting errors, the geometric mean per cent adsorption being 67.1 and 79.1 for the 2,320 and 4,960 ppm of solids, respectively.

The equation used above, $a/m = uC^n$ may also be written $a/C^n = um$. The

Table 2—Effect of Activated Sludge on Coxsackie A9 Virus

Sample Time	Average Plaque Counts/0.5 ml	Per cent Virus Reduction
Initial	54,000	—
45 min	400	99.26
1.5 hr	40	99.93
3 hr	10	99.98
6 hr	1.5	99.99

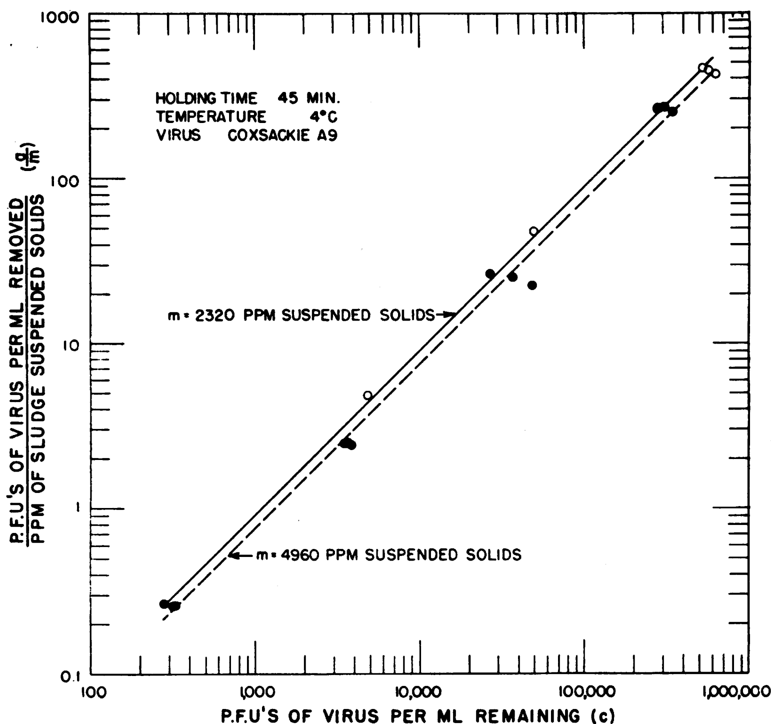

Figure 3—Relationship Between the Ratios of Plaque-Forming Units (PFU) of Virus Removed to PPM of Sludge Suspended Solids and PFU's of Virus Remaining in Supernate

experiments just described showed that the value of n is close to unity, and therefore the ratio of a/C should be directly proportional to m. To test this hypothesis, a fixed amount of virus was added to each of a number of bottles to yield an initial titer of 150,000 PFU. Activated sludge was then added to each bottle in ratios of x, x/2, x/4, x/8 ppm of suspended solids. The virus-sludge mixtures were held 45 minutes at 4° C with intermittent shaking, samples were removed, centrifuged at 1,000 rpm for ten minutes and the supernate titered for virus concentration.

The results of these tests are shown in Figure 4, by plotting on a log-log scale the ratio of the per cent of virus removed to the per cent remaining against the respective ppm of suspended solids. For comparison, corresponding values were also computed from the data of Figure 3 and are shown. Figure 4 shows some scattering of points, but the majority of points lie within the limits of sampling and counting errors. Of interest is the observation that the two points computed from Figure 3 are so located that they show a similar slope, but of somewhat lower removal efficiency. This difference could be accounted for by experimental error, but it may indicate a lower adsorption capacity of the sludge used in the earlier experiment. At any rate, the linear relationship between a/C and m further substantiates that the removal of virus by activated sludge is apparently an adsorption phenomenon.

c. Disassociation of Virus from the

Sludge-Virus Complex—In view of these data, which indicate virus removal by activated sludge is an adsorption phenomenon, a number of experiments were conducted in an attempt to disassociate adsorbed virus from sludge. These experiments were conducted by shaking sludge, to which virus had been adsorbed, in buffer systems with pH values of 4.9, 6.5, 7.2, and 8.5. In addition to these systems, virus-sludge mixtures were shaken with diethylether and 1:5,000 Versene. All extractions were conducted by vigorously shaking the sludge-virus complex in separatory funnels, allowing the sludge to settle for 30 minutes, decanting and titering the supernates for virus. Each sludge-virus complex was extracted three times. These experiments showed that only a small per cent of the virus could be extracted from the sludge-virus complex by any of these procedures, and indicate that the sludge-virus complex is extremely stable or that virus is in some manner inactivated by adsorption to activated sludge.

