APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1980, p. 253-260 Vol. 39, No. 1
0099-2240/80/01-0253/08$02.00/0

Technique for Determining Total Bacterial Virus Counts in Complex Aqueous Systems

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A direct method is described for measuring bacteriophage concentrations in complex aqueous systems. Conditions for sample clarification, phage recognition, and recovery were optimized. In contrast to the plaque assay, this procedure permits quantitation of total numbers of phages independent of bacterial host. Also, the modifications increase the sensitivity of the sedimentation assay, permitting detection of particles at a minimum concentration of 10^4 per ml. Maximal total phage concentrations in the aqueous phase of sewage and activated sludge mixed liquor were 1.3×10^6 and 4.3×10^7 per ml, respectively.

Although bacteriophages have been isolated from many habitats, including sewage (3), natural waters (20), soils (7), and the bovine rumen (1), their quantitative ecology has been poorly defined. A major limitation has been lack of a technique to determine their total numbers. The plaque assay, which is the only widely used assay for quantitating bacteriophages, cannot be expected to give information concerning the total number of bacteriophages present in a sample containing multiple virus types. Environmental factors such as pH, ionic strength, temperature, and the presence of organic cofactors and nutrients (2, 4) must be optimized for each phage-host system before the plaque assay can be considered quantitative. Even if all sensitive hosts in a system were available and used as indicator organisms, the sum of individual virus populations assayed would probably not represent the total population of viruses in the system, because each host would not necessarily be specific for a given virus type. Moreover, mutations in both hosts and phages could change host range specificities (2). Thus the plaque assay technique cannot be used to enumerate total numbers of phages in a complex system containing a mixture of virus types.

Methods for estimating total numbers of both plant and animal viruses have been developed, and these have been reviewed by Sharp (15). All employed an electron microscope for direct observation and identification of virus particles. These methods can be divided into two groups, based upon the procedure used to determine the volume from which the counted viruses were removed. This determination is made either directly from the area recorded on an electron micrograph, or indirectly by means of indicator particles. Methods employing indicator particles, the spray-droplet (21) or agar filtration technique (10), require at least 10^9 to 10^{10} particles per ml, respectively, whereas sedimentation methods are capable of detecting viruses at a concentration of 10^8 per ml (15). Sedimentation techniques require that all viruses be evenly distributed in a known volume of liquid and sedimented onto a receiving surface. Sharp (18) developed a special centrifuge rotor which permits sedimentation of viruses onto an agar block or onto a collodion film on a glass cover slip. When particles are sedimented onto agar they are subsequently removed by stripping with a collodion film. Collodion films with attached virions (pseudoreplicas) are then mounted on specimen grids and examined with an electron microscope. Randomly selected areas are photographed at the lowest magnification permitting recognition of viruses. The total number of viruses in the sample is then calculated from the number counted on the electron micrograph. This type of assay has been primarily used for quantitating animal viruses of known size and morphology in semipurified tissue extracts. To date, direct assays of the type described above have been used only to quantitate bacteriophage of a known morphology in purified preparations (8, 10).

This paper describes an adaptation of the sedimentation technique of Sharp (15) so that total numbers of bacteriophages in samples from complex aqueous systems, e.g., sewage, activated sludge mixed liquor, and bovine rumen fluid, can be quantitated.

MATERIALS AND METHODS

Test samples. All samples of sewage and activated sludge mixed liquor (the mixture of sewage and activated sludge in an aeration unit) were obtained from unit one of the Limestone Creek treatment plant at

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Gaffney, S.C. Sewage entering the plant was solely from domestic sources. Samples were kept on ice during transportation to the laboratory, and unless otherwise stated, were filtered (0.45-μm pore size) and stored in sterile containers at 4°C (1 to 2 days) until assayed.

Preparation of agar blocks. Agar blocks were prepared by pouring 15 ml of 4.5% (wt/vol) agar (Difco Laboratories) solution into a plastic petri dish (100 mm diameter) so that, after drying for 25 h at 37°C, the agar was 2 mm thick. The agar was cut into 1-cm^2 blocks, and the air-dried surface was used for receiving virus particles.

Primary clarification procedures. Samples (25 ml) of mixed liquor were centrifuged, at 3°C, for various times at various gravitational forces. The upper two-thirds of supernatants were carefully aspirated, and total bacterial cell counts were measured by using a phase contrast microscope and a Petroff-Hausser counting chamber. A sample of supernatant was diluted 10-fold with 1% (vol/vol) glycerol and then sedimented at 3°C, for 2 h, at 31,500 × *g*, onto 2-mm agar blocks (4.5% wt/vol). Three strippings of each block were made and stained with uranyl acetate, and the number of phages was counted in two randomly selected grid holes for each stripping.

Clarification of 25-ml samples of liquor was also carried out by membrane filtration (0.45-μm pore size; Millipore Corp.). Filtrates were assayed for total phage concentrations as described above for supernatants.

Sedimentation of viruses. After primary clarification, samples were centrifuged in an SU particle sedimenting rotor with a RC-2B centrifuge (Ivan Sorval, Inc.) at 3°C under the various conditions described in the results section.

Preparation of pseudoreplicas. After sedimentation of virus particles, the supernatant was carefully aspirated, the agar block was removed from the rotor chamber and placed on a glass slide which was stood on edge, and the block was air dried for 30 to 40 min. The surface of the dried agar block was covered with a drop of collodion (1%, wt/vol), the excess was removed by touching a corner of the agar block to filter paper, and the block stood on edge to dry. When dry, the collodion membrane was floated off onto the surface of a solution of 0.5% (wt/vol) uranyl acetate. Total staining time was 45 to 60 s. A 200-mesh electron microscope grid, 3.05 mm diameter (Ernest F. Fullam Inc., Schenectady, N.Y.), was placed on top of the floating membrane, convex side down. The membrane covered grid was removed from the surface of the staining solution by touching a metal washer, held with forceps, to the membrane so that the grid was located in the hole of the washer. The washer, with attached membrane and grid, was lifted from the liquid and brought down over a metal peg so that the grid rested on top of the peg with the collodion membrane uppermost. One drop of water was placed on the pseudoreplica, and the excess was drawn off with absorbent paper. The pseudoreplica was carbon coated for protection against the electron beam. When more than one agar stripping was required, the block was removed from staining solution immediately after the membrane was floated off and washed by placing one drop of deionized water on the receiving surface. The block was then returned to a glass slide, and the above stripping procedure was repeated.

Counting procedure. Pseudoreplicas were examined by using an RCA EMU 2D electron microscope. Phages were counted in randomly selected grid holes at a magnification of 10,000×. Higher magnifications were used to identify smaller particles if necessary. The total phage concentration was calculated by using the following formula: total number of phages per milliliter = $(N/F) \times (1.26 \times 10^4) \times D$, where N is the mean number of phages counted per grid hole on the first stripping of the agar block, F is the fraction of total phages recovered on the first stripping, 1.26×10^4 is the reciprocal number of volumes from which phages were sedimented onto the area of one grid hole, and D is the dilution factor of the sample.

Reagents. Collodion (1.0% wt/vol nitrocellulose in amylacetate) was purchased from Ernest F. Fullam, Inc. Uranyl acetate stain (20% wt/vol) was prepared as a 100-ml stock solution in deionized water and stored in an aluminum foil-wrapped bottle. The day of each assay, this was diluted with deionized water to give a working concentration of 0.5%, final pH 4.6 to 4.8.

RESULTS

Recognition of virus particles. Bradley and Kay (5) defined six morphological types of bacteriophages, three of which have icosahedral heads (giving a hexagonal outline in electron micrographs) with tails, two of which are icosahedrons with no tails, and one of which is a flexible filament. The majority of phages appear to be those that possess tails (5). Many plant and animal viruses are known with morphologies that give a hexagonal outline; however, none has been found which resemble the tailed bacteriophages. Therefore, the majority of bacterial viruses can be distinguished from plant and animal viruses on the basis of morphology. This was the criterion employed here. Examples of phage morphologies observed in samples of sewage and activated sludge are presented in Fig. 1 and 2, respectively.