B. Continuous Flow Type Experiments

The batch type experiments just described indicate that the activated sludge process is effective in removing Coxsackie A9 virus from sewage. However, it appeared necessary to determine the efficacy of the process as it is usually conducted, in a system in which sewage

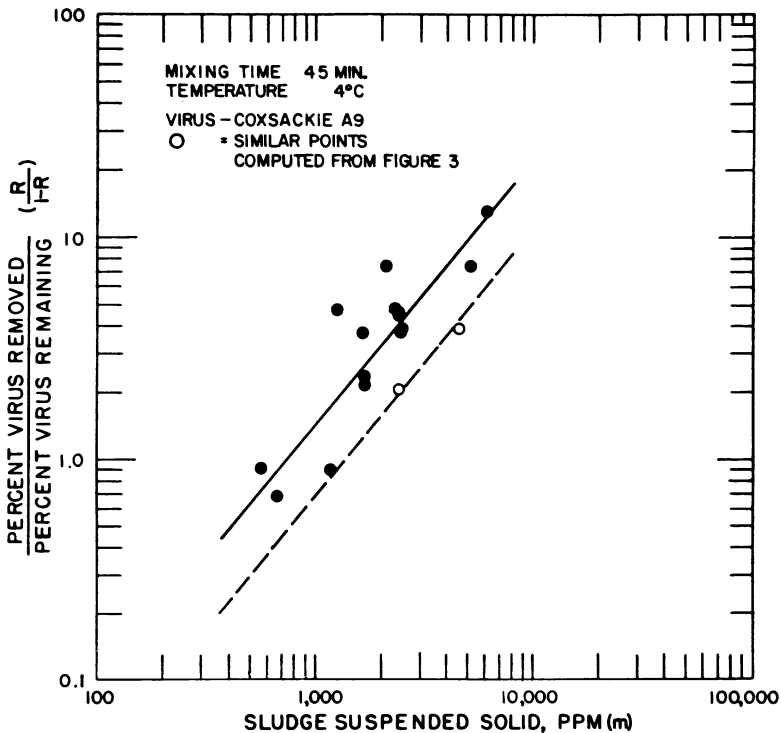

Figure 4—Relationship Between the Ratio of the Per cent of Virus Removed to the Per cent of Virus Remaining and the Concentration of Activated Sludge as Suspended Solids in PPM

Table 3—Removal of Coxsackie A9 Virus by a Bench Model Activated Sludge Unit

Experiment	Volatile Solids (mg/liter)	Initial Virus (PFU/ml)	Effluent Virus (PFU/ml)	Per cent Virus Reduction
1	600	166,000	2,200	98.8
2	650	509,000	20,000	96.1
3	1,000	167,000	1,400	99.2
4	1,100	320,000	2,800	99.1
5	1,500	321,000	8,400	97.4
6	1,500	314,000	2,000	99.4

Average retention time of virus in sludge unit, 7.0 hours (6.0-8.4).

and virus could be continually fed for 48 hours to an activated sludge unit as previously described.

In these studies both Coxsackie A9 and Type 1 poliovirus were used. Table 3 presents the results obtained in a series of experiments with Coxsackie A9 virus. It is apparent from these data that varying the amount of activated sludge (600-1,500 ppm volatile solids) used to seed the sludge unit did not significantly change the per cent of virus removal.