Several techniques were tested for increasing contrast of phages in the electron microscope without masking the identifying details of their morphology. A filtered sample of activated sludge reactor liquor was sedimented onto 1.5% (wt/vol) agar blocks in an SU particle-counting rotor at 31,500 × *g* for 30 min. Pseudoreplicas, recovered by flotation on water (13), were subjected to three treatments for increasing contrast of virus particles: (i) light shadowcasting with chromium at an angle of 15°; (ii) negative staining for 30 s with 2% (wt/vol) phosphotungstic acid (pH 5.5) as described by Brenner et al. (6); and (iii) positive staining with uranyl acetate or lead hydroxide with and without Formalin lanthanum fixation as described by Marinozzi (12). Shadow casting enhanced the contrast of bac-

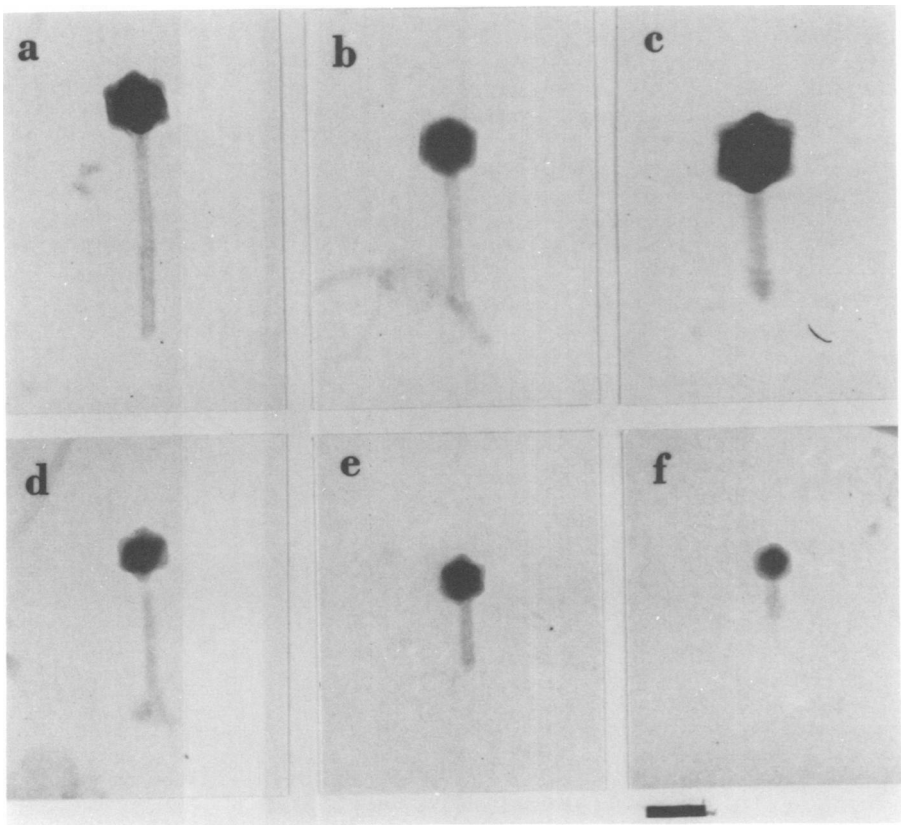

FIG. 1. *Some typical phages observed in samples of sewage. Bar represents 0.1 μm.*

teria and debris, but only the heads of large phages were evident. Small phages and the tails of large phages were either buried by the metal or obscured by the shadows of large particles. These observations confirm previous reports that shadow casting is useful for enhancing contrast of large viruses, e.g., vaccinia or T2 phage (11, 15, 17) but hides the fine structure of smaller viruses (19). Negative staining highlighted debris to such an extent that many phage particles were obscured. Lead hydroxide stain, with and without fixation, failed to reveal phage particles. However, treatment with uranyl acetate, with and without fixation, substantially increased the contrast of phages above the background, permitting easy recognition. No significant difference was noted between fixed and nonfixed preparations. Therefore, fixation was not employed in future studies. As reported by Huxley and Zubay (9), the advantage of this stain is that the nucleic acid cores of virions are preferentially stained without appreciable uptake by protein.

Optimum staining time with uranyl acetate was 45 to 60 s. Longer periods resulted in uniform uptake of stain by both phages and debris. When an agar block was subjected to multiple strippings, with flotation on 0.5% (wt/vol) uranyl acetate, stain accumulated on the preparation making it more difficult to recognize phage particles on consecutive strippings. Decreasing the concentration of uranyl acetate to 0.25% (wt/vol) gave less staining of phages without appreciably reducing build-up of background staining. It was found that build-up of background stain could be prevented during multiple strippings of an agar block by washing off excess stain after preparation of each pseudoreplica. This was accomplished by placing a drop of water on both the grid-mounted pseudoreplica and on the agar block after each stripping and immediately drawing off the water with absorbent paper.

Counting procedure. In the sedimentation technique developed by Sharp, pox viruses were counted on electron micrographs of pseudoreplicas taken at a low magnification. This procedure was practical for samples with virus concentrations greater than 10^6/ml (15). Because of the variability of size and concentration of phages in our samples, a more versatile method of counting was desired. We found it practical to count

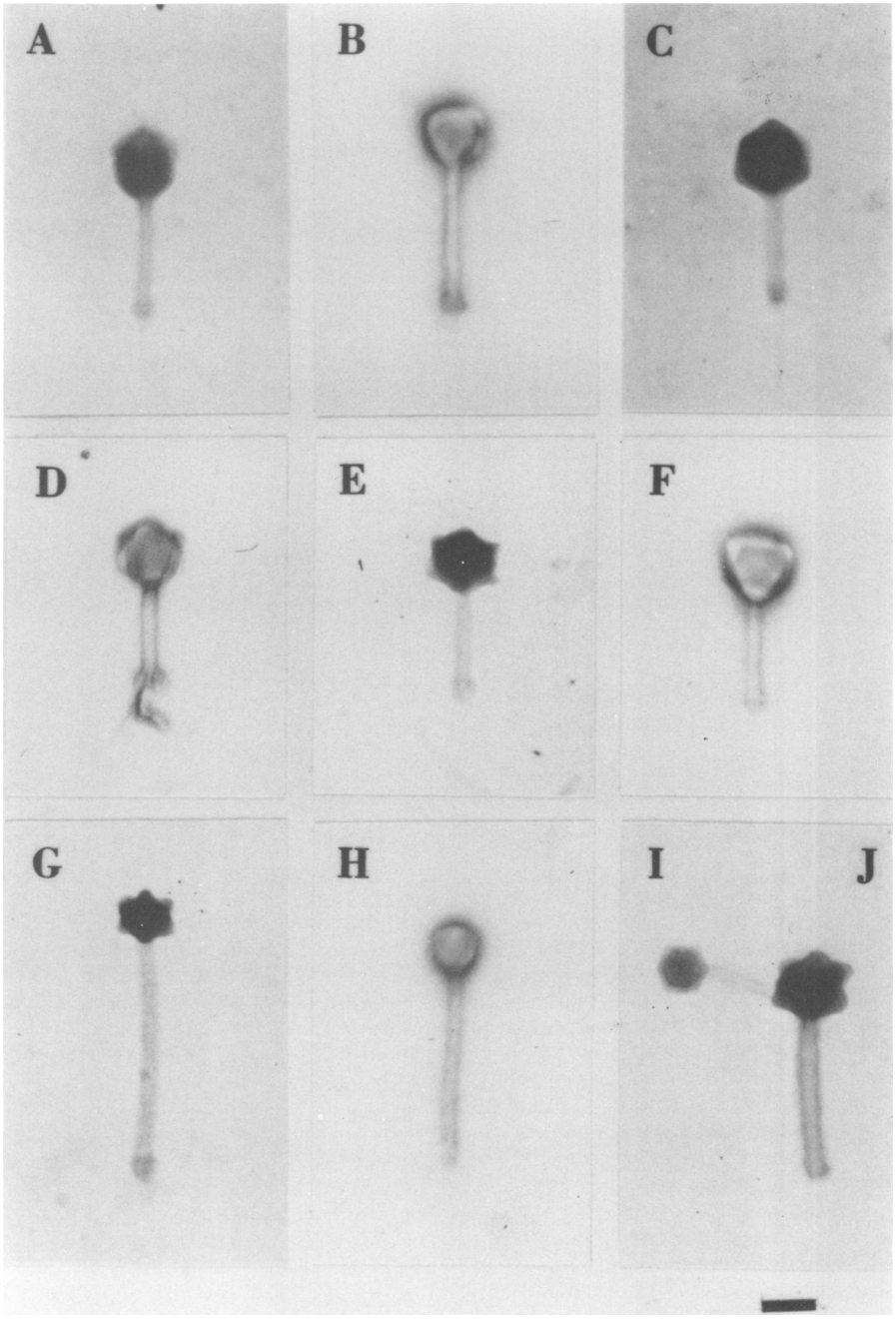

FIG. 2. *Some typical phages observed in samples of activated sludge mixed liquor. Bar represents 0.1 μm.*

phage particles directly on the pseudoreplicas by using an electron microscope. A grid hole on a 200-mesh electron microscope grid provided a suitable standard area (7.2×10^{-3} mm^2) in which to count phage particles. The coefficient of variance for a grid hole area, as measured by a light microscope equipped with an ocular micrometer, was less than 2% for holes on the same grid, holes on other grids of the same lot, and holes on grids purchased at three different times but from the same manufacturer. Generally, for each pseudoreplica two randomly selected grid holes

were scanned for phages at a magnification of 10,000×. Although most phages could be recongized at this magnification, higher magnifications were used to identify smaller phage particles. In principle, the total concentration of phages in a sample was calculated in a way similar to that used by Sharp (16). The volume (7.92×10^{-2} mm^3) from which particles were sedimented was derived from the area of the electron microscope grid hole (7.2×10^{-3} mm^2) and the height of liquid in the sedimentation chamber above the agar block (11 mm). Therefore, the number of phages per milliliter was calculated by multiplying the average number of phages per grid hole by 1.26×10^4.