Table 4 presents the results of experiments using Type 1 poliovirus. The data in this table suggest that the per cent reduction of Coxsackie A9 virus by activated sludge treatment is greater than was achieved using Type 1 poliovirus. However, in unpublished experiments we have observed that Coxsackie A9 virus is not as stable as Type 1 poliovirus, and it is possible that the greater reduction of Coxsackie A9 virus reflects the die-off of virus that occurs during the six to seven hours the virus is in contact with the sewage-sludge mixture. It is of considerable interest to note the relatively insignificant increase in poliovirus reduction as the amount of activated sludge was increased from 400 ppm to 4,000 ppm of volatile solids. The jump in amount of virus removed, from 80 to 88 per cent, when the volatile solids were increased from 200 to 400 ppm was probably due to the sludge formation and settling, which was very poor at 200 ppm in contrast to the excellent sludge formation and settling at all other levels.

It should also be noted that in all experiments, with both viruses, the per cent removal of virus was constant during the entire 48-hour test period.

In order to determine if the data of Table 4 conform to the adsorption isotherm, the ratio of the per cent of virus removed to the ppm of volatile solids is plotted against the respective per cent of virus remaining in the effluent in Figure 5. The data show a good fit of the observed points to the theoretical curve computed from the Freundlich adsorption isotherm at sludge levels between 400 and 4,000 ppm of volatile solids. Below 400 ppm of volatile solids, the curve bends rather sharply toward the point of 200 ppm of solids. The values for u and n for the straight-line section of the curve are 6.4×10^{-5} and 3.3, respectively. The results further substantiate the earlier observations that removal of virus by sludge is apparently an adsorption phenomenon.

The sharp bend in the curve between 200 and 400 ppm of solids clearly indicates that virus removal at the lower sludge level is much less than would be dictated by the isotherm. It appears

that the activated sludge process requires a certain minimal concentration of activated sludge if it is to be efficient.

Since earlier investigations had demonstrated that a high per cent of virus was "lost" by simple mixing with sewage, an experiment was conducted in which no activated sludge was used to seed the sludge unit, only "fortified" sewage and Type 1 poliovirus being fed to the unit. The results from this experiment showed that a 60 per cent reduction in virus titer was achieved by allowing sewage and virus to mix for a six to seven hour period in the bench model activated sludge unit. This loss of virus was comparable to earlier observations in which losses up to 75 per cent or greater occurred within five minutes of simple mixing of virus and sewage. It thus appears that the suspended solids and other colloidal material in sewage, and perhaps toxic substances, contributed to a substantial portion of the reduction in virus when virus is treated by the activated sludge process.

A number of investigations were also conducted with the activated sludge unit to determine its effectiveness in removing coliform and fecal streptococci organisms. A series of five experiments demonstrated a mean coliform reduction of 97.0 per cent, with a range of from 95.0 to 99.7 per cent and a mean fecal streptococci reduction of 95.6 per cent, with a range of from 93.8 to 97.0 per cent.

COD measurements were made for each experiment reported in this paper. All effluents had approximately the same COD values, with a range of from 90 to 145 mg per liter and a mean value of 109 mg per liter. Sewage feed COD measurements ranged 172-590 mg per liter, with a mean value of 307 mg per liter. Suspended solids measurements of the effluent always yielded values of less than five mg per liter.

Discussion and Summary

The data presented in this paper indicate that the activated sludge process is an effective method for removing at least two different enteric viruses from sewage. Since the mechanism of removal of virus by activated sludge appears to be an adsorption phenomenon, it is reasonable to infer that other members of the enteric virus group would also be removed from sewage by such treatment. The ultimate fate of the adsorbed or inactivated virus was not determined, although a number of various technics were tried in an attempt to release virus presumed to be adsorbed to

Table 4—Removal of Type 1 Poliovirus by a Bench Model Activated Sludge Unit

Experiment	Volatile Solids (mg/liter)	Initial Virus (PFU/ml)	Effluent Virus (PFU/ml)	Per cent Virus Reduction	Sludge Formation and Settling
1	200	67,000	14,000	79	Very poor
2	400	68,000	8,000	88	Good
3	600	64,000	6,200	90	Good
4	600	53,000	5,000	91	Good
5	1,200	100,000	8,000	92	Good
6	1,200	40,000	3,600	91	Good
7	4,000	56,000	3,600	94	Good

Average retention time in sludge unit, 6.7 hours (6.0-7.5).