Dilution of samples. As the parameters of the assay were optimized, it became necessary to dilute samples of activated sludge so that phages could be counted easily and accurately. A diluent was required that would maintain phage intergrity and would not reduce the quality of the electron microscope preparation. A 0.03 M phosphate buffer (KH_2PO_4-NaOH), pH 6.9, which was routinely used to maintain stock cultures of T2 and λ phages, formed a precipitate upon contact with the staining solution. Similarly, 0.9% (wt/vol) NaCl containing 10^{-2} M magnesium acetate produced a residue on electron microscope preparations. A 1% (vol/vol) solution of glycerol in deionized water proved to be a suitable diluent. Virions remained intact as indicated by their densely stained heads, and the glycerol did not lower the quality of pseudoreplicas.

Receiving surface. Initially, Sharp (14) used collodion-covered glass blocks as a receiving surface for influenza virus. Later, he used agar blocks because they permitted solutes in samples to diffuse into the agar while virions remained on the surface, thereby reducing contamination of the pseudoreplicas (16). During early development of the phage assay, 1.5% (wt/vol), agar blocks were used as a receiving surface. However, it was found that all phages were not removed by a single stripping with collodion. Therefore, collodion-covered glass was tested to determine whether quantitative recovery of phages could be obtained. Glass blocks (2-mm thick) were cut and trimmed to fit the base of chambers of the SU particle-sedimenting rotor. The surfaces of two blocks were covered with collodion, allowed to air dry for approximately 30 min, and placed in rotor chambers with the collodion covered surface facing the inside of the chambers. Two agar blocks (1.5% wt/vol, 2 mm thick) were placed in other chambers, and all were filled with a filtered (0.45 μm) sample of activated sludge mixed liquor and centrifuged for 1 h at 31,500 × *g*. After sedimentation, supernatant in the chambers containing glass blocks was quantitatively removed and recentrifuged for 1 h at 31,500 × *g* onto identically prepared glass blocks. Collodion membranes were floated off glass blocks onto distilled water, transferred to electron microscope grids, and stained with 0.5% (wt/vol) uranyl acetate. Pseudoreplicas were prepared from each agar block in the usual manner, with the stripping procedure repeated three times. Recovery on collodion covered glass after one centrifugation treatment was only 68% of that on 1.5% agar, with three strippings. Centrifuging the sample a second time onto a fresh collodion-covered glass surface did not improve recovery appreciably. Therefore, an agar-receiving surface was chosen.

Sharp and Beard (17) stated that agar concentrations ranging from 1 to 3% were used to recover pox virus, but they did not indicate that agar concentration had any effect on virus recovery.

Three concentrations of agar (Difco) 1.5, 3.0, and 4.5% (wt/vol) were used to prepare receiving surfaces. Duplicate blocks of agar at each concentration were placed in the rotor chambers so that the air-dried surface would receive the phages. Chambers were filled with a filtered sample of an activated sludge reactor liquor and centrifuged for 1 h at 31,500 × *g*. Five collodion strippings made from each block were stained with uranyl acetate and examined electron microscopically. Phages were counted (trial A, Table 1). Maximal recovery of phages occurred with 4.5% agar blocks. The experiment was repeated, using another sample of mixed liquor with the range of agar concentrations increased to 6% (trial B, Table 1). Phages recovered from 6% agar blocks were only 25% of those from 4.5% blocks, which was the agar concentration that again showed maximal phage recovery. In sub-

TABLE 1. *Effect of agar concentration in receiving blocks on recovery of phages from samples of activated sludge mixed liquor*[a]

% Agar concn (wt/vol)	Total no. of phages recovered per grid hole (mean of replicate agar blocks)[b]	
	Trial A	Trial B
1.5	89	136
3.0	359	1,502
4.5	978	2,025
6.0	—	501

[a] Centrifuged in SU particle sedimenting rotor for 1 h at 31,500 × *g*, 3°C.

[b] Sum of phages recovered on five pseudoreplicas per agar block. —, Not measured.

sequent experiments we found that the air-dried (18 to 24 h) surface of agar blocks gave better recovery of phages than the smooth surface which had been in contact with the bottom of the petri dish. It was also noted that highly purified agar (Ion Agar no. 2, Colab Laboratories, Inc. Chicago, Ill.) could not be used because collodion membranes were so firmly bonded to the agar surface that they could not be floated off blocks.

Effect of sedimentation time on phage recovery. The centrifugal force used was the maximum that could be achieved with the SU rotor. Therefore, the effect of sedimentation time was studied. A sample of filtered mixed liquor was sedimented for 1, 2, or 3 h onto agar blocks (4.5%). Three strippings of each block were made and stained with uranyl acetate, and the number of phages was counted. The experiment was repeated for 2 and 3 h sedimentation times using the same sample of mixed liquor but diluted 10-fold with 1% glycerol. In both experiments, maximal phage recovery was obtained after 2 h of centrifugation. More small phages were recovered from the preparations spun for 2 or 3 h than from the samples spun for 1 h. Consequently, a 2-h sedimentation time was chosen for all subsequent determinations.

Primary clarification of activated sludge samples. Experiments were done with activated sludge mixed liquor to determine the minimal centrifugal force and time to reduce the bacterial content of the supernatant to approximately 10^6 organisms per ml. This was achieved by centrifuging for 15 min at 5,000 × *g*. This concentration of bacteria was sufficiently low so as not to interfere with the phage assay. Experiments were performed to compare low-speed centrifugation and membrane filtration on the removal of phage from liquor samples. Approximately twice as many phages were routinely recovered from centrifuged samples as from filtered samples. Presumably, phages were being removed by adsorption to the filter. Centrifugation at 5,000 × *g* would remove few, if any, phages from suspension because the force is far below that required to sediment even the larger vaccinia viruses (18).