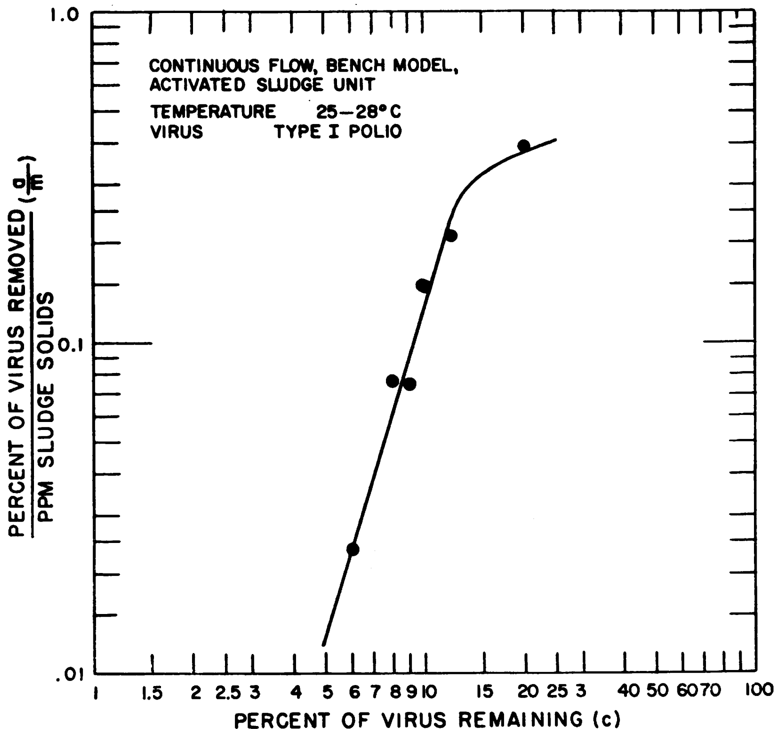

Figure 5—Relationship Between the Ratio of Per cent Removal of Virus to Concentration of Activated Sludge and the Per cent of Virus Remaining in the Effluent

the sludge. Only a very small per cent of virus could be recovered, indicating that the sludge-virus complex is extremely stable or that most of the virus is in some manner inactivated.

The bench model continuous-flow activated sludge unit used in these experiments was operated with a six to seven hour retention time and was seeded with activated sludge in amounts ranging from 200 to 4,000 ppm, expressed as volatile solids. Excepting with the 200 ppm seed, the unit removed approximately 90 per cent of Type 1 poliovirus and about 98 per cent of Coxsackie A9 virus. Coliform and fecal streptococci removals averaged about 97 and 96 per cent, respectively. It was also demonstrated that about a 60 per cent reduction of Type 1 poliovirus could be achieved by feeding sewage and virus through a bench model unit containing no activated sludge. This indicates that the suspended solids and colloidal material, and perhaps toxic substances, in raw sewage also play a role in the reduction of virus as virus is treated by the activated sludge process. Activated sludge undoubtedly removes the major portion of this sewage-virus complex, in addition to most of the remaining free virus in sewage.

Studies designed to determine the effect of primary treatment on virus demonstrated that no significant loss of virus occurred with a three-hour settling time, but that some loss occurred between six and 24 hours of settling. This

laboratory observation confirms the observation of Kelly, et al.,[1] who were able to isolate viruses from the effluent of primary treatment plants with some regularity.

The only study of which we are aware in which effluent from activated sludge treatment plants has been examined for virus was reported by Mack, et al.[6] Their report showed that enteric viruses could be isolated from unchlorinated effluents with considerable regularity. Unfortunately, the amounts of virus in neither the raw sewage nor effluent were determined, but it was observed that a higher per cent of settled sludge samples yielded virus than did raw sewage. Their report, coupled with our observations, would indicate that although activated sludge does remove 90 per cent or greater of enteric viruses added to sewage, it must be coupled with additional treatment (disinfection) if a virus-free effluent is sought.

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"As a child, studying the Bible in Sunday School, I read of the 'miracles,' and often wondered if ever it would be my lot actually to see a miracle. I can truly say near the end of a long life in the field of preventive medicine that I have not only seen, but participated in, one of the greatest miracles that the world has ever seen. 'Those who were dead are now alive.' "

(Wilson G. Smillie, M.D. Experiences in Preventive Medicine. 1957.)