Phages adsorbed to cells and debris in mixed liquor were removed by primary centrifugation of samples. Release of adsorbed phages was attempted by: (i) blending (Waring blender) 100-ml samples for 10 min; (ii) sonicating (Branson Sonifier, model 125) 25-ml samples at power setting 3 for 2 min; and (iii) incubating 3-ml samples with equal volumes of 0.1% (wt/vol) papain, pronase, or lysozyme at 29°C for 3, 10, or 30 min. Uranyl acetate-stained psuedoreplicas of blended and sonicated samples were heavily contaminated with debris, and pseudoreplicas from enzyme-treated specimens were masked with a precipitate formed by interaction of the enzymes with uranyl acetate. Thus, none of the treatments was effective, and no method for releasing adsorbed phages was discovered.

Estimation of total number of phages sedimented. Since not all the phages were recovered with the first collodion stripping of an agar block, it was necessary to determine the number of strippings required to remove all phages from the agar. Electron microscope preparations were made of samples of mixed liquor by all of the previously standardized techniques, except that 10 strippings were made from each of three agar blocks per sample. The average numbers of phages per grid hole on pseudoreplicas are given in Table 2. Although some phages were recovered on the tenth stripping, the number represented only 0.5 to 1.5% of the cumulative total. It was found that a similar fraction of the total phages was recovered on equivalent strippings of different agar blocks, a mean of 63.5 (±1.6)% being recovered on the first stripping.

Recovery of phages from a sewage sample (Table 3) was examined by using the same pro-

TABLE 2. *Recovery of mixed liquor phages from agar blocks by repeated strippings with collodion*

Sample[a]	Stripping no.	Phages/grid hole on the following agar blocks:[b]		
		Block 1	Block 2	Block 3
A	1	254 (65.0)	232 (61.0)	307 (65.1)
	2	64 (16.3)	56 (14.8)	76 (16.2)
	3	38 (9.8)	38 (10.1)	36 (7.6)
	4	8 (2.2)	13 (3.3)	12 (2.6)
	5	8 (2.2)	11 (3.0)	10 (2.1)
B	1	472 (62.1)	440 (64.0)	459 (63.6)
	2	112 (14.8)	89 (12.9)	97 (13.5)
	3	75 (9.9)	70 (10.3)	66 (9.1)
	4	30 (4.4)	22 (3.3)	25 (3.5)
	5	30 (4.4)	20 (2.8)	21 (2.9)

[a] Samples A and B were obtained from the activated sludge reactor at two different times separated by an interval of 1 month. Primary clarification was performed by centrifuging for 15 min at 5,000 × *g* and 3°C. Supernatants were 10-fold diluted with 1% glycerol, and phages were sedimented onto 4.5% agar blocks in an SU particle-sedimenting rotor for 2 h at 31,500 × *g*, 3°C.

[b] Each value represents the mean of two grid holes. Each value in parentheses represents percentage of total phages recovered by each collodion stripping, based on sum total of phages recovered on ten pseudoreplicas per block.

TABLE 3. *Recovery of sewage phages from agar blocks by repeated stripping with collodion*[a]

Stripping no.	No. of phages/grid hole on the following agar blocks:[b]		
	Block 1	Block 2	Block 3
1	65 (62.5)	58 (61.7)	62 (57.9)
2	17 (16.3)	15 (16.0)	22 (20.6)
3	16 (15.4)	15 (16.0)	15 (14.0)
4	4 (3.8)	4 (4.3)	5 (4.7)
5	2 (1.9)	2 (2.1)	3 (2.8)

[a] Sample was obtained from sewage before entering the activated sludge reactor and treated as described in footnote *a*, Table 2.

[b] Each value represents the mean of two grid holes. Each value in parentheses represents percentage of total phages recovered by each collodion stripping, based on sum total of phages recovered on five pseudoreplicas per agar block.

cedure as in the previous experiment. The concentration of phages in the sewage preparation was low, and only two to three phages could be found per grid hole after five strippings. The mean fraction recovered on the first stripping (61%) was similar to that found in the previous experiment with activated sludge mixed liquor. Therefore, it was considered valid to predict total number of phages in a sample by dividing the number of phages counted on the first stripping by 0.63, i.e., the average fraction of the total which they represented.

Efficiency of phage recovery. Samples of undiluted activated sludge mixed liquor supernatant were assayed for phage concentrations under standard assay conditions. The samples were also diluted 10-fold with 1% (wt/vol) glycerol and assayed. Variation between diluted and nondiluted samples did not exceed ±8%. Thus, the assay procedure developed was judged to be acceptable for quantitative ecological analyses.

Standardized procedure for assaying total concentration of phages in sewage and activated sludge mixed liquor. Samples, stored on ice before treatment, are centrifuged for 15 min at 5,000 × *g* and 3°C to remove large debris and to reduce the bacterial concentration to approximately 10^6 per ml. The top two-thirds of the supernatant is removed and diluted 10-fold with 1% (wt/vol) glycerol. Diluted samples are sedimented onto duplicate 4.5% (wt/vol) agar receiving blocks (2 mm thick) in an SU particle-sedimenting rotor (Ivan Sorval, Inc.), for 2 h at 31,500 × *g* and 3°C. Agar blocks are air-dried (30 to 40 min) and coated with 1% (wt/vol) collodion. When dry, collodion membranes are floated off the agar onto the surface of 0.5% (wt/vol) uranyl acetate, pH 4.6. Staining is continued for 45 to 60 s, and each pseudoreplica is transferred to a 200-mesh electron microscope grid, washed with one drop of deionized water, and carbon coated. Each pseudoreplica is examined with an electron microscope at a magnification of 10,000×, and the number of phages is counted in two randomly selected grid holes. A grid hole with 500 to 1,000 phages could be counted in approximately 20 min, whereas lower concentrations (e.g., 50 to 100 phages per grid hole) could be counted in approximately 10 min. Total phage concentration per milliliter is calculated from the equation previously given in the Materials and Methods section by using the mean number of phages per grid hole for the two pseudoreplicas and the value of 0.63 for the fraction of total phages recovered on the first pseudoreplica.

DISCUSSION

The modified sedimentation technique presented herein has several advantages over the procedure described by Sharp (15), and over techniques requiring the use of reference particles (10, 21). First, concentrations as low as 10^4 phages per ml can be assayed, whereas at least 10^6 or 10^{10} particles per ml are required for detection by the sedimentation assay of Sharp (16) and the spray droplet (20) or gel filtration assays (10), respectively. Second, errors involved in calibration of the electron microscope, which lowers the accuracy of those assays which use electron micrographs or reference particles, are not a factor in the modified technique. Third, recognition of virus particles is not limited by the fixed magnification of the electron micrograph. Fourth, preparation of electron micrographs is eliminated. Also, the selective staining of phage heads with uranyl acetate makes possible more rapid recognition of phage particles. This assay is capable of determining total bacteriophage concentrations (the sum of both viable and nonviable virions) in the aqueous phase of sewage and activated sludge mixed liquor. Maximum concentrations observed for these two systems were 1.3×10^6/ml for sewage and 4.3×10^7/ml for mixed liquor. Until demonstrated otherwise, these concentrations must be considered minimum values, since phages adsorbed to debris and bacteria were excluded from the assay by primary clarification. Future investigations should address the problem of releasing adsorbed viruses without decreasing the quality of pseudoreplicas.

The assay described in this paper is the first one adapted for direct enumeration of total bacteriophages suspended in samples of unpurified aqueous ecosystems. Unlike the plaque assay,

the total concentration of phages in a sample can be determined directly without infection of a sensitive host. Thus, the limitations of the plaque assay are avoided. It is anticipated that application of this direct assay, in conjunction with the plaque assay, will make possible a more precise analysis of the role of bacteriophages in natural ecosystems.

ACKNOWLEDGMENTS

We thank D. G. Sharp, Department of Bacteriology, University of North Carolina, Chapel Hill, for his help and instruction in the sedimentation technique for enumerating viruses.

D.L.E. thanks the trustees of the Belle W. Baruch Foundation for providing a fellowship and funds to support part of this research.

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